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MINI REVIEW

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Dynamic genome and transcriptional network-based biomarkers and drugs: precision in breast cancer therapy

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Abstract

Despite remarkable progress in medium-term overall survival benefit in the adjuvant, neoadjuvant and metastatic settings, with multiple recent targeted drug approvals, acquired resistance, late relapse, and cancer-related death rates remain challenging. Integrated technological systems have been developed to overcome these unmet needs. The characterization of structural and functional noncoding genome elements through next-generation sequencing (NGS) systems, Hi-C and CRISPR/Cas9, as well as computational models, allows for whole genome and transcriptome analysis. Rapid progress in large-scale single-biopsy genome analysis has identified several novel breast cancer driver genes and oncotargets. The exploration of spatiotemporal tumor evolution has returned exciting while inconclusive data on dynamic intratumor heterogeneity (ITH) through multiregional NGS and single-cell DNA/RNA sequencing and circulating genomic subclones (cGSs) by serial circulating cell-free DNA NGS to predict and overcome intrinsic and acquired therapeutic resistance. This review discusses reliable breast cancer genome analysis data and focuses on two major crucial perspectives. The validation of ITH, cGSs, and intrapatient genetic/genomic heterogeneity as predictive biomarkers, as well as the valid discovery of novel oncotargets within patient-centric genomic trials, encouraging early drug development, could optimize primary and secondary therapeutic decision-making. A longer-term goal is to identify the individualized landscape of both coding and noncoding key

mutations. This progress will enable the understanding of molecular mechanisms perturbating regulatory networks, shaping the pharmaceutical controllability of deregulated transcriptional biocircuits.

KEYWORDS
biomarkers, breast cancer, drugs, intrapatient heterogeneity, next-generation sequencing systems, precision therapy, regulatory networks

1 | INTRODUCTION

Over the past decade, Modern Oncology and Pharmacology have been based on linear static experimentation, single-biopsy tumor analysis, and single-gene transcription.[1] Although breast cancer represents a prime paradigm of advancing progress in targeted and personalized treatment, reflected by improved oncological outcomes, a significant proportion of patients remains at risk of late relapse and death.[2] Currently, conventional research, as well as commercial and funding interest are focused on the concept of static interpatient genetic heterogeneity as an already fruitful approach.[3] Whether scientific perspectives should shift toward promising comprehensive dynamic structural intrapatient genomic heterogeneity,[4–6] as well as temporal regulatory networks controlling gene expression in the human genome[7–9] and nonlinear transcription-based drug development,[10] is currently under debate.

Remarkable progress in the integration of next-generation sequencing (NGS) technologies and breakthrough NGS systems into patient-derived sample genome analysis over the past years has produced evidence on the emergence of genomic and transcriptional heterogeneity in time and space.[11,12] Personalized structural mutational landscapes, including dynamic intratumor heterogeneity (ITH) before and after neoadjuvant treatment (NAT)[5] and NGS of serial circulating cell-free DNA (cfDNA) samples (cfDNA-NGS),[13] could be used as predictive biomarkers guiding precise therapeutic targeting of key druggable mutations.[6] Moreover, the validity in the identification of functional noncoding regulatory mutations,[14] coupled with computational, mathematical, and genome-editing tools,[9,15] could enhance our understanding on the controllability of dysregulated transcriptional networks.[8]

On the basis of data analysis of valid genomic studies, we propose a novel design for breast cancer clinico-genomic trials applying NGS systems. The aims of these trials include the evaluation of the predictive power of dynamic ITH and circulating genomic subclones (cGSs), as well as the valid identification of new targetable mutations. In a more distant perspective, we discuss the potential and challenges in the future integration of the personalized comprehensive functional, in addition to the structural, mutational landscape, which could enhance our understanding of regulatory networks as the foundation for future pharmaceutical controllability of dysregulated transcriptional biocircuits (Figure 1).

2 | MODERN ONCOLOGY: ADVANCES AND CHALLENGES

Remarkable progress in breast cancer research has been translated into standardization of clinical treatment, including surgery, radiotherapy, chemotherapy, endocrine, and targeted treatment. Following the strict criteria for evidence-based medicine, multiple phase III randomized controlled trials and meta-analyses guiding therapeutic decision-making have progressively led to substantial improvements in survival and cancer-related death rates.[2,16]

Breast cancer represents an excellent example of rapid research advances toward interpatient heterogeneity-based personalized treatment. Primary therapeutic decision-making, crucial for reducing the risk of relapse and death, takes into consideration both the clinico-pathologic (age, TNM-staging, histological grade) and the molecular

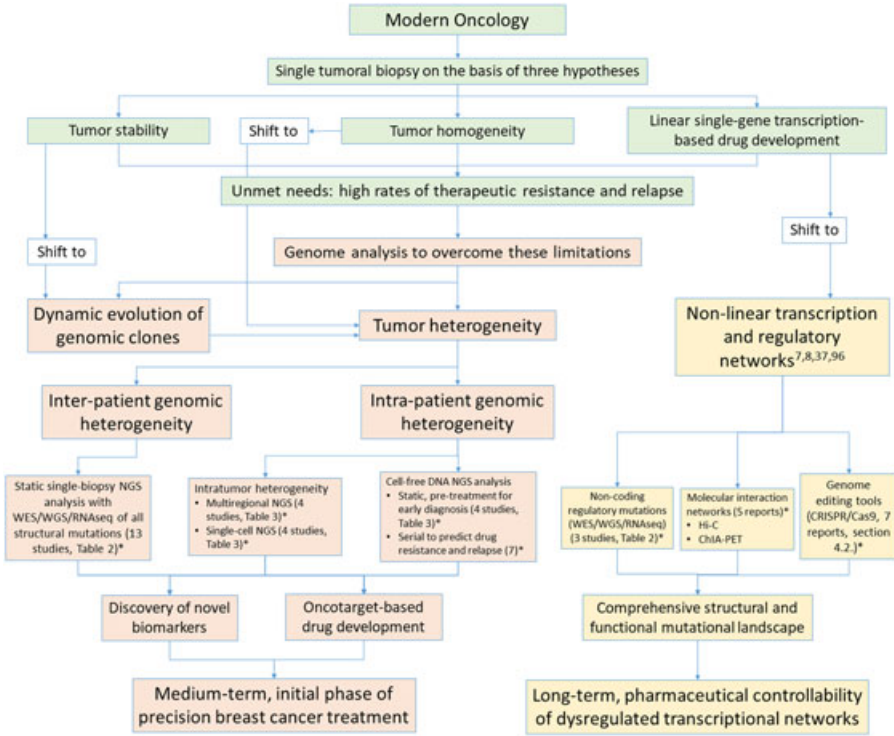

FIGURE 1 Integrating genome analysis and editing technologies into appropriate clinico-genomic studies to overcome unmet needs. Step-wise delineation of the shift from current single-tumoral biopsy approach, on the basis of static tumor homogeneity and linear transcription-based drug development, to spatiotemporal genome, transcriptome and regulatory network exploration. This strategy could lead to optimization of precise individualized prediction-based breast cancer therapy. NGS, next-generation sequencing; RNAseq, RNA sequencing; WES, whole-exome sequencing; WGS, whole-genome sequencing. *Optimal selection of appropriate genomic studies among published reports [Color figure can be viewed at wileyonlinelibrary.com]

(ER/PR/HER2, *BRCA1/2* status) characteristics for each individual patient.[2] Table 1[17–30] summarizes the growing list of targeted agents for the different molecular subtypes of breast cancer in the adjuvant, neoadjuvant, and metastatic settings, including HER2-positive, hormone receptor (HR)-positive/HER2-negative, triple-negative, and *BRCA*-mutated/HER2-negative tumors, from the approval of trastuzumab in 2001 to multiple recent drug approvals, namely pertuzumab,[20,21] neratinib maleate,[23] and palbociclib.[26] More specifically for early stage, HER2-positive breast cancer, disease-free survival (DFS) has increased from approximately 80% at 4 years following adjuvant treatment with trastuzumab plus chemotherapy[31] to 94.1% at 3 years after adjuvant dual HER2 inhibition plus chemotherapy[20] and over 90% 5-year DFS with the addition of neratinib after trastuzumab chemotherapy.[23] These impressive results were achieved independently of ER status.[20] In the neoadjuvant setting, the addition of pertuzumab to trastuzumab-docetaxel in the NeoSphere trial significantly increased pathological complete response rate (pCR), from 29% to 45.8%[32] and improved progression-free and disease-free survival.[21]

Despite these advancements, no targeted therapy has been developed and approved against triple-negative breast cancer (TNBC), with chemotherapy remaining the only available option for systemic treatment. TNBCs carry 1.68 somatic mutations per Mb of coding regions corresponding to approximately 60 somatic mutations in each tumor.[33] Remarkably, 10% of TNBC patients carry BRCA1/2 germline mutations, which are associated with an increased 60% to 70% lifetime risk of breast cancer.[34] Lately, a preclinical experimental study on patient-derived xenografts has reported exciting results after combinatorial inhibition of PTPN12-regulated receptor tyrosine

TABLE 1 Targeted drugs for breast cancer according to the stage and molecular subclass

Molecular subtype	M0	Locally advanced/M1
HER2 positive	Trastuzumab (as adjuvant or neoadjuvant therapy, in conjunction with chemotherapy)[17,18]	Trastuzumab[19]
	Pertuzumab (as adjuvant or neoadjuvant therapy, in conjunction with trastuzumab)[20,21]	Pertuzumab (in conjunction with trastuzumab and chemotherapy)[22]
	Neratinib maleate (extended adjuvant treatment after trastuzumab)[23]	Lapatinib (in conjunction with chemotherapy or endocrine therapy)[24]
		Ado-trastuzumab emtansine (after trastuzumab, lapatinib, and taxanes)[25]
HR positive/HER2 negative	Not available	Palbociclib (in conjunction with chemotherapy or endocrine therapy)[26]
	Not available	Ribociclib (in conjunction with endocrine therapy)[27]
	Not available	Abemaciclib (in conjunction with endocrine therapy)[28]
Triple negative	No targeted treatment has been approved, promising results have been reported with combinatorial RTK inhibition of PTPN12-regulated receptors with crizotinib-sunitinib[29]	
HER2 negative, BRCA mutated	Not available	Olaparib (after chemotherapy)[30]

Abbreviations: HER2, human epidermal growth factor receptor 2; HR, hormone receptor; M0, nonmetastatic disease; M1, metastatic disease; RTK, receptor tyrosine kinase.

kinases (RTKs). More specifically, the combination of crizotinib and sunitinib, inhibiting MET and PDGFRβ RTKs, respectively, achieved significant levels of therapeutic response, leading to 50% tumor regression.[29] Should these findings be validated within phase I/II clinical trials, more effective therapies could at last become a reality for TNBC and confirm the clinical utility of umbrella and basket trial designs.[35] For instance, crizotinib and sunitinib have already received approval for non-small–cell lung cancer and gastrointestinal stromal tumors, pancreatic adenocarcinoma and renal-cell carcinoma, respectively. Moreover, a recent report suggests potential therapeutic utility of HER kinase inhibition with neratinib[23] in patients with HER2/3 mutated cancers, such as breast, cervical, and biliary tumors.[36]

2.1 | Unmet needs

Despite substantial progress in single-biopsy linear single-gene transcription research, reflected by the development and approval of several targeted agents, as well as high survival rates, particularly for early stage, HER2-positive breast cancer, major challenges remain unresolved. First, despite the positive results provided by adjuvant dual HER2 blockade regarding early recurrence, DFS at 10-year follow-up after adjuvant trastuzumab chemotherapy remains 69%.[17] In the neoadjuvant setting, although pertuzumab plus trastuzumab chemotherapy significantly increased pCR, the rate is still only 46% for HER2-positive patients.[32] Thus, despite exciting data in the control of recurrence during the 5-year follow-up, late relapse rates remain high and suggest both intrinsic and, particularly, acquired therapeutic resistance, confirming the general concept of the temporary efficacy of all targeted drugs. Second, 5-year DFS for the triple-negative molecular class after adjuvant chemotherapy remains at 77%.[37] Moreover, although the combination of dual HER2 blockade (pertuzumab and trastuzumab) and docetaxel has improved overall survival for metastatic HER2-positive tumors to over 50% at 3 years, 3-year progression-free survival is only 20% approximately.[22] Third, the modern single-biopsy approach, based on the hypothesis of tumor stability and homogeneity, comes in direct conflict to current evidence on spatiotemporal tumor evolution,

TABLE 2 Static single-biopsy next-generation sequencing analyses on breast cancer

Number of samples	Technology	Findings	Clinical implications	References
Structural coding genomic alterations				
992	WES	This report focuses on the characterization of signature 3 and its association to homologous recombination repair (HRR) deficiency	Deleterious mutations leading to HRR deficiency could form the basis for future clinical trials	Polak et al[46]
914	RNAseq	• This study focuses on subtype-specific expression at the isoform level • Isoform-level information could enable comprehensive molecular classifications	Isoform-level data on key genes could offer insight on therapeutic response prediction	Vu et al[57]
892 (4742 from 21 cancer types)	WES	1 novel cancer driver gene identified	Valid identification of novel cancer driver genes and oncotargets requires large-scale genomic analyses and $P < 0.01$	Lawrence et al[43]
680 (ductal)	WGS	*TP53* mutations/*MYC* amplifications and PIK3CA mutations were associated with a higher and lower histologic grade respectively, in 83% of tumors	Histologic grade could in vivo predict cancer driver events and potentially guide personalized treatment	Ping et al[53]
560	WGS	• Five novel cancer driver genes were found (*MED23*, *FOXP1*, *MLLT4*, *XBP1*, *ZFP36L1*) • Inactivating *BRCA1/2* mutations correlated to increased genomic rearrangements • Activated fusion genes and noncoding driver mutations were rare	Further studies are required to clarify the frequency and significance of noncoding mutations	Nik-Zainal et al[54]
216 (metastatic)	WES	• 12 cancer driver genes were identified, 2 novel (*ESR1* and *RB1*) • Mutations of *ESR1* were drivers of metastasis • Several actionable mutations were detected (*TSC1*, *TSC2*, *ERBB4*, *NOTCH3*, *ALK*, *BRAF*)	Genomic analysis could harbor therapeutic implications for oncotarget-based targeted therapy	Lefebvre et al[47]
195 (familial or early onset)	WES	*RECQL* was identified as a novel susceptibility gene	If validated, *RECQL* could complement screening and risk assessment	Cybulski et al[48]
144 (familial)	WES	This study focuses on non-founder mutations in familial breast cancer in the Polish population	Polish women with familial breast cancer, negative for founder mutations in *BRCA1*, *CHEK2* and *NBS1* should be fully screened for mutations in *BRCA1*, *BRCA2*, and *PALB2*	Cybulski et al[49]

(Continues)

TABLE 2 (Continued)

Number of samples	Technology	Findings	Clinical implications	References
108	WES and/ or WGS	• *CBFB* transcription factor gene was identified as a novel recurrently mutated gene • A novel, recurrent, potentially targetable gene fusion was detected (*MAGI3-AKT3*)	Akt inhibitors could be evaluated for the treatment of TNBCs with this novel fusion	Banerji et al[50]
93 (advanced stage)	WGS	This study assessed the HRDetect algorithm to predict outcomes on platinum-based chemotherapies	HRDetect score correlated with clinical improvement and overall survival and could guide future clinical trials	Zhao et al[55]
79	WES and tNGS	This study focuses on rare and aggressive breast cancer histologies, including micropapillary, metaplastic, and pleomorphic lobular tumors	NGS could reveal novel oncotargets in these rare tumors but further studies are required	Dieci et al[44]
77	46 WGS/ 31 WES	• *GATA3* mutations were identified as potential positive predictive markers for AI response • Transcriptional associations to Ki67 response comprise a network controlled by several key genes including *MYC*, *FYN*, and *MAP* kinases	• Targeting key genes could improve therapeutic response of resistant tumors • Larger studies are required, implementing gene expression	Ellis et al[51]
68 ER+	tNGS and RNAseq	• Tumors resistant to prolonged neoadjuvant letrozole exhibited a gene expression signature of E2F4-target activation, including 20 E2F4-regulated genes • Preoperative palbociclib significantly decreased expression of 24/47 most upregulated genes in letrozole-resistant tumors, including 18/20 E2F4 targets • After long-term estrogen deprivation, palbociclib downregulated all E2F4-target genes and P-RB levels	This reports suggests a potential benefit of CDK4/6 inhibition in patients with ER + breast cancer resistant to preoperative estrogen deprivation	Guerrero-Zotano et al[45]
Functional noncoding regulatory mutations				
930	WES and RNAseq (117 WGS)	• Recurrent mutations were identified in non-coding regulatory regions of cancer-associated genes (*NBPF1*, *PIK3CA*, *TP53*) • Three coding (*CDH1*, *MAP3K1*, *TP53*) and two noncoding variants (*CRTC3*, *STAG2*) correlated to prognosis in ER-positive/HER2-negative tumors	• Functional noncoding mutations may have clinical significance, but further studies are required • Identification of the comprehensive coding and noncoding mutational landscape could have therapeutic implications for targeted therapy	Gyorffy et al[52]

(Continues)

TABLE 2 (Continued)

Number of samples	Technology	Findings	Clinical implications	References
360	WES	• This study focuses on recurrently mutated promoters • Three gene promoters were recurrently mutated (*FOXA1*, *RMRP*, *NEAT1*) • Promoters were altered in similar frequencies as coding regions • *FOXA1* mutations could drive therapeutic resistance and progression	• Further research is required to expand the catalog of noncoding promoter regions with potential clinical utility • Precision Cancer Medicine requires the identification of the full repertoire of functional mutations, including promoters	Rheinbay et al[14]
98	WGS, RNAseq, 3C and CRISPR/Cas9	• 7% of *ESR1*-positive breast cancers harbored somatic mutations within the set of regulatory elements targeting *ESR1*, modulating transcription factor binding, including a recurrently mutated enhancer • Mutations affecting distinct regulatory elements do not need to directly target DNA recognition motifs	The breakthrough combination of WGS, RNAseq, 3C, and CRISPR/Cas9 could set the basis for the future understanding of regulatory networks paving the next-generation avenue of transcriptional network-based drug development	Bailey et al[56]

Abbreviations: 3C, chromosome conformation capture; AI, aromatase inhibition; ER, estrogen receptor; RNAseq, RNA sequencing; tNGS, targeted NGS; WES, whole-exome sequencing; WGS, whole-genome sequencing.

producing tumor heterogeneity.[5,6,13] Fourth, the linear transcription dogma[38] is in contrast with functional noncoding genome functionality and complex regulatory networks in health and disease.[7–9,39] These unmet needs could potentially be overcome through the exploration of genomic and transcriptional heterogeneity in time and space for the development of dynamic predictive biomarkers, as well as the discovery of novel therapeutic targets and nonlinear transcriptional network-based drugs.

3 | STRUCTURAL GENOME ANALYSIS: DATA AND TRANSLATIONAL IMPLICATIONS

The validity of NGS in the characterization of human genome elements in health[39] and disease, particularly in cancer,[40] has revolutionized biomedical research. Two large-scale international cancer projects,[41,42] aiming to complete the cancer driver gene and mutation catalog for multiple cancer types, have already reported significant basic and translational research progress. Evidence on extensive genomic and transcriptional heterogeneity,[12,43] and the shift from static, single-biopsy genome analysis to spatiotemporal identification of genomic clones in multiple tumoral and liquid biopsies[6] shape the framework for personalized treatment. Considering the ENCODE project on cell-specific genomic variability in the healthy human genome,[7,39] as well as transcriptional heterogeneity in cancer,[12] including transcription factors (TFs), TF-binding site mutations and transcriptional networks, basic research on regulatory networks is crucial for the future pharmaceutical controllability of deregulated transcriptional biocircuits.[10]

The high incidence of breast cancer among women, combined with the relatively simple acquisition of patient-derived samples, have led to a large number of NGS studies. Static analyses implement targeted NGS (tNGS),[36,44,45] whole-exome (WES),[14,43,44,46–52] whole-genome (WGS),[50,51,53–56] and/or RNA (RNAseq)[45,52,56,57] sequencing, based on a single-tumoral biopsy approach (Table 2).[14,43–57] Breakthrough NGS analysis (Table 3),[5,13,58–74] includes the static or spatiotemporal exploration of ITH either with NGS analysis of multiregional (MR) tumor samples (MR-NGS)[5,58–60] or single-cell DNA/RNA NGS,[61–64] the identification of cGSs through cfDNA-NGS at a single[65–68] or multiple serial time points,[13,69–74] as well as the comparative analysis of the above for the identification of comprehensive intrapatient genetic/genomic heterogeneity (IPGH) in patients without relapse or metastasis.[75] Among those with metastasis or recurrence IPGH refers to the comprehensive comparisons between ITH, cGSs, and genetic/genomic alterations of the relapsed or metastatic tumors.[6] This strategy can provide solid evidence on "resistant" subclones through cfDNA-NGS, responsible for relapse or metastasis.

3.1 | Static, single-biopsy structural genome analysis

The recent widespread application of tNGS in laboratory and clinical research has enabled the evaluation of targeted therapies within clinical trials of the umbrella and basket designs. Remarkably, such trials have provided promising preliminary results. For instance, Hyman et al,[36] explored the clinical significance of HER2 and HER3 mutations in a variety of cancers and the effectiveness of the pan-HER kinase inhibitor neratinib against those tumors. Highest efficacy, while still lower than approved targeted therapies, was observed for HER2 mutant breast, cervical and biliary cancers, suggesting the potential for combinatorial targeted treatment and providing proof of concept for advancing genome-based oncology through molecularly driven clinical trials. No responses to neratinib were observed in patients with HER3-mutant tumors, in contrast to previous findings.[76] Beyond targeted sequencing of known-gene panels, single-biopsy WES, WGS, and RNAseq are used for the valid detection of new cancer driver genes, robust biomarkers, and oncotargets. Large static genomic analyses (Table 2)[14,43–57] have recently identified approximately 10 novel cancer driver and susceptibility genes that could potentially complement and further enhance current genetic screening.[43,47,48,50,54] Several actionable mutations were identified, including a novel *MAGI3-AKT3* gene fusion, putatively targetable by existing drugs, such as *BRAF* and Akt

TABLE 3 Breast cancer studies exploring spatiotemporal genomic clonal evolution and tumor heterogeneity with integrated next-generation sequencing systems

Number of patients/samples	Sample type/ molecular subclass	Technology	Findings	Clinical implications	References
Static intratumor heterogeneity					
50/303	BC	29 samples WGS/290 tNGS	• Variable degrees of intratumor heterogeneity were identified • 13/50 cancers harbored subclonal targetable mutations	Validation is required for the clinical benefits of targeting subclonal actionable mutations	Yates et al[5]
Dynamic identification of intratumor heterogeneity					
18	Pre-NAT and post-NAT MR-NGS of the PT	Breast MR-tNGS	• In 6/18 pts, mutations were subclonal indicating subclone persistence despite chemotherapy • In 5/18 pts, a subclone was identified in the post-NAT samples, which was not evident in the pretreatment samples • Amplifications of *CDK6*, *FGFR2* and *MYC* and a deletion within *RUNX1* were identified as driver mutations of resistant subclones	The finding that 28% of pts featured new subclones after NAT suggests the need for MR-NGS before and after NAT with potential diagnostic and therapeutic implications	Yates et al[5]
11	Matched PT and MT samples	WGS	• Concordance for chromosomal rearrangements between matched PT and MT was 89% (61%-100%), suggesting stability over the disease course	Further studies are required to confirm the pre-existence and stability of chromosomal rearrangements within the PT	Tang et al[58]
10/51	Matched PT and MTs	WES and CNV (multiregional PT samples from 7 pts)	• Two modes of metastasis were observed: monoclonal seeding from a common ancestor within the PT and multiple seedings from different PT clones • Metastasis-to-metastasis spread was commonly seen	Genomic analysis of metastases could offer additional information for therapeutic decision-making in the metastatic setting	Brown et al[59]

(Continues)

TABLE 3 (Continued)

Number of patients/samples	Sample type/ molecular subclass	Technology	Findings	Clinical implications	References
9 treatment naive	Matched PT and MT samples	WES	• Somatic mutational landscapes between PT and MT were 60% shared • Pathogenic mutations in epithelial-mesenchymal transition-related genes (*SMAD4*, *TCF7L2*, *TCF4*), were found to be restricted to or enriched in the MTs	Synchronous breast primary and metastatic tumors are genetically different even in the absence of systemic therapy, with potential future implications for targeted treatment	Ng et al[60]
Single-cell NGS					
12/1000	TNBC	CNV	• Minor subpopulations of nonclonal cells with high metastatic potential were identified • Copy number aberrations were early evolutionary events which remain stable in time, potentially guiding invasion, metastasis and chemotherapy resistance	N/A	Gao et al[61]
332/2	ER + BC	CNV	Subclonal heterogeneity supports dynamic diversification over the disease course	Potential spatiotemporal therapeutic strategies may further improve treatment effectiveness	Baslan et al[62]
200/2	BC and LM	CNV	• 3 clonal subpopulations were detected • Metastatic potential was identified as a late evolutionary event • This study supports punctuated clonal evolution, as opposed to gradual tumor evolution	N/A	Navin et al[63]
179/2	1 ER+ and 1 TNBC	CNV and SNV	Aneuploid rearrangements occurred early in tumor evolution and remained highly stable, while point mutations evolved gradually, producing extensive subclonality	Dynamic evolution of point mutations suggests the potential for effective targeted drug development	Wang et al[64]

(Continues)

TABLE 3 (Continued)

Number of patients/samples	Sample type/ molecular subclass	Technology	Findings	Clinical implications	References
Static cfDNA-NGS					
1627 (878 pts and 749 controls)	358 pts with invasive BC (82% stage I/II)	tNGS, WGS, WGBS	• This report is a substudy of the first registered clinical trial applying cfDNA-NGS with approximately 15,000 participants, for early noninvasive diagnosis of various cancers • Three prototype sequencing assays were performed on cfDNA: tNGS, WGS, and WGBS • WGBS returned the highest sensitivity: 58% for TNBC, 40% for HER2+, and 15% for HR+/HER2− • Sensitivity was 44% for symptomatic vs 10% for screen-detected breast cancer	• Though promising, blood-based tests for early stage cancer detection still lack accuracy • This clinical trial could form the basis for future clinico-genomic trials	Liu et al[65]
1005 (and 812 controls)	Samples from 8 resectable cancer types	tNGS and protein biomarkers	• The study utilized a panel on 16 genes and the levels of 8 circulating proteins • CancerSEEK test sensitivity and specificity were 70% and >99% respectively for 8 cancer types • Tumor localization was feasible in 83% of pts	The novel CancerSEEK test, following validation, could be used for noninvasive early diagnosis	Cohen et al[66]
164	Metastatic TNBC	CNV analysis	• CNV profiles were highly similar between matched PT and MT • Tumor fraction in cfDNA >10% correlated to poor survival	CNVs could be used as prognostic biomarkers	Stover et al[67]
128	Early onset familial BC	WGS	Novel deletions in susceptibility genes (*BRCA1*, *TP53*, *PALB2*, *PTEN*, and *RAD51C*) were identified	Identification of deletions could have diagnostic implications within blood-based tests	Guo et al[68]

(Continues)

TABLE 3 (Continued)

Number of patients/samples	Sample type/ molecular subclass	Technology	Findings	Clinical implications	References
Serial cfDNA-NGS					
30/141	Metastatic BC	tNGS on 44 samples from 11 pts	Circulating tumor DNA provided the earliest measure of therapeutic response	This study provided proof-of-concept for the potential use of serial liquid biopsies as a monitoring tool during treatment	Dawson et al[69]
20/93	14 metastatic and 6 non-metastatic BCs	WGS	• Serial ctDNA-NGS was highly accurate for postsurgical discrimination between patients with (93%) and without (100%) clinical recurrence • Detection by ctDNA-NGS preceded clinical diagnosis of metastasis in 86% of pts by an average of 11 mo • Levels of ctDNA were a significant predictor of poor DFS and OS	Monitoring by ctDNA-NGS in the adjuvant setting can accurately detect occult metastasis, months before clinical diagnosis	Olsson et al[70]
18/52	HER2-positive metastatic BC under HER1/2 TKI	tNGS	• This is a registered clinical trial (NCT01937689) demonstrating tumor clonal evolution • Primary therapeutic resistance was identified in 6/14 pts with progressive disease, secondary resistance in 8/14, *HER2* amplifications were found in 4/6 and 7/8, respectively • *TP53* and PI3K/mTOR pathway mutations were additional markers of resistance • Molecular identification of therapeutic resistance correlated to shorter PFS	Dynamic *HER2* CNV detection in cfDNA could guide secondary decision-making on targeted therapy, following validation	Ma et al[71]

(Continues)

TABLE 3 (Continued)

Number of patients/samples	Sample type/ molecular subclass	Technology	Findings	Clinical implications	References
15	Locally advanced BC	tNGS	• At diagnosis, the amount of plasma SNVs did not correlate with clinical stage • In 2 pts, ctDNA disappeared after the 1st NAC cycle achieved a pCR • The amount of ctDNA correlated with residual tumor volume	Serial ctDNA-NGS could monitor drug response and predict therapeutic resistance, but validation is required	Kim et al[72]
11	Metastatic BC	tNGS	4/11 pts showed cfDNA changes over time, of which two acquired an *ESR1* mutation	Identification of ESR1 mutations could predict secondary resistance to endocrine therapy	Guttery et al[73]
10	ER-positive BC	tNGS on PT and serial cfDNA before and during tamoxifen therapy and at progression	• Among 6 pts with disease progression, 3 and 9 variants were identified only in blood and PT specimens respectively • Specific missense mutations were identified both in cfDNA at progression and in the PT (*PIK3CA*, *TP53*, *SMAD4*, and *CREBBP*)	• *CREBBP* and *SMAD4* could be associated with resistance to endocrine therapy • Further validations studies are required to explore the prognostic and predictive significance of cfDNA-tNGS	Jansen et al[74]
6 (2 breast)	Samples at various time points	WES	• Emergence of acquired therapeutic resistance was associated with increased representation of mutant alleles • Specific mutated genes were associated with specific therapies	If validated, serial cfDNA-WES could be used as a predictive biomarker	Murtaza et al[13]

Abbreviations: BC, breast cancer; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CNV, copy number variation; DFS, disease-free survival; ER, estrogen receptor; LM, liver metastasis; MR, multiregional; MT, metastatic tumor; mTOR, mechanistic target of rapamycin; NAT, neoadjuvant treatment; NAC, neoadjuvant chemotherapy; NGS, next-generation sequencing;
OS, overall survival; pCR, pathologic complete response; PFS, progression-free survival; PI3K, phosphoinositide 3-kinase; PT, primary tumor; pts, patients; SNV, single-nucleotide variation; TKI, tyrosine kinase inhibitor; tNGS, targeted NGS; TNBC, triple-negative breast cancer; WES, whole-exome sequencing; WGS, whole-genome sequencing; WGBS, whole-genome bisulfite sequencing

inhibitors.[47,50] Moreover, genome analysis has uncovered novel biomarkers predicting therapeutic response to aromatase inhibition, namely GATA3 mutations[51] and activation of E2F4-regulated genes,[45] although the predictive power of these tools remains to be validated. Additionally, recent reports imply that NGS could be used for the stratification of patients based on specific prognostic biomarkers.[47,53]

3.2 | Genome exploration in time and space

Considering the dynamic evolution of genomic clones by the Darwinian principles[4] and temporal emergence of ITH[5] as major causes of intrinsic and acquired therapeutic resistance,[6] integrated NGS systems have been developed to explore spatiotemporal tumor evolution. Thus, a shift from static single-biopsy to multiple tumoral and liquid biopsy analysis of genome evolution in time and space is essential to overcome therapeutic resistance. Furthermore, detection of cGSs, which have escaped from the primary tumor and into the circulation, provides valuable data for early diagnosis, understanding and predicting tumor recurrence, primary and secondary decision-making, as well as patient monitoring.[13,66] Table 3[5,13,58–74] provides an overview of published data from breakthrough NGS studies, including reports on ITH and cGS identification.

3.2.1 | Intratumor heterogeneity

Exploration of static and dynamic ITH could provide crucial clinical implications in the field of prognostic and predictive biomarkers to guide more effective personalized systemic therapy.[6] Yates et al,[5] in a very influential MR-NGS study, have reported dynamic clonal evolution in response to NAT with a significant proportion of patients harboring subclonal targetable alterations. The emergence of resistant subclones after NAT bares great clinical significance for post-NAT therapeutic decision-making on available or new to be developed targeted agents against the identified resistant oncotargets. Moreover, single-cell NGS analysis further supports dynamic diversification of genomic clones during the course of breast cancer,[62–64] with more detailed analysis suggesting that point mutations are responsible for spatiotemporal clonal evolution and ITH.[64] In contrast, large genome changes, namely chromosomal rearrangements and copy number alterations, appear to pre-exist within the primary tumor and remain stable over the disease course.[61,64] These findings are consistent with the data provided by Tang et al through MR-NGS, similarly to other cancer types.[58,75] These results require confirmation by appropriately designed large-scale genomic trials.

3.2.2 | Circulating genomic subclones

The breakthrough concept of cGS detection through cfDNA-NGS has recently been extensively investigated as a powerful platform to develop noninvasive blood tests to complement and further enhance current screening and diagnostic strategies. Cohen et al[66] developed a multianalyte blood test based on plasma genetic and protein biomarkers named CancerSEEK, which promises the detection of eight surgically resectable cancer types with sensitivity and specificity of 70% and greater than 99%, respectively. However, sensitivity in the diagnosis of early breast cancer was only 33%, rendering it ineffective as a diagnostic tool in its current form for breast cancer.[66] Similarly low sensitivity rates for breast cancer have also been reported by the first large-scale clinical trial evaluating cfDNA-NGS as a diagnostic biomarker (NCT02889978), in a preliminary substudy with 810 participants.[65] Ongoing and new clinical trials, in conjunction with basic research progress in this field, could elucidate on the low sensitivity rates for breast as compared with other cancer types. Other smaller studies have also investigated the diagnostic[68] and prognostic[67] significance of cGSs, with findings requiring validation through large and appropriately designed studies.

Besides potential diagnostic utility, a series of innovative small-scale studies have investigated liquid biopsies at consecutive time points as a monitoring tool to predict secondary therapeutic resistance and recurrence before

clinical-imaging diagnosis. Since the breakthrough study of Murtaza et al[13] which introduced the concept of serial liquid biopsies by cfDNA-NGS and highlighted its putative predictive capacity, multiple independent reports have demonstrated both the prognostic,[70,71,74] as well as the predictive[13,69–74] capabilities of temporal noninvasive NGS. Higher levels of circulating tumor DNA (ctDNA) were once again associated with poor disease-free and overall survival,[70] while ctDNA was nondetectable in patients with pCR to neoadjuvant chemotherapy.[72] Specific mutations were markers of primary and secondary therapeutic resistance and short progression-free survival, including *HER2* amplifications, TP53, and phosphoinositide 3-kinase (PI3K)/mechanistic target of rapamycin (mTOR) pathway mutations.[71]

Additionally, all studies underlined two major key points. The first is the potential capacity of serial cfDNA/ctDNA-NGS as a predictive biomarker. Liquid biopsy-based patient monitoring has demonstrated highly promising results on the prediction of resistance to systemic therapy, including endocrine and targeted treatment, proposing markers of resistance, such as *HER2*, *TP53*, PI3K/mTOR, *CREBBP*, and *SMAD4* mutations.[71,73,74] Two studies have suggested that molecular detection of therapeutic resistance could precede clinical diagnosis of disease relapse,[69,70] with Olsson et al[70] reporting identification of metastasis at a median of 11 months before clinical diagnosis. Furthermore, a registered clinical trial by Ma et al (NCT01937689)[71] on metastatic HER2-positive breast cancer underlined the potential for patient monitoring by ctDNA copy-number analysis, guiding secondary decision-making on therapy. The second is the universal support of the Darwinian model in the evolution of breast cancer.[13,69–74] Spatiotemporal evolution of genomic clones due to selective pressure, such as systemic therapy, more convincingly explains the clinical course of breast cancer, characterized by excellent short-term and medium-term oncological outcomes and late emergence of therapeutic resistance and recurrence, in contrast to the theory of pre-existence of a minor aggressive cell subpopulation within the primary tumor, which could apply to the TNBC subtype[61] or other more aggressive tumors.[77] Nevertheless, studies implementing dynamic liquid biopsies are still scarce, small-scale, and lack a strict clinically-centered protocol, warranting further future investigation.

4 | FUNCTIONAL, NONCODING MUTATIONAL LANDSCAPE, AND REGULATORY NETWORKS

4.1 | Noncoding regulatory mutations in breast cancer

Most cancer-associated mutations have already been localized in noncoding parts of the genome by genome-wide association studies.[78] Consequently, noncoding functional mutations have gained significant research spotlight due to their impact on gene regulation and expression and potential subsequent clinical relevance (Table 2).[14,41–55] Noncoding alterations have been recurrently identified in breast cancer within regulatory regions of cancer-related genes, including promoters and enhancers,[14,52,56] possibly at similar frequencies as coding mutations.[14] Noncoding variants of two genes (*CRTC3* and *STAG2*) were identified as prognostic factors in a specific subset of breast cancer patients (ER-positive/HER2-negative),[52] while Rheinbay et al[14] highlighted mutations affecting *FOXA1* expression, coding and noncoding, as potential markers of therapeutic resistance and disease progression. Taken together, these results open new exciting doors towards understanding the intricacy with which regulatory networks control gene expression.

4.2 | Transcriptional network interactions and genome editing

The exploration of complex interactions between regulatory elements within transcriptional networks has posed a great research challenge. New, enhanced high-throughput methods, such as Hi-C and ChIA-PET, have been developed, improving upon the chromosome conformation capture (3C) technique, which can reveal the physical interactions between enhancers and promoters, beyond functional correlations probed by genomic studies.[9,79] Hi-C studies have reported the spatial organization of the genome into topologically associated domains.[80] Normally,

promoter-enhancer interactions take place within these domains but not between them.[80] However, disruption of domain boundaries and long-range interplay between distant elements has been associated with disease,[81] including breast[79] and other cancers.[82] ChIA-PET can construct chromatin interaction maps with even greater detail, further facilitating the match of TF-binding sites to the respective target genes.[83] Interestingly, these studies suggest that cell-type specificity is not limited to gene expression, but also regulatory element interactions, leading to cell-specific transcriptional activation.[83] Nevertheless, chromatin interaction assays only characterize the spatial architecture of the genome, not distinguishing functional from nonfunctional relations.

Recently, genome editing tools, especially transcription activator-like effectors and the highly versatile CRISPR/Cas9, have provided unprecedented potential in the exploration of noncoding genome functionality, with their ability to accurately alter single nucleotides in the genome and observe the phenotypic results through reverse genetics.[9] These systems are utilized by basic research to determine the functional impact of specific mutations in disease-associated loci through the identification of functionally relevant genetic variations in several diseases, including neurodevelopmental disorders[84] and cancer.[85] Genome editing screens target both protein-coding genes and noncoding elements with the latter being distinguished in two major categories based on design. The first focus on regions proximal to specific genes under investigation,[86] including regulatory elements and their interplay, such as promoter-promoter interactions,[87] aiming to correlate regulatory mutations with phenotypic events such as therapeutic resistance and progression, as demonstrated by Sanjana et al[88] for melanoma. The second target selected TF-binding sites, such as enhancers, regulating cancer-related genes, as for instance *TP53*.[89] Moreover, these tools allow for selective perturbation of the activity of targeted regulatory elements through epigenome editing,[90] providing an additional means to investigate gene regulation and transcriptional networks.

Thus, genome editing systems are a powerful platform for the identification of novel therapeutic targets by uncovering genetic vulnerabilities in genes essential for tumor cell viability, metastasis, and drug resistance.[91] In this regard, Hart et al[92] recently generated an extended list of over 1500 such essential genes in cancer cell-lines, five times more than previously reported. Furthermore, highly innovative studies have lately reported the use of CRISPR-based screening and single-cell RNA-NGS in conjunction, demonstrating high precision in the correlation of genes to biological processes and promising accurate and efficient dissection of complex cellular responses.[93] However, CRISPR-based analyses are limited by the small number of targets and expansion to the whole-genome level is required before the completion of the regulatory element catalog becomes a realistic goal.

During the past decade and following the completion of the human genome sequence, tremendous effort has been concentrated towards understanding how mutations within regulatory networks affect network interactions and promote pathogenesis, aiming to translate the vast amount of data generated by genomic studies into clinical benefit.[94] However, experimental, as well as computational network reconstruction has been hindered mainly by the complexity of genotype-to-phenotype relationships between diseases and their associated genes.[94] Based on the eight established hallmarks of cancer[95] and exploiting genome sequencing data, a cancer hallmark network framework has been proposed to predict complex phenotypic events, such as tumorigenesis, relapse, and metastasis.[96,97] Although the temporality of cellular networks presents as a fundamental advantage putatively enabling network manipulation,[8] traditional statistical tools are unsuitable to reliably characterize the intricate intra- and intercellular network comprising the interactome.[98] Thus, network reconstruction requires the design of enhanced computational algorithms implementing interaction matrix and temporal data,[99] with both medical and pharmaceutical interest towards next-generation biomarker and drug development.[94,98]

5 | FUTURE OUTLOOK

Over the past decade, rapid progress in single-gene linear transcription-based drug development has been successfully integrated into clinical practice improving oncological outcomes of breast cancer patients. However,

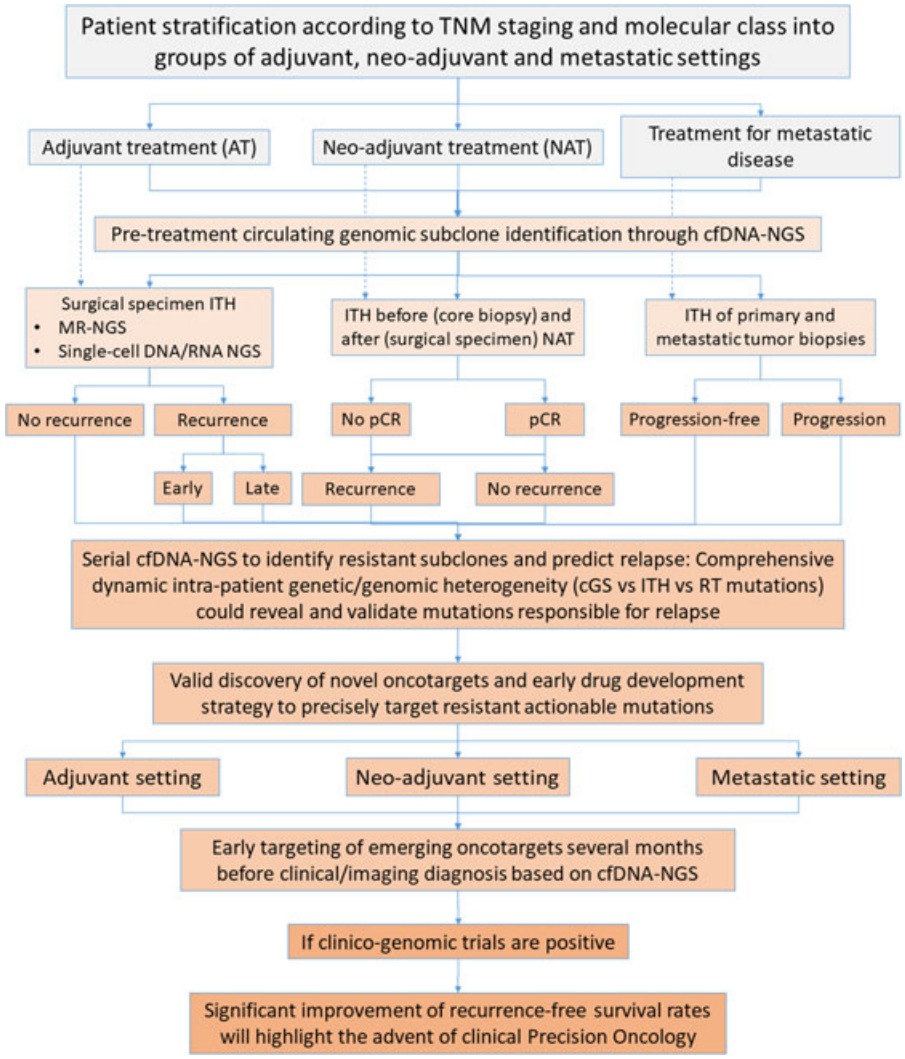

FIGURE 2 Patient-centric genomic trials in a step-wise strategy to achieve precise prediction-based individualized breast cancer therapy. Potential establishment of ITH, cGSs, and IPGH as robust predictive biomarkers, as well as oncotarget-based drug development, could enable precise prediction-based individualized therapy. Spatiotemporal emergence of resistant genomic subclones could be detected by pre and posttreatment patient monitoring through serial liquid biopsies, considering ITH and RT alterations, to predict and promptly target resistant mutations before clinical relapse, therefore improving oncological outcomes. cfDNA, cell-free DNA; cGSc, circulating genomic subclones; IPGH, intrapatient genetic/genomic heterogeneity; ITH, intratumor heterogeneity; MR, multiregional; NGS, next-generation sequencing; pCR, pathologic complete response; RT, relapsed tumor [Color figure can be viewed at wileyonlinelibrary.com]

personalized prediction of acquired therapeutic resistance and late relapse remains challenging. Integration of genome analysis, including NGS systems, Hi-C, and CRISPR/Cas9, into basic and translational research, coupled with computational strategies, has provided exciting results on the spatiotemporal characterization of structural and functional cancer genome and transcriptome elements. Breast cancer genome analysis has achieved several goals, including at least a dozen novel cancer genes and oncotargets for future drug development. Promising data on early noninvasive diagnosis has been reported.[65,66] Moreover, exciting but still inconclusive data have emerged regarding the spatiotemporal diversification of genomic clones with the dynamic emergence of tumor

heterogeneity.[4–6,12,70,100,101] During the next decade, the roadmap for overcoming resistance and relapse includes two major goals, patient-centric genomic trials and understanding how noncoding genome functionality affects regulatory networks and gene expression.

5.1 | Clinico-genomic trials

Despite high sensitivity in the early diagnosis of some cancer types excluding breast cancer,[65,66] much more sophisticated trial designs on spatiotemporal exploration of tumor evolution are essential to accurately predict intrinsic and particularly acquired therapeutic resistance.[75] A step-wise process includes patient stratification into groups of adjuvant or NAT and metastatic setting according to recent guidelines for diagnosis, molecular classification, and personalized treatment. The innovative strict protocol, abiding by clinical and genomic recommendations, could reveal the clinical implications of IPGH and, for the first time, provide evidence on resistant cGSs to predict primary and acquired therapeutic resistance. Should these studies provide positive results, ITH, cGSs, and IPGH could be validated as prognostic and predictive biomarkers to accurately predict therapeutic resistance and relapse. Serial cGS detection, considering ITH of the primary tumor and IPGH validating circulating "resistant" subclones responsible for relapse, could not only predict recurrence several months before clinical diagnosis but also prolong time to relapse through early precise targeting of circulating druggable genomic alterations (Figure 2).

5.2 | Potential controllability of transcriptional networks

The promising findings on functional noncoding mutations in promoters and enhancers targeting cancer-related genes,[14,52,56] derived from WES/WGS and RNAseq studies, coupled with dropping costs, will allow for the completion of a breast cancer-specific catalog of both functional noncoding TF-binding site mutations and TFs. Considering noncoding genome functionality and transcriptional networks controlling gene expression in the healthy human genome,[7,39] the exploration of temporal perturbated regulatory networks in disease is imperative. Nevertheless, one of the greatest future challenges is the delineation of molecular mechanisms and principles orchestrating the perturbation of regulatory networks. To achieve this goal, further technological refinements are required, including the integration of interaction mapping and genome editing tools, as well as computational systems and network reconstruction models, into innovative studies exploring genome, and interactome mapping in time and space. On this basis, a translational framework is shaped, aiming to pharmaceutically control intricate dysregulated transcriptional biocircuits by next-generation drugs disrupting nonlinear networks. However, lack of financial support from the private sector due to nondirect profit represents a major hurdle in speeding up the advent of nonlinear transcription-based drug development.[10]

6 | CONCLUSIONS

Recent evidence on extensive genomic and nonlinear transcriptional heterogeneity, due to spatiotemporal clonal evolution rather than minor pre-existing genomic clones drives two major research directions. The first medium-term evidence-based strategy is centered on the conduction of patient-centric genomic trials to establish dynamic ITH, cGSs, and IPGH as biomarkers for the individualization of therapy. Both single and multiple biopsy-based trials could discover novel valid oncotargets guiding an early drug development strategy. Prediction of drug response encourages optimized targeted agent combinations from a future comprehensive drug bank. However, the ultimate optimization of precise therapy will require a two-step approach. The first step is the completion of a comprehensive catalog of all breast cancer-specific structural and functional noncoding mutations. The second and much more complex is the discovery of molecular mechanisms and

principles driving the perturbation of regulatory networks, opening the new horizon of pharmaceutical controllability of temporal transcriptional networks. These advancements will realize the shift from inexact medicine to precision life science, including accurate individualized prediction-based therapy.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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