

| PATIENT | | SAMPLE | | ORDERING PHYSICIAN | |
|--------------|--|---------------------|-------------|--------------------|--|
| Name: | | Specimen ID: | MDX-PT-281 | Name: | |
| ID: | | Date of collection: | | Address: | |
| Report date: | | Type: | Neoadjuvant | Contact: | |

TEST DESCRIPTION

The **Multiplex8+** breast cancer test assesses RNA-based biomarkers by conducting a **VISUALIZATION TEST** that uses RNA fluorescent in situ hybridization (RNA-FISH) to visualize a panel of biomarkers. Based on the expression of these biomarkers and the tissue histology, laser capture microdissection is used to dissect out regions of interest. With these tumor-enriched samples, a **SEQUENCING TEST** that utilizes total RNA next generation sequencing to survey gene expression in a spatially resolved manner, is further carried out. Analytical validation of **Multiplex8+** was conducted on a large retrospective cohort of 1 082 breast tumors.



THE TEST PROVIDES INFORMATION ABOUT:

- RECEPTOR STATUS:** for RNA expression of the estrogen receptor, progesterone receptor, Her2 receptor, and Ki67 measured and cross-validated by the two tests.
- MOLECULAR SUBTYPE:** based on RNA gene expression tumor biology.
- GENE SIGNATURES:** personalized for patients' tumor biology and clinical status.

INTERPRETATION GUIDE

In the following report, each gene/gene signature is given a percentile score, which ranks the expression level in the context of the patients included in our retrospective cohort. For the four main breast cancer biomarkers, estrogen receptor (*ESR1*), progesterone receptor (*PGR*), Her2 receptor (*ERBB2*), and Ki67 (*MKI67*), these percentile rankings are in the context of all 1 013 eligible patients. For all other genes/gene signatures, the percentile rankings are in the context of other patients belonging to the same **MOLECULAR SUBTYPE**. For example, for patients classified as Luminal A, the genes and gene signature will receive a percentile score compared to all Luminal A samples in our retrospective validation. The percentile scores do not necessarily imply a given level of sensitivity or resistance to a therapy.

| Sample Percentile |
|-------------------|
| Low (1-33) |
| Medium (<33-66) |
| High (<66-100) |

Percentile groups and ranges

| Subtype | # of patients |
|--------------|---------------|
| Luminal A | 432 |
| Luminal B | 313 |
| HER2 | 87 |
| Basal-like | 181 |
| All patients | 1 013 |

Number of patients in each molecular subtype and total retrospective cohort that are used to determine percentile rankings

RESULTS SUMMARY

A SUMMARY IS PROVIDED BELOW AND ADDITIONAL DETAILS ARE PROVIDED IN THE FOLLOWING PAGES.

RECEPTOR STATUS

| Sample | ESR1 | PGR | ERBB2 | MKI67 |
|--------|------|-----|-------|-------|
| A | - | - | - | + |
| | | | | |

MOLECULAR SUBTYPE

| Intrinsic subtype | TNBC subtype |
|-------------------|-----------------------|
| Basal-like | Immunomodulatory (IM) |

RELEVANT TREATMENT

| THERAPY | KEY FINDINGS | PREDICTED CLINICAL BENEFIT* |
|---|--|---|
| Pembrolizumab | Molecular subtype, prognostic risk, gene expression signature | Higher possibility of benefit |
| Anthracycline/taxane chemotherapy | Molecular subtype, prognostic risk, gene expression, gene expression signature | Higher possibility of benefit |
| Gemcitabine and Capecitabine | Gene expression | Higher possibility of benefit |
| 5-fluorouracil (5-FU) | Gene expression | Higher possibility of benefit |
| Sacituzumab govitecan (Trodelvy) | Gene expression | Higher possibility of benefit |
| ADCs in clinical trials | Gene expression | Higher possibility of benefit (off-label, clinical trial) |
| PARP inhibitor (Veliparib), Carboplatin | Gene expression signature | Lower possibility of benefit |
| Trastuzumab deruxtecan (Enhertu) | Gene expression | Lower possibility of benefit |
| Bevacizumab (Avastin) | Gene expression, gene expression signature | Lower possibility of benefit |
| Methotrexate | Gene expression | Lower possibility of benefit |

*Predicted Clinical Benefit is based on inferences from various data sources. The relationship of Multiplex8+ results to actual Clinical benefit has not been validated.

LASER CAPTURE MICRODISSECTION



Based on histological assessment and RNA-FISH biomarker expression one sample (**Sample A**) was laser capture microdissected for further analysis.

RECEPTOR STATUS

| Sample | ESR1 | PGR | ERBB2 | MKI67 |
|--------|------|-----|-------|-------|
| A | - | - | - | + |
| | | | | |

Receptor status was determined using both the **VISUALIZATION TEST** and **SEQUENCING TEST**: the table shows results after cross-validation.

INTERPRETATION

- The results from the Multiplex8+ are concordant with the immunohistochemistry findings.

MOLECULAR SUBTYPE

| Intrinsic subtype | TNBC subtype ²⁻⁴ |
|-------------------|-----------------------------|
| Basal-like | Immunomodulatory (IM) |

Based on the **SEQUENCING TEST**, we used a consensus subtyping approach consisting of our proprietary 293 gene molecular subtyping signature, a research-based PAM50 test and the AIMS method to classify the intrinsic molecular subtype¹. TNBC subtype, if applicable, was classified according to Lehmann²⁻⁴.

INTERPRETATION

- The biology of the Basal-like tumor type is consistent with the immunohistochemical and clinical designation.
- The basal-like subtype is similar to TNBC because it often lacks expression of hormone and HER2 receptors. Although patients with basal-like breast cancer respond better to chemotherapy, they are more susceptible to early relapse and have poorer prognosis.
- The immunomodulatory TNBC subtype shows enriched immune gene signatures, including checkpoint inhibitor genes, association with high grade, and shows favorable prognosis²⁻⁴.

GENE SIGNATURE

- Based on the assigned molecular subtype, and TNBC subtype (if applicable), we evaluated several individual genes and gene signatures that demonstrate prognostic and predictive potential in early and advanced/metastatic settings.

| Treatment type/ Pathway | Gene signature | Description | Sample A Percentile | |
|-------------------------|-----------------------|---|---------------------|--|
| Prognosis | HR+_ Prognostic | The prognostic signature is derived from a consensus of three research-based prognostic signatures, including the 21-gene signature GENE21 ⁵ , the 70-gene GENE70 signature ⁶ , and the 50-gene risk of relapse based on subtype alone (ROR-S) signature ⁷ . The prognostic signatures are intended for early-stage breast cancer patients with ER+/Her2- IHC, lymph node-negative, or 1-3 positive lymph nodes. The score is reported as high, intermediate, or low. Patients with high signature scores are at a greater risk of relapse and may benefit from adjuvant chemotherapy, while patients with low scores have lower risk of relapse and may not benefit from adjuvant chemotherapy. | N/A | |
| | TNBC_ Prognostic_ MDX | MultiplexDX (MDX) 60-gene signature for recurrence-free survival risk prediction for TNBC patients. The signature was derived from the MDX-BRCA retrospective cohort ¹ , comprising 159 TNBC patients and validated on 508 TNBC patients from two large international cohorts spanning Asia, Europe, and the USA. | High risk | |

GENE SIGNATURE

| Treatment type/ Pathway | Gene signature | Description | Sample A Percentile | |
|-------------------------|------------------------|---|---------------------|--|
| Luminal signatures | ESR1 | The ESR1 and PGR genes encode for the estrogen (ER) and progesterone (PR) hormone receptors, respectively, which are involved in growth, metabolism, and reproductive functions. High ER/PR is predictive of endocrine therapies and low or negative ER/PR is associated with poor prognosis ⁸ . | Low (8) | |
| | PGR | | Low (20) | |
| | ESR1_PGR average | The average gene expression of ESR1 and PGR. Higher levels of hormone receptors are predictive markers for endocrine therapies. | Low (10) | |
| | E2F4_score | This gene signature assesses activity of the E2F4 transcription factor and its targets. A high E2F4 signature is associated with endocrine resistance to aromatase inhibitors and may predict sensitivity to CDK4/6 inhibitors ⁹ . | Medium (65) | |
| Her2 | ERBB2 | The ERBB2 gene is translated into Her2, a receptor tyrosine kinase involved in cell growth/proliferation and is both a prognostic marker and predictive of response to Her2 targeted therapies ⁸ . | Low (5) | |
| | MUC4 | Mucin 4 (MUC4) is a glycoprotein that is implicated in resistance to trastuzumab through interactions with the Her2 receptor. High MUC4 is associated with reduced sensitivity to trastuzumab ¹⁰ . | High (79) | |
| | NRG1 | NRG1 codes for neuregulin 1, a ligand of the Her3 receptor. In the phase II NeoSphere trial, high NRG1 gene expression was associated with reduced response to neoadjuvant trastuzumab, but not combination trastuzumab-pertuzumab ¹¹ . | Medium (48) | |
| | pSTAT3-GS | A signature that predicts phosphorylation of STAT3 and was found to be predictive of trastuzumab resistance in the FinHer study ¹² . | High (84) | |
| | Her2 amplicon_MDX | Proprietary MDX 43-gene signature used to assess Her2 status. | Low (12) | |
| | Module7_ERBB2 | Her2-signaling signature predictive of response to multiple anti-Her2 treatments in the I-SPY2 trial ¹³ . | Low (16) | |
| | T-DM1_pred | The trastuzumab emtansine (T-DM1) predictive signature is a Research Use Only classifier that combines 19 genes/gene signature involved in the mechanism of action of T-DM1 and was shown to predict response in the T-DM1 arm of the I-SPY2 trial (https://www.nature.com/articles/s41467-024-55583-2). | Low (4) | |
| Proliferation | AURKA | Aurora Kinase A (AURKA) is a protein coding gene involved in cell proliferation and is an independent prognostic marker in breast cancer. | Low (33) | |
| | MKI67 | MKI67 codes for the marker of proliferation Ki67 protein, a marker of poor prognosis in ER+/Her2- tumors, but not Her2+ or TNBC tumors. Ki67 levels are also predictive of sensitivity to neoadjuvant endocrine and chemotherapies ⁸ . | High (97) | |
| | Module11_proliferation | Proliferation index used in I-SPY2 trial broadly predictive of pathological complete response in hormone receptor positive patients ⁴ . | Medium (58) | |
| | Proliferation_MDX | Proprietary MDX 7-gene signature used to assess cellular proliferation and cross-validate MKI67 expression levels. | High (69) | |
| CDK4/6 inhibitors | CDK4 | Cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) are important proteins that regulate cell cycle progression from G1 to S phases. They are the main targets of CDK4/6 inhibitors such as palbociclib (Ibrance), ribociclib (Kisqali), and abemaciclib (Verzenio); however, it is unclear whether their expression level predicts CDK4/6 inhibitor sensitivity. | Low (16) | |
| | CDK6 | | Medium (53) | |
| | CCNE1 | Elevated expression of the G1/S cell cycle regulators, CCNE1, CCND3, and CDKN2D, was associated with resistance to palbociclib (Ibrance) in the single-arm phase II neoadjuvant trial (NeoPalAna) ¹⁴ . | Medium (57) | |
| | CCND3 | | Low (13) | |
| | CDKN2D | | Medium (37) | |
| PIK3CA mutations | PIK3CA-GS | A gene signature that is predictive of mutations in the PIK3CA gene and consequently the PI3K inhibitor alpelisib (Piqray). A high PIK3CA-GS score is also associated with activation of the PI3K/AKT pathway and loss of mTORC1 signaling, which may be relevant for response to mTOR inhibitors (e.g., everolimus) ¹⁵ . | High (67) | |

GENE SIGNATURE

| Treatment type/ Pathway | Gene signature | Description | Sample A Percentile | |
|----------------------------|---|---|------------------------|--|
| Chemotherapy | TOP1 | The gene encoding DNA topoisomerase I, an enzyme critical for DNA transcription, is a target for anticancer drugs. | High (96) | |
| | TOP2A | The gene encoding DNA topoisomerase IIa, an enzyme critical for DNA transcription, is a target for anticancer drugs. | Medium (61) | |
| | RAD51 | The DNA repair protein RAD51 homolog 1 (RAD51) is involved in the repair of damaged DNA and is associated with resistance to chemotherapy. | Medium (50) | |
| | ERCC1 | The DNA excision repair protein ERCC-1 (ERCC1) is involved in the repair of DNA damage and is associated with resistance to chemotherapy. | High (70) | |
| | TYMS | The Thymidylate Synthetase (TYMS) gene encodes a protein involved in DNA biosynthesis and is the target of the antimetabolite chemotherapy, 5-Fluorouracil ¹⁶ . | High (72) | |
| | SLC29A1 | SLC29A1 codes for the equilibrative nucleoside transporter 1 (ENT1) protein, which is a nucleoside transporter that is involved in transporting gemcitabine and capecitabine ¹⁷ . | Medium (44) | |
| | DHFR | Dihydrofolate reductase is an enzyme coded by the DHFR gene and is involved in folate metabolism and cell growth. It is the target of the antimetabolite chemotherapy, methotrexate ¹⁸ . | High (73) | |
| | SLC19A1 | SLC19A1 codes for the reduced folate carrier 1 (RFC1) protein, which transports methotrexate into the cell ¹⁸ . | Medium (63) | |
| | CDK12 | The protein product of the Cyclin Dependent Kinase 12 (CDK12) gene regulates transcription, DNA repair pathways, and cell cycle ¹⁹ . | Low (29) | |
| | Chemotherapy_ MDX | MultiplexDX (MDX) 45-gene signature predicting pathological complete response (pCR) to neoadjuvant chemotherapy for TNBC patients, developed and validated on 938 TNBC patients from 20 diverse cohorts, including randomized Phase 2 and 3 trials. | pCR positive | |
| Immune system | PDCD1 | PDCD1 codes for the immune checkpoint marker PD-1. PD-1 is the target of pembrolizumab (Keytruda), an immunotherapy approved for the first-line treatment of metastatic TNBC. | High (78) | |
| | CD274 | CD274 codes for the immune checkpoint marker PD-L1. PD-L1 is the target of atezolizumab (Tecentriq), an immunotherapy approved for the first-line treatment of metastatic TNBC. | High (80) | |
| | CTLA4 | Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an immune checkpoint marker. | Medium (56) | |
| | Immunotherapy_ _MDX | MultiplexDX (MDX) 5-gene signature predicting pathological complete response (pCR) to neoadjuvant immunotherapy for TNBC patients, developed and validated on 60 TNBC patients from the I-SPY2 pembrolizumab and durvalumab arms. | pCR positive | |
| DNA damage and repair | VCpred_TN | DNA damage repair / immune signature predictive of response to veliparib (PARP inhibitor) and carboplatin (I-SPY2 trial) ¹³ . | Medium (61) | |
| Angiogenesis/ hypoxia | VEGFA | A gene coding for vascular endothelial growth factor, a protein involved in angiogenesis, vasodilation, and endothelial cell growth. VEGF is the target of the drug bevacizumab (Avastin). | Medium (56) | |
| | Hypoxia / Angiogenesis / Inflammatory_ MDX | Proprietary MDX 7-gene signature used to assess hypoxia, angiogenesis, and inflammation. Signature includes genes known to be predictive of response to bevacizumab (Avastin) in the neoadjuvant GeparQuinto trial ²² . | Low (18) | |

| Treatment type/ Pathway | Gene signature | Description | Sample A Percentile | |
|--|----------------|---|------------------------|--|
| ADC (antibody- drug conjugate) targets | ERBB2 | ERBB2 codes for the protein receptor Her2, which is a target for classical anti-Her2 treatments. Low and ultralow levels of Her2 can be eligible for treatment with the antibody-drug conjugate, trastuzumab deruxtecan (Enhertu) ²³ . | Low (5) | |
| | TACSTD2 | TACSTD2 codes for Tumor-associated calcium signal transducer 2, also called Trop-2, which is the target of sacituzumab govitecan (Trodelvy), an antibody-drug conjugate approved for metastatic HR+/HER2- or TNBC ²⁴ and also the drug datopotamab deruxtecan (Datoway), an ADC being investigated in clinical trials for metastatic HR+/HER2- breast cancer ²⁵ . | High (90) | |
| | NECTIN4 | Nectin Cell Adhesion Molecule 4 (NECTIN4) is a cell adhesion molecule that is a target for antibody-drug conjugates in clinical trials for breast cancer. | Medium (59) | |
| | ERBB3 | ERBB3 codes for a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. It is under investigation in clinical trials for the antibody-drug conjugate patritumab deruxtecan. | Medium (40) | |
| | FOLR1 | FOLR1 encodes the protein Folate Receptor Alpha, which is an antibody-drug conjugate target under investigation in several phase 1 and 2 clinical trials for breast cancer. | High (83) | |
| | F3 | F3 codes for tissue factor, coagulation factor III a target of several antibody-drug conjugates in phase 1 and 2 clinical trials. | Medium (52) | |
| | SLC39A6 | The SLC39A6 genes encodes for the zinc transporter LIV-1, which is highly expressed in luminal breast cancers and is under investigation in several phase 1 and 2 clinical trials. | Low (22) | |
| | CD276 | This gene codes for an immune checkpoint marker called CD276 (also known as B7-H3). It is the target of the antibody-drug conjugate (Mirzotamab clezutoclax (ABBV-155) that is in a phase 1 and 2 clinical trial for advanced solid cancers, including breast cancer. | Medium (52) | |
| | VTCN1 | V-Set Domain Containing T Cell Activation Inhibitor 1 (VTCN1 also called B7-H4) is an immune checkpoint marker and the target of the antibody-drug conjugate, SGN-B7H4V, which is under investigation in a phase 1 and 2 clinical trial for advanced solid cancers, including breast cancer. | High (92) | |
| | CEACAM5 | A gene that encodes CEA Cell Adhesion Molecule 5 protein, a target of the antibody-drug conjugate Tusamitamab ravtansine (SAR408701) that is under investigation in a phase 2 clinical trial for advanced solid cancers, including breast cancer. | High (78) | |

INTERPRETATION*

- The patient is classified as high risk by the TNBC_Prognostic_MDX signature. In our interim analysis on 177 patients included in a real-world retrospective study of neoadjuvant TNBC patients, high-risk patients have poorer outcomes relative to low-risk patients as assessed by the probability of 2-year recurrence-free survival (RFS, see Table 4). High-risk patients also have lower pCR rates to both neoadjuvant chemotherapy and chemoimmunotherapy (see Tables 1-3).
- The patient is classified as Immunotherapy_MDX pCR positive. In our interim analysis on 177 patients included in a real-world retrospective study of neoadjuvant TNBC patients, patients with a positive Immunotherapy_MDX signature have higher pCR rates to neoadjuvant chemoimmunotherapy (i.e., the KEYNOTE-522 regimen) compared to patients with a negative Immunotherapy_MDX signature (see Tables 1-3).
- The patient is classified as Chemotherapy_MDX pCR positive. In our interim analysis on 177 patients included in a real-world retrospective study of neoadjuvant TNBC patients, patients with a positive Chemotherapy_MDX signature have higher pCR rates to neoadjuvant chemotherapy compared to patients with a negative Chemotherapy_MDX signature (see Tables 1-3). However, note the high expression of the chemotherapy resistance marker ERCC1.
- Expression levels of nucleoside transporters like SLC29A1 are tied to sensitivity to gemcitabine and capecitabine, with medium/high levels predicting sensitivity. Also, high levels of TYMS may predict response to 5-fluorouracil and chemotherapies that are metabolized to 5-FU (e.g., capecitabine).

*Supporting literature can be found in the relevant gene description above. This report and its citation coverage are currently being revised.

INTERPRETATION*

- The sample has high expression of TACSTD2. TACSTD2 codes for Trop-2, which is the drug target of sacituzumab govitecan (Trodelvy), an ADC approved for the treatment of metastatic TNBC. In the biomarker analysis of the pivotal phase III ASCENT trial, patients with high/medium expression of Trop-2 benefited from sacituzumab govitecan. The cytotoxic payload of this ADC, SN-38, targets TOP1, which is expressed at high levels. Expression of both the ADC target and cytotoxic payload target suggest that sacituzumab govitecan may be effective in the advanced/metastatic setting.
- The sample shows high expression of several targets for antibody-drug conjugates, including FOLR1, VTCN1, and CEACAM5. These are currently being investigated in clinical trials for triple negative breast cancer.
- In the I-SPY2 study, a high VCpred_TN signature score, which reflects immune activation as well as lack of DNA damage repair, was shown to predict response to veliparib and carboplatin, a finding that was also validated in the BrighTNess study. However, medium expression of the VCpred_TN gene signature suggests the patient may not benefit from PARP inhibitors (veliparib) and carboplatin.
- Since the sample is classified as ERBB2-negative and ERBB2 expression is low overall, the patient is unlikely to benefit from the antibody-drug conjugate (ADC) trastuzumab deruxtecan (Enhertu).
- A low expression of Hypoxia/Angiogenesis/Inflammatory_MDX and medium expression of VEGFA suggest the patient would not benefit from bevacizumab (Avastin).
- Low expression of CDK12 suggests the patient would not benefit from methotrexate.

*Supporting literature can be found in the relevant gene description above. This report and its citation coverage are currently being revised.

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