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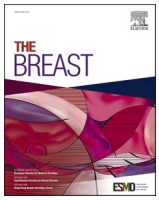

Comprehensive genomic profiling can predict response to neoadjuvant chemotherapy in triple-negative breast cancer

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ABSTRACT

Background: The rate of pathological complete response (pCR) after neoadjuvant chemotherapy (NACT) in triple-negative breast cancer (TNBC) varies, and adjuvant therapy treatment for residual cancer remains a challenge. The aim of our study was to assess the added value of FoundationOne®CDx (F1CDx) testing in the non-metastatic TNBC in predicting responses to NACT and disease outcomes.

Methods: Ninety-three eligible patients with stage II-III TNBC were treated with NACT without immunotherapy. Response to NACT was evaluated postoperatively. Comprehensive genomic profiling with NGS-based molecular test F1CDx was performed on diagnostic biopsies (N = 93). Hierarchical clustering and logistic regression were applied for data analysis.

Results: Genomic profiling and data clustering revealed heterogeneous genetic landscapes of TNBC with subsets displaying multilayered co-amplifications of oncogenes and overlapping changes in crucial signaling pathways. *TP53* mutations were detected in 95 % of all TNBCs. *BRCA1/BRCA2* mutations were significant molecular factors in predicting favorable responses to NACT (OR = 0.09, p = 0.002), while CCNDs co-mutations with FGFs (OR = 13.4, p = 0.016) and PI3Ks family mutations in AR-positive cases (OR = 6.1, p = 0.008) – poor responses. Low tumor mutational burden (TMB) $\leq$ 3 (OR = 9.4, p = 0.009) was a significant factor for the disease progression after NACT.

Conclusions: This study suggests that comprehensive CDx testing can be explored as a prognostic tool in early-stage TNBC to predict responses to NACT and disease progression. Based on these results, genomic analysis should be performed early in the patient journey, possibly guiding adjuvant treatment choices and participation in randomized clinical trials, mainly when pCR is not achieved, as the ultimate goal is improving patient outcomes.

1. Introduction

Breast cancer, with over 2.3 million new cases estimated in 2020, is the most commonly diagnosed cancer globally [1]. In Western countries, TNBC accounts for approximately 10–15 % of all breast cancer cases [2]. The overall incidence of TNBC can vary depending on various factors, including geographical location, ethnic and racial background, and population demographics [3].

Current guidelines for stage II-III TNBC recommend neoadjuvant chemotherapy and immunotherapy with pembrolizumab [4,5]. Response to neoadjuvant treatment remains one of the most significant prognostic factors of disease-free and overall survival, leading to the individualization of adjuvant therapy [6]. No reliable tool exists to predict an individual tumor's response to treatment and disease outcome.

Comprehensive genomic profiling (CGP) - based tests are mainly designed for molecular profiling of the tumors and serve as the predictive tools for targeted- and immune-therapy selection. However, through a detailed examination of cancer-specific genetic changes, these tests could provide additional prognostic information on the behavior of

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the tumor during neoadjuvant therapy and afterward. Being able to accurately predict response rate and recurrence risk, along with identification of the tumor-specific driver mutations, CGP tests may provide a more accurate picture of the disease and aid medical oncologists in selecting appropriate individualized therapy [7]. This study aimed to determine whether the companion diagnostics-adapted CGP test could have added value as a prognostic tool for assessing response to NACT in non-metastatic TNBC.

2. Materials and methods

2.1. Study population

The prospective observational study included a mono-institutional cohort of 93 eligible patients with stage II-III TNBC diagnosed between 2019 and 2022. All patients gave written informed consent regarding participation in the study. The study received approval from the Institutional Scientific Review Board of the National Cancer Institute (NCI) of Lithuania (protocol code II-2018-1) and the Vilnius Regional Bioethics Committee (Approval number 2019/2-1084-589). All patients received standard NACT at the NCI, undergoing 12 cycles weekly of paclitaxel (80 mg/m^2) and carboplatin (AUC2), followed by four 3-weekly cycles of AC (doxorubicin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2). Subsequently, surgery was performed on all patients, and radiotherapy was administered following local guidelines. Adjuvant treatment with capecitabine was administered to 18 (38.3 %) of 47 patients with residual disease. As shown in the Flow diagram (Fig. S1), the follow-up data cutoff was on March 31, 2023. The patient's clinical and pathologic characteristics are summarised in Table 1.

2.2. Pathological assessment

Pathological diagnosis based on standard immunohistochemical (IHC) staining was performed on primary tumor tissue samples collected using a core needle biopsy. All tumors were considered to have negative hormonal receptor status if they had less than 1 % stained cells for estrogen and progesterone receptors. The human epidermal growth factor receptor 2 (HER2) status was evaluated as negative with an IHC score of 0/1+; for HER2 equivocal cases (scored 2+), fluorescence *in situ* hybridization (FISH) analysis revealed no HER2 gene amplification. Tumors were considered androgen receptor (AR) positive if they had more than 1 % stained cells. According to the median score, Ki67 expression was rated high if ≥ 60 % of cells were positive on IHC. Biopsie cores were stained for CD8 tumor-infiltrating lymphocytes and digitized at 20× magnification. The HALO® AI (Indica Labs, USA) Densenet v2 classifier was created to separate tumor structures from the stroma. After tissue classification, HALO® Multiplex IHC analysis was used to segment CD8$^+$ positive cells. Finally, the area occupied by CD8$^+$ cells in the stromal compartment was estimated (Fig. S2).

Breast pathologists evaluated the response to NACT postoperatively. Pathological complete response (pCR) was defined as the absence of invasive cancer in the breast and lymph nodes (ypT0/*in situ,* ypN0). Incomplete response (non-pCR) refers to residual cancer cells in breast tissue and/or lymph nodes. A residual cancer burden (RCB) score and class were calculated using a validated methodology and the Residual Cancer Burden Calculator [8–10]. The patients were separated into three groups according to response to treatment and further assessment of prognostic factors (pCR, RCB-1, and RCB-2/3). Patients in pCR and RCB-1 were defined as having a favorable response to NACT, whereas RCB-2/3 had a poor response.

2.3. Comprehensive genomic profiling (CGP) of primary tumors

CGP of primary tumors was performed on FFPE samples from all our patients using NGS-based molecular profiling with F1CDx. The technology analyses the entire regions of 324 genes relevant to cancer.

Table 1
Clinicopathologic characteristics of the patients.

Patient number (percent)	93 (100 %)
Patient age at diagnosis, years	
Average (± standard deviation)	54.53 (±11.78)
Median (range)	56 (25–75)
Body mass index (BMI)	
Normal (≥18.5 and ≤25)	36 (38.7 %)
Overweight (≥25 and < 30)	31 (33.3 %)
Obese (≥30)	26 (28.0 %)
Menopausal status, patient number (percent)	
Premenopausal	37 (39.8 %)
Postmenopausal	56 (60.2 %)
Clinical stage, patient number (percent)	
II	59 (63.4 %)
III	34 (36.6 %)
Tumour category (cT), patient number (percent)	
x	1 (1.1 %)
1	16 (17.2 %)
2	62 (66.7 %)
3	4 (4.3 %)
4	10 (10.7 %)
Node positivity (cN), patient number (percent)	
0	32 (34.4 %)
1	35 (37.6 %)
2	21 (22.6 %)
3	5 (5.4 %)
Histologic grade (G), patient number (percent)	
G1	2 (2.2 %)
G2	12 (12.9 %)
G3	79 (84.9 %)
Area (mm^2) % of CD8$^+$ in stroma (CD8$^+$)	
Data available for patients number; median, (range)	86 (92.5 %); 5.6 (0.04–24.08)
Androgen receptor expression (AR), patient number (percent)	
Data available for patients number; median, (range)	83 (89.2 %); 0 (0–100 %)
≥1 %	25 (30.1 %)
<1 %	58 (69.9 %)
Proliferation index Ki67 (%), patient number (percent)	
<60 %	38 (40.7 %)
≥60 %	55 (59.1 %)
Median % (range)	60 % (3–95 %)
BRCA1/2 mutations, patient number (percent)	
Intratumoral	23 (24.7 %)
Confirmed germline	15 (16.1 %)
Tumour mutational burden (TMB), patient number (percent)	
Data available; median, (range)	91 (97.8 %), 4 (0–16)
<4	45 (49.5 %)
≥4	46 (50.5 %)
Treatment, patient number (percent)	
Neoadjuvant chemotherapy	93 (100 %)
Surgery	93 (100 %)
Adjuvant chemotherapy (capecitabine)	18 (19.4 %)
Adjuvant radiotherapy	77 (82.8 %)
Pathological response, patient number (percent)	
Complete response (CR)	46 (49.5 %)
Partial response (PR)	41 (44.1 %)
Stable disease (SD)	3 (3.2 %)
Locally progressive disease (PD)	3 (3.2 %)
Residual cancer burden (RCB class), patient number (percent)	
0	46 (49.5 %)
1	15 (16.1 %)
2	21 (22.6 %)
3	11 (11.8 %)
Survival data, patient number (percent)	
Disease progression	16 (17.2 %)
Deceased	11 (11.8 %)
Follow-up, months	0–49.8
Median overall survival (OS), months	32.3
Median progression-free survival (PFS), months	32.3

F1CDx provides a report of detected primary classes of genomic alterations as well as complex biomarkers, including tumor mutational burden (TMB) measured as mutations per megabase (mut/MB) of DNA and microsatellite instability (MSI) [11]. Likely pathogenic alterations were not analyzed.

Our study analyzed 93 biopsy and 18 resected tumor samples using F1CDx at the laboratory service center (Foundation Medicine GmbH,

Nonnenwald 2, 82377 Penzberg, Germany). The data were provided in a standard report format.

2.4. Study data analysis

Clustering using Ward's method and Euclidean squared distances (Fig. S3) and OncoPrint [12] were carried out using R [13]. In the OncoPrint, cases clustered based on the similarity of mutated genes were subsequently combined with clinicopathologic and demographic data, enabling the visualization of these characteristics' distribution across the resulting (sub)clusters. Descriptive statistics, binary/ordinal logistic regression, and Kruskal-Wallis H-test were performed using IBM SPSS Statistics 21. For all 134 mutated genes, only the significant or relevant results of the logistic regression are shown in tables. A 2-sided p-value <0.05 was considered statistically significant.

3. Results

3.1. Main characteristics of study group

Table 1 presents clinicopathologic and demographic data for the study group. The mean age of the patients with TNBC was 54.5 years, with 60 % of them being postmenopausal. The predominant clinical stage diagnosis was II, tumor category cT2, and histological grade 3, with moderate lymph node involvement (predominantly 0–1).

No patients experienced disease progression clinically or radiologically during NACT. Following neoadjuvant therapy and surgery – which all cases underwent - almost half of the patients (46 cases; 49.5 %) showed pCR. Among those who did not achieve pCR, the majority had the class of RCB-2/3 (32 cases; 34 %). Fifteen cases were classified as RCB-1. A subset of patients (83 %) received adjuvant radiotherapy. The minority of the patients with residual disease were treated with adjuvant chemotherapy with capecitabine according to national guidelines. The follow-up period for the study group was almost 50 months. During this time, 16 cases were diagnosed with the distant disease progression, and 11 of these patients died.

Table 2
Univariate and multivariate logistic regressions of the prognostic factors for response to NACT.

Variables (N)	Univariate	Multivariate
	OR (95 % CI), p-value	OR (95 % CI), p-value
mutant *BRCA1* (16)	0.27 (0.09–0.95), **0.04**	
mutant *BRCA2* (7)	0.35 (0.07–1.9), 0.26	
mutant *BRCA1/BRCA2* (23)	0.26 (0.09–0.7), **0.01**	0.14 (0.03–0.64), **0.01**
mutant *CCND1* (5)	7.5 (0.8–71), 0.08	
mutant *CCND2* (3)	5.3 (0.5–54), 0.16	
mutant *CCND3* (4)	1.4 (0.2–11), 0.75	
mutant *CCNDs* (10)	2.9 (0.8–11), 0.10	
mutant *FGFs* (10)	2.2 (0.7–7.7), 0.20	
mutant FGFs + mutant *CCNDs* (7)	5.7 (1.1–30), **0.04**	9.3 (1.2–72), **0.03**
mutant *PIK3CA* (18)	1.1 (0.4–2.9), 0.81	
mutant *PIK3C2B* (5)	1.4 (0.3–7.4), 0.70	
mutant *PIK3R1* (9)	0.8 (0.2–3.2), 0.78	
mutant *PI3Ks* (24)	1.1 (0.5–2.7), 0.80	
AR	2.0 (0.8–5.1), 0.12	
mutant *PI3Ks* + AR (15)	2.5 (0.9–7.5), 0.09	5.6 (1.4–23), **0.02**
TMB	2.7 (1.2–6.0), **0.01**	2.0 (0.7–5.6), 0.16
Ki67	0.8 (0.4–1.8), 0.65	
Age	0.98 (0.95–1.01), 0.21	0.96 (0.92–1.0), 0.10
BMI	0.97 (0.60–1.57), 0.97	
$CD8^+$	0.90 (0.83–0.97), **0.009**	0.92 (0.84–1.0), 0.10
Menopausal status	0.7 (0.3–1.6), 0.39	
Disease stage	1.8 (0.8–4.0), 0.15	
cN	2.0 (0.9–4.7), 0.09	2.3 (1.2–4.2), **0.01**
cT	1.6 (0.95–2.6), 0.08	1.5 (0.8–2.8), 0.20

The dependent variable (response to NACT) represented categories as follows: pCR, RCB-1, RCB-2/3. Cases with no mutant gene tested are selected as the reference group unless otherwise indicated. BRCA1/2 – pooled cases with mutant *BRCA1* or *BRCA2*; FGFs – pooled cases with mutant *FGF 19, FGR 3, FGF 4, FGF 6, FGF 10, FGF 12, FGF 14, FGF 23*; CCNDs – pooled cases with independently registered and co-occurring mutated *CCND1*, *CCND2*, *CCND3*; mutant FGFs + mutant CCNDs represents pooled cases with the mutant genes at 11q.13 and 12q.13; PI3Ks - pooled cases with independently registered and co-mutated *PIK3CA, PIK3R1, PIK3C2B*. For AR, cases with values < 1 % are selected as the reference group for cases with values ≥ 1 %. PI3Ks + AR – PI3Ks with positive AR, reference for this group, are all other cases that do not have a PI3Ks + AR combination. For TMB, cases with values > 3 are selected as the reference group for values ≤ 3. For cN, cases with values $= 0$ are selected as the reference group for values ≥ 1. cT and Age selected as covariates for the model. OR – Odds ratio; CI - confidence interval; TMB - tumor mutational burden; AR - androgen receptor; BMI – body mass index; $CD8^+$ - area (mm^2) % of $CD8^+$ in stroma.

3.2. Main mutation targets, types, and rates

Mutations of 134 genes of the F1CDx panel were detected in 93 TNBC tumors. The median number of mutated genes per case was 5 (range 1–18). The median TMB was 4 (range 0–16). *TP53* mutations were detected in 95 % of tumors (88/93). Other frequently mutated genes included *MYC* (23 %), *RAD21* (22 %), *PIK3CA* (19 %), *BRCA1* (17 %), *PTEN* (15 %), *FGFR1* (12 %), *NSD3* (12 %), *ZNF703* (11 %), *NF1* (11 %), *PIK3R1* (10 %), *RB1* (9 %), and *BRCA2* (9 %). Structural variants (SVs) were the major mutation type, accounting for 62 % of all mutations registered. Amplifications predominated, accounting for 81 % of SVs and 50 % of all identified mutations. Point mutations accounted for 32 %, splice site mutations 4 %, and indels 2 %. Of all mutations in these genes, point mutations were most common in *TP53* (92 %), *BRCA2* (88 %) and *BRCA1* (73 %). *MYC*, *RAD21*, *FGFR1*, *NSD3*, and *ZNF703* exclusively had amplified variants. *PIK3CA*, *EGFR*, *FGFR2*, *NOTCH3*, and *BRD4* showed heterogeneous alterations, including amplifications, SVs, and point mutations. Co-occurring amplifications markedly influenced the mutation profile, with large amplicons involving the *FGFR1/ZNF703/NSD3* (8p11.23), *JAK2/PD-L1/PD-L2* (9p24.1), *CCND1/FGF19/FGF3/FGF4* (11q.13), *CCND2/FGF23/FGF6/KDM5A* (12p.13), *CCND3/VEGFA*(6p12-21), *MYC/RAD21*(8q24), *CCNE1/AKT2*(19q12-13), and *PIK3C2B/MDM4* (1q32) genes. Of the 23 *BRCA1/BRCA2* mutations detected in the tumor cells, 15 cases were confirmed as germline testing blood leucocytes in our local genetic laboratory. Eighteen resected tumor samples were analyzed with F1CDx; no significant changes were detected post-NACT.

3.3. Classification of TNBC-specific mutations

Three clusters of TNBC were identified based on gene mutation and co-mutation patterns (Fig. S3). Cluster 1 (78 cases) was bifurcated into two branches and nine sub-clusters (1A-1I). The first branch (1A-1F) showed a complex pattern with dominant mutations in *BRCA2*, *NF-1*, PI3Ks, *BRCA1*, and *JAK2/PD-L1/PD-L2*. The second branch (1H and 1I) was characterized by amplified *RAD21* and/or *MYC*. Cluster 2 (5 cases) had amplifications at the 11q.13 locus (*CCND1/FGF19/FGF3/FGF4*) and various co-mutations, while cluster 3 (10 cases) – amplicon at 8p11.23 (*FGFR1/ZNF703/NSD3*) with additional co-mutations. TMB values were comparable across sub-clusters. However, the lowest median number of mutated genes per case was in 1A and 1B. Higher numbers were observed in 1G, 1H, 1I, and clusters 2 and 3 (Table S1).

3.4. Pathological and demographical features of (sub-)clusters

Relationships between genetic mutations within (sub-)clusters and pathological features were analyzed using logistic regression. AR-positive tumors were prevalent in sub-clusters 1D/1E, characterized by high mutant *PIK3CA* and *PIK3R1* genes (Fig. 1, Table S1). High Ki67 expression predominated in sub-cluster 1G (100 % of cases) and sub-clusters 1H/1I (81 % of cases). Ki67-high cases were the least abundant in sub-clusters 1D/1E (17 %; Fig. 1, Table S1). Sub-clusters 1G, 1D/

(caption on next page)

Fig. 1. Oncoprint of genomic alterations across TNBC patients.
The oncoprint displays genomic alterations across 93 tumor samples for selected genes. Each row represents a gene, and each column corresponds to an individual tumor sample. The color-coded cells indicate the type of genomic alterations: in the study group, this was a mutation set: Structural variant: amplification (red), structural variant: deletion, duplication, rearrangement, fusion (light blue). Point mutations (dark green): *missense* mutations involved amino acid changes (f.e. R175H, Y234C), *frameshift* mutations (1–3 bp change), and *nonsense* mutations where one amino acid is replaced by a stop codon (f.e. Q1928). *Splice mutations* (yellow) affected pre-mRNA splicing in a deletion-insertion way (f.e. spice site 376-9_382del16), nucleotide was replaced by another nucleotide at + position (f.e. splice site 1566+2C > T) or deletion of specific nucleotides section (f.e. Splice site 783-1_792delGTGGTAATCTA). *Indels* (violet) involving genomic rearrangements, such as insertions or deletions (f.e. del ex 12). Lastly, *subclonal* (salmon or bright green) mutations presented in a subset of tumor cells contribute to intratumor heterogeneity. The top bars (above the heatmap) represent the percentage of samples with an alteration in any of the genes displayed, providing a quick overview of the mutational burden per sample. The sidebar (to the right of the heatmap) showcases the frequency at which each gene is altered across all samples. The bottom bar represents the patient's ID; BMI – body mass index; $CD8^+$ - area (mm^2) % of $CD8^+$ in stroma.

E, and cluster 3 were predominant in postmenopausal cases (Fig. 1, Table S1), while sub-clusters 1F, 1C, and cluster 2 had the highest frequency of premenopausal cases (Table S1). Age differences were insignificant among (sub-)clusters, but 1F had the youngest patients, with a median age of 47. No significant differences were observed between sub-clusters in the disease stage.

3.5. Clinical and genetic determinants of response to NACT and disease progression

Sub-clusters 1F and 1G showed favorable responses (pCR/RCB-1) in 80 % and 100 % of cases, respectively (Table S1). In contrast, cluster 2 and sub-clusters 1D/1E had the highest RCB-2/3 instances at 80 % and 50 %, respectively. The OR for poor response was 16.6 (p = 0.03) in cluster 2 and 5.3 (p = 0.06) in sub-clusters 1D/1E compared to 1F. Using cluster 2 as a reference, sub-cluster 1G had a significantly higher OR for good response (OR = 26.2, 95 % CI 1.2–597, p = 0.04), and sub-clusters 1A/1B showed a tendency for good response (OR = 10.4, 95 % CI 1.0–109, p = 0.05). Sub-cluster 1B (mutant *BRCA2*) had a higher OR for a good response (OR = 19.0, 95 % CI 1.2–295, p = 0.04) than cluster 2.

Logistic regression analysis for 134 mutated genes showed that *BRCA1* and *BRCA2* mutations were significant in the pCR group (24 % and 13 %, respectively) and low in the RCB-2/3 group (9 % and 3 %, respectively). Only mutant *BRCA1* was significantly associated with a good response (Table 2). The pooled *BRCA1/BRCA2* group had a lower OR for poor response (OR = 0.26, 95 % CI 0.09–0.7, p = 0.01). Mutated PI3Ks tended to show a worse response when AR ≥1 %. However, AR as a single factor, was not a significant predictor of response to NACT, even when the different cut-off values for AR were evaluated (Tables S2–S3). Although there was no significant association between TMB values and the (sub)clusters of mutant genes, TMB ≤3 was linked to poor response, while high TMB (10–16) indicated a favorable response. *FGFR2* amplification with mutations in *QKI*, *CDK6*, *BRAF*, or *LYN* was found only in RCB-2/3 cases, while mutations in *NTRK1*, *PARP1*, *REL*, *RAD51B*, or *GNAS* were found in pCR cases. In terms of clinicopathologic characteristics, a higher number of tumor-infiltrating $CD8^+$ lymphocytes significantly correlated with pCR (Table 2, Table S2).

During the follow-up, 16 out of 93 patients experienced disease progression, and 11 died. Regression analysis revealed no significant difference in disease progression across (sub-)clusters (Table S1). Sub-cluster 1H/1I had the highest incidence of progression, while 1B, 1D/E, and 1F had none. Postmenopausal status and advanced age were associated with decreased ORs for progression, while stage III increased the OR (Table 3). Tumors with TMB ≤3 or amplifications of *CCNE1* or *AKT2* were significantly correlated with progression. Mutations in *BRAF* and *SPEN* were also associated. Two cases with amplified *LYN* genes showed poor response to NACT and subsequent progression, with premenopausal status and TMB ≤3.

The multivariate analysis confirmed *BRCA1* and *BRCA2* mutations as significant predictors of favorable NACT response (Table 2). Co-mutations of *CCND1/CCND2,* FGFs and PI3Ks with AR positivity predicted poor NACT response. cN was also a significant factor. TMB ≤3 and stage III were linked to disease progression (Table 3).

Table 3
Univariate and multivariate logistic regressions of the prognostic factors for disease progression.

Variables (N)	Univariate	Multivariate
	OR (95 % CI), p-value	OR (95 % CI), p-value
mutant *CCNE1* (6)	5.7 (1.0–31), 0.046	7.8 (0.7–86), 0.09
mutant *AKT2* (5)	8.2 (1.6–41), 0.025	
mutant *CCNE1/AKT2* (4)	5.4 (0.7–41), 0.11	
mutant *BRAF* (3)	10.9 (0.9–128), 0.06	
mutant *SPEN* (3)	10.9 (0.9–128), 0.06	
mutant *PI3Ks* (24)	0.28 (0.1–1.3), 0.10	
Disease stage	3.7 (1.2–11), 0.02	5.9 (1.5–24), 0.01
cN	2.6 (0.7–9.9), 0.16	
cT	1.3 (0.7–2.5), 0.38	
TMB	9.9 (2.1–47), 0.004	9.4 (1.7–51), 0.009
Ki67	3.4 (0.90–13), 0.07	
Age	0.95 (0.91–0.99), 0.03	0.9 (0.8–1), 0.2
BMI	0.68 (0.34–1.36), 0.27	
$CD8^+$	0.97 (0.88–1.1), 0.59	
Menopausal status	0.3 (0.1–0.99), 0.048	0.7 (0.07–7.2), 0.77

The dependent variable represented categories as follows: progressed disease and no-progressed disease. Cases with no mutated gene tested are selected as the reference group unless otherwise indicated. PI3Ks - pooled cases with independently registered and co-mutated *PIK3CA, PIK3R1,* and *PIK3C2B*. Premenopausal status was selected as a reference group for postmenopausal status. Disease stage II was selected as a reference group for stage III. For TMB, cases with values > 3 are selected as the reference group for values ≤ 3. Age was selected as a covariate for the model.
OR – Odds ratio; CI - confidence interval; TMB - tumor mutational burden; BMI – body mass index; $CD8^+$ - area (mm^2) % of $CD8^+$ in stroma; N- group size for the analyzed mutant gene.

4. Discussion

In our study, the prediction of NACT response and disease progression was based on a comprehensive F1CDx test and clinicopathological data. To identify the complex mutational landscape and clinically relevant subtypes, we aimed to cluster samples according to similar patterns of mutated genes and identified (sub)clusters associated with clinical outcomes.

Response to treatment is pivotal for prognosis, underscoring the necessity for tailored therapeutic strategies. Anthracycline, cyclophosphamide, and taxane-based chemotherapy are primary TNBC regimens, achieving a pCR rate of 30 %–40 % [14]. Platinum compounds have shown efficacy in increasing response rates in neoadjuvant setting. In our study, a locally approved regimen at the time of patients' treatment of paclitaxel and carboplatin, followed by anthracycline plus cyclophosphamide, yielded a pCR rate of 49.5 %, aligning with general observations of a response rate of 52.1 % [15,16]. In the KEYNOTE-522 trial, pembrolizumab combined with NACT increased the pCR rate to 64.8 % [17]. Immunotherapy has a significant impact on disease-free and overall survival. European Medicines Agency approved pembrolizumab plus chemotherapy as neoadjuvant treatment, then continued as adjuvant monotherapy after surgery for locally advanced or early-stage TNBC at high risk of recurrence based on event-free survival benefit in 2022. Currently, this is the standard approach for the

patients in this setting [18,19].

The F1CDx test is widely utilized for targeted therapy selection. Meanwhile, we evaluated its predictive value of conventional chemotherapy in TNBC. Over 40 % of F1CDx panel genes were mutated in TNBC tissues, with a median of 5 mutated genes per case. *TP53* mutations were the most prevalent (95 %) in our study. *TP53* is a key driver of breast carcinogenesis, with a reported mutation rate of about 60 % in TNBC [20,21].

In our study, genetic mutations were also frequent in *BRCA1/2* and other genes from the DNA damage response (DDR) pathway. *BRCA1/BRCA2* mutations were mainly found in 1F and 1B sub-clusters with favorable responses to NACT. Mutant *BRCA1* was the single prognostic factor of good response to NACT, and in multifactorial analysis, mutant *BRCA1* or *BRCA2* gene was also a significant prognostic factor of favorable response to therapy. The role of *BRCA* status in early breast cancer treatment regimen selection remains controversial. However, data on *BRCA* status guiding the use of platinum agents in the metastatic setting are well established [16,22]. Germline *BRCA* mutations in TNBC are tested, tailoring adjuvant treatment decisions in women with locally advanced TNBC with residual cancer after neoadjuvant treatment [23–25]. In our study, only a subset of cases with intratumoral *BRCA* mutations in diagnostic biopsies was confirmed as germline mutations in blood leucocytes, primarily due to the specific type of mutations. Only germline mutations are relevant for PARPi therapy in breast cancer patients [26]. Notably, in sub-cluster 1F, which is enriched for *BRCA1* gene mutations, there were also some mutations in *PIK3CA*.

AR-positive tumors also have frequent *PIK3CA* mutations [27,28]. In our study, the 1D/1E sub-clusters were enriched in AR-positive cases with mutations in *PIK3CA* and *PIK3R1* genes. Multifactorial analysis revealed that somatic mutations in genes of the PI3K family in AR-positive tumors are statistically significant indicators of poor tumor response to NACT. AR-positive cases, especially those with genetic alterations in the genes of the PI3K family, should be considered a specific TNBC subgroup for whom separate treatment regimens are desired [29].

In our study, structural variants, especially amplifications, accounted for the most co-mutations detected. The amplicons identified were genes of high interest in the field of oncology, like *MYC* (8q24) [30,31], *FGFR1* (8p11.23) [32], *JAK2* (9p24.1) [33,34], *CCND1* (11q.13) [35,36], *CCND2* (12p.13) and *CCND3* (6p12-21) as well as *CCNE1* (19q12-13) [37], which together with co-amplified genes, including programmed death-ligands or growth factors, designed specific genomic and pathologic profile of TNBC sub-clusters. Two small TNBC clusters were distinguished by amplifications of large genomic regions - 11q.13 locus in cluster 2 and 8p11.23 locus in cluster 3. In addition, the amplicons in TNBC clusters 2 and 3 were also characterized by genomic alterations in the *FGF/FGFR* axis. The cases with mutated 8p11.23 amplicon showed a diverse response to NACT. Genes linked to driver mutations in this amplicon, notably *FGFR1* [38] and *ZNF703* [39], are associated with early relapse and poor survival. However, reported response rates to targeted *FGF/FGFR* therapies are lower compared to other driver-positive tumors, suggesting the need for further exploration of specific mechanisms [40,41]. The 11q13 locus is an alternative amplicon encompassing *FGFs* and the cyclin D gene *CCND1* and is considered a key determinant of therapeutic resistance accelerating the aggressive evolution of breast cancer [42,43]. In our study, TNBC with co-amplified *FGFs* and D-type cyclins showed a poor response to NACT. Tailored combinations targeting the cyclin pathway, and the extended co-amplification network may be indispensable to tackling resistance mechanisms [44]. Among these, cluster 1 included two branches with multiple subclusters, all characterized by changes in specific molecular pathways controlled by *BRCA*, *PI3K*, PD-Ls, or *MYC*.

In addition, cN was a significant factor for predicting TNBC response to NACT, while disease stage and TMB were significant prognostic factors for disease progression. The use of TMB as a biomarker for cancer response to chemotherapy remains controversial [45]. Still, it is considered a biomarker for immune checkpoint inhibitors [46], and the FDA has approved pembrolizumab for cancers with TMB >10 mut/MB [47]. In our study, TNBC cases with high TMB (10–16) responded well to NACT, but TMB ≤3 was a significantly prevalent factor in cases of progressed disease. In general, the early-stage TNBC cohort had a median of 4 mut/MB, whereas other studies show a median of 2.6 mut/MB in breast cancer [48]. Evidence suggests that *TP53* mutations correlate with higher TMB [49], and this is consistent with our study, where 95 % of mutated *TP53* were enrolled.

Although the study had some limitations, including a relatively small sample size and the patients received conventional chemotherapy without immunotherapy with pembrolizumab, our results showed the relevance of F1CDx for the prognosis of TNBC response to NACT.

In conclusion, our study shows that predictive tests like F1CDx have significant potential in guiding targeted therapies and predicting responses to neoadjuvant chemotherapy. These findings contribute to the evolving knowledge of the landscape in TNBC biology, emphasizing the multifaceted benefits of early comprehensive genetic testing of these tumors.

CRediT authorship contribution statement

Monika Drobniene: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dominyka Breimelyte:** Visualization, Conceptualization. **Ieva Sadzeviciene:** Writing – review & editing, Methodology. **Rasa Sabaliauskaite:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Ruta Barbora Valkiuniene:** Investigation. **Raimundas Meskauskas:** Investigation, Conceptualization. **Daiva Dabkeviciene:** Writing – review & editing, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Sonata Jarmalaite:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declarations of competing interest

MD received honoraria for lectures in educational events, participation in advisory boards, and support for attending international educational meetings from Novartis, Roche, Pfizer, Lilly, Amgen, AstraZeneca, Ewopharma (Eisai), MSD and Gilead.

Other authors have no declarations of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2025.104423.

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