TP53 mutations are associated with primary endocrine resistance in luminal early breast cancer

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Abstract
Background: Whereas the genomic landscape of endocrine-resistant breast cancer has been intensely characterized in previously treated cases with local or distant recurrence, comparably little is known about genomic alterations conveying primary non-responsiveness to endocrine treatment in luminal early breast cancer.

Methods: In this study, 622 estrogen receptor-expressing breast cancer cases treated with short-term preoperative endocrine therapy (pET) from the WSG-ADAPT trial (NCT01779206) were analyzed for genetic alterations associated with impaired endocrine proliferative response (EPR) to 3-week pET with
INTRODUCTION

About 75% of breast cancers (BCs) belong to the luminal type with estrogen-dependent tumor cell growth. Hormonal blockade with tamoxifen or aromatase inhibitors (AI) therefore provides an effective growth suppressive therapy for the majority of BCs. However, in a considerable proportion of luminal BC, that is, about 20%–30%, local or distant recurrence during or after endocrine treatment indicates resistance to endocrine therapy.1

Two general patterns of endocrine therapy resistance are recognized clinically: primary, intrinsic resistance, whereby estrogen receptor-expressing (ER-positive) cancers never adequately respond to endocrine treatment, and secondary, acquired resistance, which develops following an initial response. A major mechanism of acquired secondary resistance is provided by activating mutations in the ERα gene (ESR1).3 Over 30% of long-term treated luminal BC display ESR1 mutations. ESR1 mutations are enriched in metastatic BC and with significant differences in metastatic site.4,5 In bone metastasis, 14% of ER-positive cases were found to be ESR1-mutated.6 Mechanisms of intrinsic resistance may partly overlap but are currently far from being understood.

Efforts have been undertaken to identify the genetic characteristics of endocrine-resistant BC.1 Razavi et al. analyzed 1501 luminal BC cases with resistance to endocrine therapy by next-generation sequencing. The majority of cases in this study (87.5%) had been exposed to prior therapy in the adjuvant and/or metastatic setting.7 The number of treatment-naïve cases was too small to allow a sufficiently powered analysis of primary genetic alterations. Genetic alterations occurring in more than 5% of metastases in this study included mutations of ARID1A, ESR1, ERBB2, and TP53.7

TP53 gene mutation is frequent in the triple-negative BC with up to 80% of cases showing this mutation.7,8 In luminal BC, TP53 mutation is encountered in about 12%–29% of cases.8 There appears to be an association with the luminal B phenotype.9-11 The majority of TP53 mutations are somatic because selection for familial BC cases yielded lower proportions of TP53-mutated cases indicating that germ-line mutations (Li-Fraumeni syndrome) are considerably rarer than somatic changes.12 Among different somatic mutations, TP53 alterations were most frequent in metastatic luminal BC (29%).13 Whether TP53 mutations evolve in the context of clonal evolution in recurring BC or are acquired during progression is not clear.14 In therapy-naïve primary metastatic BC, TP53 mutation was found in primary tumors as well as in metastatic deposits.15 Previous studies on the role of TP53 in BC treatment response and survival have been summarized as contradictory and inconclusive.16-18

Neoadjuvant therapy of BC has generated new endpoints to evaluate therapy efficacy. In triple-negative and HER2-positive early BC, complete pathological remission after neoadjuvant therapy is commonly used as a surrogate marker for therapy responsiveness and favorable results.
prognosis. In 70%–80% of BCs, this surrogate marker is not readily available because ER-positive, luminal BCs will usually not regress completely when exposed even to long-term preoperative endocrine therapy. Endocrine responsiveness, however, is indicated very precisely by a decrease in tumor cell proliferation, even after a short-term exposure of only 2–3 weeks of endocrine therapy. The proliferation response in vivo can be assessed by the proliferation marker Ki67 and has been shown to have clinical relevance with regard to outcome under endocrine therapy. Effective endocrine therapy, either by hormone depletion or by receptor blockade, leads to growth arrest of tumor cells evidenced by a decrease in Ki67 labeling index. The Ki67 nuclear protein is expressed by cycling cells from G1 to M phase and provides a commonly used immunohistochemical method to assess the growth fraction in BC. A sustained high Ki67 index despite hormonal blockade is thought to identify ER-independent tumor cell proliferation.

In this study, luminal early BCs from the prospective WSG-ADAPT trial which did not respond to short-term preoperative endocrine therapy (pET) were analyzed for alterations of genes which had recently been implicated in endocrine resistance in metastatic BC including ARID1A, CCND1, ERBB2, ESR1, FGFR1, PAK1, PIK3CA, and TP53.

## 2 MATERIALS AND METHODS

### 2.1 Cases and tumor tissue

Pre- and postmenopausal patients with ER- and/or PR-positive, HER2-negative early BC as determined by local pathologic assessment, were treated with short-term pET in the West German Study Group (WSG) ADAPT trial (NCT01779206). The details on study design were published previously. pET was applied for 3 weeks. Premenopausal women were mostly treated by tamoxifen and the majority (>90%) of postmenopausal women were treated by aromatase inhibitors (letrozole, anastrozole, or exemestane). All cases were subjected to central pathology review (MHH). Formalin-fixed, paraffin-embedded (FFPE) tumor tissue from the diagnostic core needle biopsies at baseline was submitted for recurrence score (RS) testing at the laboratory of Genomic Health Inc. The case characteristics are shown in Table 1.

Tumor specimens correspond to $n = 301$ unselected consecutive BC cases from the run-in phase of the WSG-ADAPT trial. To increase the number of specimens, we also included $n = 400$ consecutive cases from the main phase of the WSG-ADAPT trial. A total of $n = 79$ cases were excluded due to (i) missing Ki67 at baseline or post-pET, (ii) unavailable tissue blocks (returned to local

| TABLE 1 Characteristics of the study cohort |
|---------------------------------------------|
| **All cases** | **pET** | **TAM cohort** | **AI cohort** |
| **n = 622** | **n = 286** | **n = 334** |
| Age at diagnosis | Median (range) in years | 54 (28–76) | 47 (28–68) | 62 (43–76) |
| pT stage | | | |
| pT1 | 371 (59.6) | 166 (58.0) | 204 (61.1) |
| pT2 | 223 (35.9) | 109 (38.1) | 113 (33.8) |
| pT3 | 24 (3.9) | 9 (3.1) | 15 (4.5) |
| pT4 | 2 (0.3) | 0 (0.0) | 2 (0.6) |
| n.a. | 2 (0.3) | 2 (0.7) | 0 (0.0) |
| pN stage | | | |
| pN0 | 541 (87.0) | 245 (85.7) | 296 (88.6) |
| pN1+ | 79 (12.7) | 39 (13.6) | 38 (11.4) |
| n.a. | 2 (0.3) | 2 (0.7) | 0 (0.0) |
| Histological grade, baseline | | | |
| G1 | 46 (7.4) | 26 (9.1) | 20 (6.0) |
| G2 | 399 (64.1) | 180 (62.9) | 218 (65.3) |
| G3 | 177 (28.5) | 80 (28.0) | 96 (28.7) |
| pET | | | |
| Tamoxifen | 286 (46.0) | 286 (100.0) | 0 (0.0) |
| Aromatase inhibitors | 334 (53.7) | 0 (0.0) | 334 (100.0) |
| n.a. | 2 (0.3) | 0 (0.0) | 0 (0.0) |
| ER status, baseline | | | |
| Negative | 1 (0.2) | 0 (0.0) | 1 (0.3) |
| Low expression | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Positive | 620 (99.6) | 286 (100.0) | 332 (99.7) |
| n.a. | 1 (0.2) | 0 (0.0) | 1 (0.3) |
| ER status, post-pET | | | |
| Negative | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Low expression | 1 (0.2) | 0 (0.0) | 1 (0.3) |
| Positive | 620 (99.6) | 285 (99.6) | 333 (99.7) |
| n.a. | 1 (0.2) | 1 (0.4) | 0 (0.0) |
| PR status, baseline | | | |
| Negative | 46 (7.4) | 11 (3.8) | 35 (10.5) |
| Low expression | 27 (4.3) | 10 (3.5) | 17 (5.1) |
| Positive | 549 (88.3) | 265 (92.7) | 282 (84.4) |
| n.a. | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| PR status, post-pET | | | |
| Negative | 137 (22.0) | 14 (4.9) | 123 (36.8) |
| Low expression | 65 (10.5) | 13 (4.5) | 51 (15.3) |
| Positive | 420 (67.5) | 259 (90.6) | 160 (47.9) |

(Continues)
centers upon clinical request), (iii) divergent histological subtype at baseline and post-pET, (iv) triple-negative hormone receptor status, and (v) insufficient DNA amount and/or quality. The total number of specimens available for statistical analysis was $n = 622$ (Table 1).

### 2.2 Immunohistochemistry and fluorescence in situ hybridization

Immunohistochemistry (IHC) for estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 was performed in the central reference pathology unit of the ADAPT trial (in the years 2012–2016; prospective assessment) using a Benchmark Ultra automated stainer (Ventana). Immunological reagents and central IHC scoring methods are summarized in the (Table S1).

Ki67 index assessment was supported by digital quantification as follows: First two experienced pathologists independently scored the Ki67 index by eyeballing in a minimum of 500 tumor cells (semiquantitative assessment, 5% increment steps). Next, Ki67-positive tumor cell nuclei were quantified using the digital pathology platform iScan Coreo (Ventana) and Virtuoso quantification software (v5.3, Ventana) as described previously.25 Next, a consensus Ki67 index was defined based on the three evaluations (2x semiquantitative 1x Virtuoso). In most cases, the semiquantitative Ki67 index that was nearest to the digital Ki67 index was accepted as the definite consensus Ki67 index.

Endocrine proliferative response (EPR) was determined by the post-pET consensus Ki67 index. The EPR was categorized in four categories corresponding to optimal (post-pET Ki67 <10%) versus slightly, moderately, and severely impaired proliferative response (post-pET Ki67 10%–19%, 20%–34%, and ≥35%, respectively) (Figure S1). These provisional cutoffs were chosen only for the present exploratory molecular analysis and aimed for an utmost stringent definition of an optimal EPR.

HER2 was scored as 0, 1+, 2+, or 3+, in accordance with the Dako HercepTest. BCs with a HER2 score of 2+ or 3+, were subjected to HER2 fluorescence in situ hybridization (FISH). FISH categorization was in accordance with the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2018 guidelines.29

### 2.3 DNA extraction

Depending on tumor size 6–8 sections (8 μm) were taken. Genomic DNA was extracted from FFPE specimens with the Maxwell® RSC DNA FFPE Kit on a Maxwell® RSC instrument (Promega) according to the manufacturer’s recommendations. DNA concentration was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the dsDNA high sensitivity kit (Thermo Fisher Scientific).

### 2.4 Mutational analysis

Within this study, we analyzed candidate endocrine resistance genes (ARID1A, BRAF, ERBB2, ESR1, HRAS, KRAS, NRAS, and TP53) that were identified by Razavi et al. by comparing alterations in ER-positive metastatic BCs with primary tumors.7 In addition, PIK3CA mutation status was determined because of its potential as a target for selective inhibition.30
potential marker for sensitivity toward aromatase inhibitors, was also included.10

Mutational analysis of matched resection specimen (post-pET) from all cases was carried out retrospectively by next-generation sequencing (NGS) in the years 2018–2020. Targeted sequencing was performed using two customized amplicon-based panels. Library preparation was performed with Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher Scientific). For quantification of prepared libraries, the Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) was used. Sequencing was performed on an Ion S5 instrument (Thermo Fisher Scientific). The first panel covered the complete protein-coding sequence (as well as 10 base pairs of flanking intron sequence to cover the splice sites) of ERBB2, ESR1, PIK3CA, and TP53. Mean mapped reads per case was 141,732 (range 5,718–2,910,098) and mean depths per base was 9460 (range 55–191,139). The second panel covered the complete protein-coding sequence of ARID1A, BRAF, GATA3, HRAS, KRAS, and NRAS. Mean mapped reads per case was 282,100 (range 33,486–7,149,110) and mean depths per base was 1,213 (range 163–29,306) (Table S2).

Evaluation of sequencing data and variant annotation was performed with the ANNOVAR software and database tools (http://annovar.openbioinformatics.org/en/latest/).31 Variants with unknown significance were predicted as deleterious, when they were considered as pathogenic according to Molina-Vila et al. (classification as disruptive vs. non-disruptive).32–35

Altering in the TP53 gene can have various consequences for the functionality of the mutant p53 protein. Functional classification of TP53 variants was performed according to two different nomenclatures. These included TP53 variant classification according the International Agency for Research on Cancer (IARC, classification as [partially] functional vs. non-functional) and the classification according to Molina-Vila et al. (classification as disruptive vs. non-disruptive).32–35

2.5 DNA copy number analysis

DNA copy number analysis of CCND1, FGFR1, and PAK1 was performed using digital PCR (dPCR) in all tissue specimens with a tumor cell amount >50% (n = 199).

TaqMan™ Copy Number Assays (CCND1 assay ID: Hs02559587_cn; FGFR1 assay ID: Hs05052584_cn and PAK1 assay ID: Hs01931361_cn; Thermo Fisher Scientific) were used with 15.0 ng of FFPE DNA input. If less than 15.0 ng of DNA was present, the maximum input volume of 7.13 µl was used. The range of DNA input was 2.21–15.0 ng. Two reference gene assays were used for each target assay to determine the found amplifications. For detection of CCND1 and PAK1 amplifications RPPH1 (catalog no. 4403326; Thermo Fisher Scientific) and PMP22 (customized; Thermo Fisher Scientific) were used as reference assays. The reference assays for FGFR1 amplifications were TERT (catalog no. 4403315; Thermo Fisher Scientific) and RPPH1.

All reactions were performed with QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific), 3D PCR Master Mix v2 (Thermo Fisher Scientific), and 3D PCR 20K Chip Kit v2 (Thermo Fisher Scientific). PCRs were performed on a flat block thermocycler. The reaction conditions were as follows: hot start at 96°C for 10 min, annealing at 56°C for 2 min, and denaturation at 98°C for 30 s for a total of 39 cycles, followed by a final extension step at 60°C for 2 min. Data were analyzed with QuantStudio™ 3D AnalysisSuite™ Software (Version 3.1.6; Thermo Fisher Scientific). The threshold for amplification was a ratio of target-gene calls to reference-gene calls of 2.2. Within this study, only those cases that showed amplification with both reference assays are considered to be amplified (Table S2).

2.6 Statistics

For statistical evaluation of the association between genetic alterations and pathologic parameters, we focused on candidate genes with a mutation frequency of ≥2.5%. The two-sided Fisher’s exact test and the Chi-squared test for trends were used for contingency analysis. The results were considered to be statistically significant if p ≤ 0.05.

Multivariate logistic regression was used to analyze the independent ability of TP53 status (mut vs. wt and classification according to IARC, respectively), pET (AI vs. tamoxifen), pT stage (pT2 vs. pT1 and pT3/4 vs. pT1), pN stage (pN1+ vs. pN0), histological grade (G3 vs. G1/2), Oncotype DX RS (26–100 vs. 0–25), baseline Ki67 (10%–19% vs. <10%, 20%–34% vs. <10%, and ≥35% vs. <10%), baseline ER status (% expression, continuous variable), and baseline PR status (% expression, continuous variable) to predict impaired EPR (post-pET Ki67 ≥10%). Statistical analysis was performed with GraphPad Prism software Version 5.00 (GraphPad Software) and Stata/IC Volume 16.1. (Stata Corp).

3 RESULTS

3.1 Case characteristics

We performed targeted sequencing of 622 HR+/HER2-negative early BCs from patients, enrolled in the WSG-ADAPT trial (NCT01779206). Tumor specimens corresponded to
3.2 Type and frequency of genetic alterations

Genetic alterations in candidate endocrine resistance genes (ARID1A, BRAF, ERBB2, ESR1, HRAS, KRAS, NRAS, and TP53) were determined by next-generation sequencing in resection specimens following short-term pET.7 PIK3CA was included because the therapeutic potential of PI3K inhibitors in PIK3CA-mutated BCs.30 The GATA3 mutation status, a potential marker for sensitivity toward AIs, was also included.10 To evaluate gene amplification as a potential mechanism of resistance toward endocrine therapy, copy number analysis by digital PCR (dPCR) was performed for CCND1, FGFR1, and PAK1 in a subgroup of cases (n = 199).7 Besides this, overexpression and/or amplification of ERBB2 were determined conventionally by immunohistochemistry and FISH in the context of the central pathology review.

Somatic mutations or gene amplification were present in 66.2% (n = 412). A subset of these cases revealed more than one genetic alteration under study (18.3%, n = 114). Figure 1 shows an overview of the observed genetic alterations. Mutations of ARID1A were found in 5.3% of cases. Other somatic mutations with rates >5% of cases were GATA3 (16.6%), PIK3CA (43.3%), and TP53 (9.0%). Mutations of BRAF, ERBB2, ESR1, HRAS, KRAS, and NRAS occurred less frequently. Some genes like PIK3CA were mainly affected by missense mutations, whereas in other genes like ARID1A or GATA3 truncating mutations were more frequent. In a total of 36 tumors (18.1%) CCND1, FGFR1, and/or PAK1 amplifications were observed (Figure 1). Amplifications of CCND1 (n = 32), FGFR1 (n = 6), and PAK1 (n = 5) were detected by dPCR in 16.1%, 3.0%, and 2.5% of cases under study, respectively (Figure 1). All tumors with PAK1 amplification harbored a co-amplification with CCND1. In addition, central pathology review revealed a positive HER2/ERBB2 status (IHC 2+/FISH-positive or IHC 3+/FISH-positive) in a small subset of tumors (n = 8), which had been classified as HER2-negative by local assessment (data not shown). These cases are termed ERBB2-amplified herein. For subsequent statistical analysis, we focused on genes, which were altered in ≥2.5% of all cases and also included the ERBB2-amplified tumors.

3.3 Association of genetic alterations with Oncotype DX RS

For comparing the observed genetic alterations with the Oncotype DX RS, cases were grouped into three categories. In total, 22.8% (n = 142) were in the group with low
RS (RS 0–11). The majority (58.2%, n = 362) had intermediate RS (RS 12–25) and 16.2% (n = 101) were categorized as high-risk RS (RS ≥26). Figure 2A shows that TP53 mutations were significantly more frequent in cases with high-risk RS (15.8%, n = 16), than in cases with low RS 0–11 (5.6%, n = 8) (p = 0.0083). Nevertheless, in absolute numbers, the majority of TP53 mutations were in the low and intermediate RS groups (Table S3). In contrast, PIK3CA mutations occurred significantly more frequent in cases with low RS (45.8%, n = 65) and were rarer in cases with high RS (23.8%, n = 24) (p = 0.0027). Mutations of ARID1A, ERBB2, and GATA3 were equally distributed among RS groups (Figure 2A).

Amplification of FGFR1 (0 to 9.5%) and ERBB2 (0 to 4.0%) was significantly more frequent in cases with high RS (p = 0.0122 and p = 0.0020, respectively) (Figure 2B). CCND1 amplifications were slightly more common in the high-risk group, but this was not statistically significant. PAK1 amplifications appeared to be equally distributed (Figure 2B).

### 3.4 | Relation between genetic alterations and EPR

Endocrine proliferative response (EPR) to short-term pET was determined by the post-pET Ki67 index. In the present molecular analysis, EPR was categorized in four categories corresponding to optimal response (post-pET Ki67 <10%), versus slightly, moderately, and severely impaired proliferative response (post-pET Ki67 10%–19%, 20%–34%, and ≥35%, respectively) (Figure S1). In total, 52.6% of BC cases (n = 327) showed an optimal EPR (group I, median baseline Ki67 15.0%, median post-pET Ki67 5.0%). In total, 29.9% of cases (n = 186) displayed a slightly impaired EPR (group II, median baseline Ki67 15.0%, median post-pET Ki67 15.0%). In total, 14.0% and 3.5% of BC cases (n = 87 and n = 22) showed moderately and severely impaired EPR (group III and IV, median baseline Ki67 25.0% and 42.5%, median post-pET Ki67 20.0% and 42.5%, respectively) (Figure S1).

Two of the tested genetic alterations were significantly associated with impaired EPR (Figure 3). The frequency of TP53 mutations increased significantly with increasing post-pET Ki67 category. In detail, the frequency of TP53 mutations increased from 4.9% in BC cases with optimal EPR (group I) to 22.7% in BCs with severely impaired EPR (group IV, p < 0.0001) (Figure 3A). This increase was independent from the mode of pET and occurred both with tamoxifen- as well as with AI-based pET (p = 0.0005 each, Figure 3A).

TP53 mutations can be associated with variable loss of p53 transcriptional activity. According to IARC, the functional impact of TP53 mutations is categorized as (partially) functional or non-functional. Other classifications distinguish between non-disruptive and disruptive TP53 mutations. Within this study, we detected 47 different TP53 mutations in 56 patients. All detected TP53 mutations were located in the p53 DNA-binding domain (Figure 4A). Most of the detected mutations (77%; n = 43) were missense mutations. Truncating mutations were present in 18% (n = 10) of the patients and 5% (n = 3) harbored splicing mutations or in-frame deletions. The majority of these TP53 mutations (72%, 34/47) encoded for p53 mutants that are classified as non-functional, according to IARC (Figure 4A). The minority of the detected TP53 mutations was unclassified (6%, 3/47, including splicing mutations and in-frame deletions), or (partially) functional (21%, 10/47), according to IARC (Figure 4A). The (partially) functional TP53 mutations were more common in TP53-mutated BCs with optimal EPR (group I) compared to TP53-mutated BCs with severely impaired EPR (group IV, Figure 4B).

For completeness, we also conducted a refined statistical analysis considering the IARC classification to compare wild-type TP53 and TP53 mutation encoding for a non-functional p53 protein (Figure 4C). As expected, non-functional TP53 mutations were strongly associated with impaired EPR (Figure 4C). In detail, the frequency of non-functional TP53 mutations increased from 2.8% in BC cases with optimal EPR (group I) to 18.2% in BCs with severely impaired EPR (group IV, p < 0.0001, Figure 4C). This increase was independent from the mode of pET and occurred both with tamoxifen- as well as with AI-based pET (p = 0.0011, respectively p < 0.0001, Figure 4C). Similar results were obtained for disruptive TP53 mutations, as defined by Molina-Vila et al. (Table S5).

In addition, tumors with positive HER2/ERBB2 status (IHC +/FISH-positive or IHC3+/FISH-positive, as determined by central review) were also associated with EPR. Despite the small number of ERBB2-amplified cases (n = 8) in this study, we observed a statistically significant correlation between ERBB2 amplification status and impaired EPR (p = 0.0015, Figure 3B).

Activating mutation of the ERBB2 gene occurred in n = 18/622 BC cases (all except one had a negative HER2/ERBB2 status by IHC/FISH) and was not associated with impaired EPR (p = 0.6345, Figure 3C). Alterations of ARID1A, CCND1, FGFR1, GATA3, PAK1, and PIK3CA were not significantly associated with EPR (Table S4). However, PIK3CA mutation was associated with low baseline Ki67 and TP53 mutation was also associated with high baseline Ki67 (Table S4).
3.5 | TP53 mutation is an independent predictive parameter for impaired EPR

Using multivariate logistic regression, we analyzed multiple factors for their ability to predict impaired EPR (Figure 5). To this end, we focused on the following parameters such as TP53 mutation status, type of pET (tamoxifen vs. AI), pT stage, pN stage, baseline histological grade, Oncotype DX RS group, baseline Ki67, baseline ER status (% expression, continuous variable), and baseline PR status (% expression, continuous variable). Independent predictive parameters for impaired EPR included TP53 mutation status, type of pET, RS group, baseline Ki67 index, baseline ER status, and baseline PR status (Figure 5).

Tumors with TP53 mutations (any type) had higher odds of impaired EPR (OR = 2.6, 95% CI = 1.2–5.5) compared to TP53 wild-type tumors. Risk of impaired EPR was 3.5 times higher in cases with RS 26–100, than in cases with RS 0–25. Cases with high baseline Ki67 also had higher odds for an impaired EPR. pT stage, pN stage, and histological grade did not independently predict impaired EPR (Figure 5).

For completeness, we used a refined multivariate logistic regression considering the IARC classification to compare wild-type TP53 and non-functional TP53 mutations (Figure 5B). In this refined analysis, risk of impaired EPR was 3.7 times higher in tumors with TP53 mutations (95% CI = 1.5–9.4), than in tumors without TP53 mutations. Hence, TP53 mutation predicts impaired EPR independently from Oncotype DX RS and other clinicopathological parameters.

4 | DISCUSSION

The greatest part of our knowledge regarding endocrine resistance in BC stems from experimental in vitro studies using cell lines and animal models. Over the last decades, tamoxifen sensitivity has been the subject of many, primarily cell biological studies, but none of the many potential markers had found its way into the clinic. The major reason for this deficit in translation is the lack of clinical studies suitable to validate the in vitro findings.36

A novel approach to understand endocrine resistance was enabled by large-scale mutational analysis of relapsing and metastatic BC which had been treated with adjuvant endocrine therapy after surgery.7,13 In the study of Razavi et al. including 1500 luminal BC, the majority of cases had been exposed to prior therapy (87.5% of the biopsied metastatic tumors).7 Also, the tumors which were entered into the study of Bertucci et al. had received prior cytotoxic treatment.12 Treatment interferes with clonal evolution and selection of subclones in BC as has been obvious from ESR1 mutation which is far more frequent in previously AI-treated luminal cancers than in treatment-naïve cases.37 Whole exome sequencing of 507 primary BC showed that only three gene mutations occurred at >10% incidence across all BCs, these were GATA3, PIK3CA, and TP53.8 Overall TP53 mutation was found in 37% of BC, with a frequency of 12% in luminal A, and 29% in luminal B type of BC, respectively.8 Interestingly, luminal/ER-positive BC proved to be the most heterogeneous in terms of gene expression, mutation spectrum, copy number variations, and outcomes.8

Despite the huge number of BC cases which have undergone next-generation sequencing, there are no prospective clinical trials in primary, non-metastatic BC which exploit the available bulk of sequence data in order to stratify cases according to their endocrine responsiveness. Currently, gene expression profiles like Oncotype DX RS are utilized to achieve this goal.38 There appear to be two reasons to explain the missing utilization of available DNA sequence data for this purpose. One reason is provided by the striking heterogeneity of primary BC, in particular luminal cancers.8 Second, in particular with regard to the recent studies on metastatic cancer, there is
no certainty whether the numerous genetic alterations discovered represent primary or secondary aberrations induced by or selected during treatment.\(^7\)

With regard to luminal BC, identification of the resistant sub-cohort by impaired EPR after pET could enable recognition of primary aberrations associated with...
endocrine resistance. In previous smaller studies which have used this approach, TP53 mutation has not been clearly identified as a potential source of endocrine resistance.\textsuperscript{10,17,39,40} Previously, Gellert et al. have hypothesized that poor responders were more likely to have TP53 mutations compared with good responders.\textsuperscript{17} However, they had to reject this hypothesis after statistical analysis of their limited tumor collection from the POETIC clinical trial (n = 66, their p = 0.8).\textsuperscript{17} Ellis et al. reported correlations between TP53 mutations and higher Ki67 levels at baseline and at surgery, as well as a correlation with the luminal B subtype.\textsuperscript{10} In AI non-responders they found an increased prevalence of genetic alterations in p53 signaling pathway (including TP53, ATR, APAF1, and THBS1 combined), but predictive relevance of TP53 mutations per se was not evaluated.\textsuperscript{10} Giltinan et al. could not find any correlation between TP53 mutation and impaired EPR in a limited tumor collection from a clinical trial at the Vanderbilt University (n = 140).\textsuperscript{39} Using gene expression profiling Gao et al. showed that a TP53 dysfunction signature was associated with impaired EPR after 2 weeks of pET with AI, but TP53 gene mutation status was unknown.\textsuperscript{40} Consequently, TP53 mutation is currently not considered as a determinant of endocrine resistance.\textsuperscript{1} In the WSG-ADAPT trial, there was, for the first time, a clear-cut association between impaired EPR and TP53 mutation, in AI- as well as tamoxifen-treated BC. Different reasons may account for the fact that TP53 mutation was associated with EPR in our study, but not in previously reported studies. One possible reason is the considerably larger sample sizes in the present study.\textsuperscript{17,39,40} Another possible reason is that previous studies had included HER2-positive along with HER2-negative HR-positive BCs.\textsuperscript{10,17,40} In our study, HER2-positive BCs were almost completely excluded as per clinical study inclusion criteria. A third reason might be different criteria to define EPR.\textsuperscript{10,17,39,40} In the present study, impaired EPR was defined as post-pET Ki67 $\geq$10% and optimal EPR was defined as post-pET Ki67 <10%. This is consistent with the definition of EPR in a recent analysis of the POETIC trial.\textsuperscript{24} EPR cutoffs utilized in previous studies varied between 2.7% and 10%.\textsuperscript{10,39} Moreover, TP53 analysis was based on slightly different methods.\textsuperscript{10,40} In one study, TP53 mutation status was assumed based on a gene expression signature.\textsuperscript{40}

In the present study, the complete coding sequence of the TP53 gene was analyzed for mutations by NGS.

The overall frequency of TP53 mutations in the BC collection analyzed in this study was comparatively low (9%).\textsuperscript{11} However, this may be related to the characteristics of BC patients preferably enrolled in the WSG-ADAPT HR-positive /HER2-negative trial. The frequencies of other genetic alterations in our cohort were mostly very similar to published data for primary BC.\textsuperscript{8,39,41} With 0.6% instead of 3%, we found fewer ESRI mutations than expected.\textsuperscript{36}

There have been conflicting data regarding TP53 mutation as a predictive marker in BC, indicating either increased sensitivity to cytotoxic drugs or on the contrary potential resistance.\textsuperscript{16} Analyzing the METABRIC data, the influence of TP53 mutation on survival of BC cases was studied. Thereby, two effects of the mutation could be observed. First, TP53 mutant cancers displayed a superior overall survival when treated by chemotherapy and irradiation. Second, TP53 wild-type cases revealed substantial benefit when ER-expressing cancers were treated with endocrine therapy. Overall survival in this study was worse in endocrine-treated cases with TP53 mutation than in TP53 wild-type cancers, corresponding to our findings that TP53 might be an effector of endocrine resistance.\textsuperscript{16} Further studies are warranted to corroborate the relationship between TP53 and endocrine responsiveness in more detail. In vitro cell models of HR+BC cell lines harboring TP53 mutations introduced by the CRISPR-Cas9 method might help to clarify whether or not sensitivity to estrogen deprivation is directly dependent on the TP53 status. Furthermore, large-scale sequencing studies in clinical cohorts with long-term follow-up may document the relevance of TP53 mutation for primary endocrine resistance.

Besides of TP53 mutation, only RS group, type of pET (associated with age), and baseline Ki67 were associated with impaired EPR. For recurrence score, Paik et al. have similar findings in cases treated with tamoxifen.\textsuperscript{42} The POETIC trial showed that short-term pET with AIs (2 weeks of letrozole or anastrozole) does not improve outcome (BC recurrence). However, it was also shown that cases with high Ki67 at baseline and after preoperative therapy have a higher risk of recurrence.\textsuperscript{24} pET could be used to select an appropriate adjuvant therapy based on the observed Ki67 response, because impaired EPR might

\[ \text{FIGURE 4} \text{ Relation between non-functional TP53 mutations and endocrine proliferative response (EPR). (A) The lollipop plot shows the distribution of mutations within the functional domains of the TP53 gene. All alterations were observed in the DNA-binding domain (aa 95–288). No alterations could be observed in the transactivation domain (TA, aa 6–29) and the tetramerization domain (TD, aa 318–358). (B) The bar chart shows the classification of mutations with the IARC TP53 database according to post-preoperative endocrine therapy (pET) Ki67 category. Variants that were not assessed were excluded for this illustration (splicing variants and in-frame deletions, n = 3). (C) Relation between cases with non-functional classified TP53 mutations and EPR. Depicted are the frequencies according to post-pET Ki67 category. For (B) and (C) subsets treated with either tamoxifen (TAM) or aromatase inhibitors (AI) are shown in the right panels} \]
be an early indication for primary endocrine resistance. Unfortunately, we did not find associations between impaired EPR with targetable alterations, like \textit{FGFR1} amplifications and \textit{PIK3CA} mutations.

In conclusion, the current WSG-ADAPT translational study demonstrates that impaired EPR is suitable to identify genetic mechanisms of primary endocrine resistance already early during the course of early luminal BC. The presence of \textit{TP53} mutations indicates primary endocrine resistance in about 10% of luminal early BC cases. As \textit{TP53} mutations have also been implicated with conveying sensitivity toward conventional chemotherapy, further studies are needed in order to clarify the clinical consequences of our findings.

**CONFLICT OF INTEREST**

OG has minority ownership interest in WSG GmbH, received honoraria from Genomic Health, Pfizer Pharmaceuticals, Roche, and her institution received research funding from Agendia, Amgen, Celgene, Genomic Health, NanoString Technologies, Roche, Sanofi.

SK has minority ownership interest in WSG GmbH, received personal fees from Lilly, Roche, Genomic Health, Novartis, Amgen, Celgene, Daiichi Sankyo, AstraZeneca, SOMATEX Medical Technologies, MSD, Pfizer, Puma Biotechnology, PFM medical, and non-financial support from Roche, Daiichi Sankyo, Sonoscope.

MB received honoraria from AstraZeneca, Exact Sciences, Novartis, Pfizer, Roche, Teva, travel support from AstraZeneca, Celgene, Medac, Novartis, Roche and served in consulting/advisory role for AstraZeneca, Exact Sciences, Novartis, Puma, Roche.

BA reports a potential financial conflict of interest as follows: Pfizer Pharma GmbH, Roche Pharma AG, Novartis Pharma GmbH, AstraZeneca GmbH, PharmaMar GmbH, MSD Merck Sharp & Dohme GmbH, Onkowissen.de GmbH, Lilly Deutschland GmbH, ProMedicis GmbH.

RK served in consulting/advisory role for West German Study Group, and reports for an immediate family member: ownership interest in WSG GmbH, honoraria from Amgen, AstraZeneca, Genomic Health, Novartis, Pfizer, Pierre Fabre, Roche, Zodiac Pharma, consulting/advisory role for Agendia, AstraZeneca, Celgene, Daiichi Sankyo, Lilly, Merck Sharp & Dohme, Novartis, Odonate Therapeutics, Pfizer, Pierre Fabre, Roche/Genentech.
Sandoz, Seattle Genetics, and research funding from Lilly, Merck Sharp & Dohme, Novartis, Pfizer, Roche/Genentech.

RW served in consulting/advisory role as well as on speakers’ bureau for and received travel support from Agendia, Amgen, Aristo, AstraZeneca, Boehringer Ingelheim, Carl Zeiss, Celgene, Clinsol, Daichi-Sankyo, Eisai, Genomic Health, Glaxo Smith Kline, Hexal, Lilly, Medstروم Medical, MSD, Mundipharma, NanoString, Novartis, Odonate, Onkowissen, Paxman, Palleos, Pfizer, Pierre Fabre, Puma Biotechnology, Riemser, Roche, Sandoz/Hexal, Seattle Genetics, Tesaro Bio, Teva, Viatris.

NH has minority ownership interest in WSG GmbH, received honoraria from Amgen, AstraZeneca, Genomic Health, Novartis, Pfizer, Pierre Fabre, Roche, Zodiac Pharma, served in consulting/advisory role for Agendia, AstraZeneca, Celgene, Daichi Sankyo, Lilly, Merck Sharp & Dohme, Novartis, Odonate Therapeutics, Pfizer, Pierre Fabre, Roche/Genentech, Sandoz, Seattle Genetics, an immediate family member served in consulting/advisory role for West German Study Group, and her institution received research funding from Lilly, Merck Sharp & Dohme, Novartis, Pfizer, Roche/Genentech.

IG, SB, IK, LB, HC, MGro, MR, UL, CzE, EMG, CS, KHL, MGra, MC, and HK, declare no potential conflict of interest.

ETHICAL APPROVAL STATEMENT
The study design is following the guidelines of the local ethics committee (“Ethics committee of the Medical School Hannover/Ethik-Kommission der Medizinischen Hochschule Hannover,” head: Prof. Dr. Albrecht).

DATA AVAILABILITY STATEMENT
Data generated or analyzed during this study are included in this published article and its additional files.

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SUPPORTING INFORMATION

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