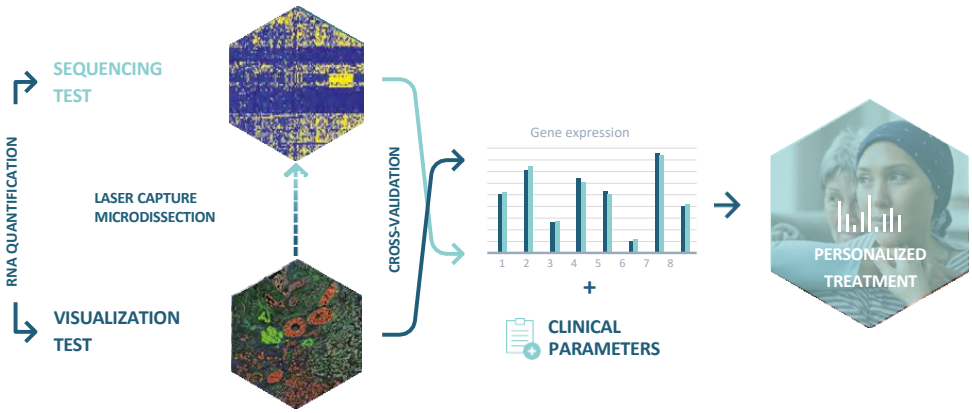
Multiplex8+ RESULTS

PATIENT	SAMPLE	ORDERING PHYSICIAN
Name:	Specimen ID: MDX-PT-11	Name:
ID:	Date of collection:	Address:
Report date:	Type:	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 1 080 breast tumors.



THE TEST PROVIDES INFORMATION ABOUT:

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

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

RESULTS SUMMARY

RECEPTOR STATUS

Sample	ESR1	PGR	ERBB2	MKI67
A	+	–	+	–

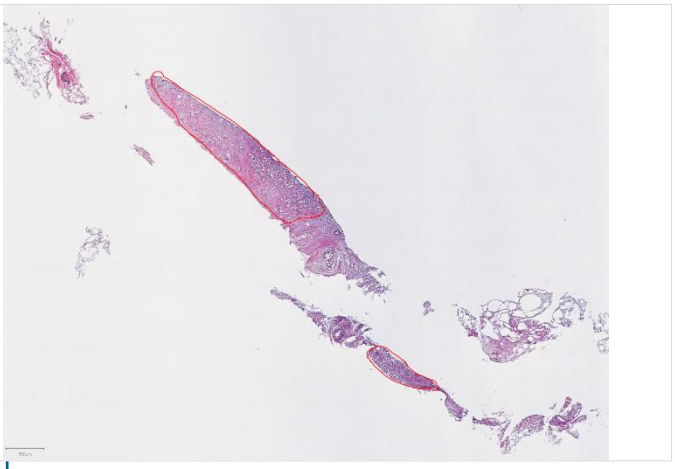
MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype
Luminal A	-

RELEVANT TREATMENT

THERAPY	KEY FINDINGS	CLINICAL BENEFIT
Anti-Her2	Gene expression, gene expression signature	Predicted benefit
Endocrine therapy	Gene expression, gene expression signature, molecular subtype	Predicted benefit
Chemotherapy	Gene expression, gene expression signature	Uncertain benefit

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 both RNA-FISH and RNA-SEQ were consistent with the immunohistochemical findings.

MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype [2-4]
Luminal A	-

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 subtype [1]. TNBC subtype, if applicable, was classified according to Lehmann [2-4].

INTERPRETATION

- The molecular classification of the subtype as Luminal A is inconsistent with the immunohistochemical and clinical designation. This is not uncommon. For example, in the METABRIC cohort, 18% of ER+/PR-/Her2+ samples were classified as Luminal A by PAM50.
- Luminal A tumors are characterized by expression of ER and/or PR and either negative or low expression of Her2 and the proliferation marker KI67. Luminal A tumors are low grade, have favorable prognosis, and respond well to endocrine therapies such as tamoxifen or aromatase inhibitors.

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	Consensus prognostic signature	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**	

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].	**Medium (40%)**	
	PGR		**Low (21%)**	
	ESR1_PGR average	The average gene expression of ESR1 and PGR. Higher levels of hormone receptors are predictive markers for endocrine therapies.	**Medium (37%)**	
	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].	**Low (12%)**	
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].	**High (90%)**	
	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].	**Low (25%)**	
	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 (62%)**	
	pSTAT3-GS	A signature that predicts phosphorylation of STAT3 and was found to be predictive of trastuzumab resistance in the FinHer study [12].	**Medium (47%)**	
	Her2 amplicon_ MDX	Proprietary MDX 43-gene signature used to assess Her2 status.	**High (99%)**	
	Module7_ ERBB2	Her2-signaling signature predictive of response to multiple anti-Her2 treatments in the I-SPY2 trial [13].	**High (100%)**	
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 (8%)**	
	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].	**Low (1%)**	
	Module11_ proliferation	Proliferation index used in I-SPY2 trial broadly predictive of pathological complete response in hormone receptor positive patients [4].	**Low (27%)**	
	Proliferation_ MDX	Proprietary MDX 7-gene signature used to assess cellular proliferation and cross-validate MKI67 expression levels.	**Low (14%)**	
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.	**High (75%)**	
	CDK6		**Medium (60%)**	
	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].	**Low (26%)**	
	CCND3		**Low (8%)**	
	CDKN2D		**Low (25%)**	
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].	**Low (28%)**	

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 (88%)**	
	TOP2A	The gene encoding DNA topoisomerase IIa, an enzyme critical for DNA transcription, is a target for anticancer drugs.	**Low (16%)**	
	RAD51	The DNA repair protein RAD51 homolog 1 (RAD51) is involved in the repair of damaged DNA and is associated with resistance to chemotherapy.	**Low (28%)**	
	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 (69%)**	
	TYMS	The Thymidylate Synthetase (TYMS) gene encodes a protein involved in DNA biosynthesis and is the target of the antimetabolite chemotherapy, 5-Fluorouracil [16].	**Low (2%)**	
	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 (40%)**	
	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].	**Low (33%)**	
	SLC19A1	SLC19A1 codes for the reduced folate carrier 1 (RFC1) protein, which transports methotrexate into the cell [18].	**Low (27%)**	
	CDK12	The protein product of the Cyclin Dependent Kinase 12 (CDK12) gene regulates transcription, DNA repair pathways, and cell cycle [19].	**Medium (65%)**	
	MAPs_Mitotic_kinases_neoadj_chemo118	A 118-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**Medium (35%)**	
	MAPs_Mitotic_kinases_neoadj_chemo17	A 17-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**Low (1%)**	
	Early_Relapse_ER.Neg	Chemoresistance gene signature predicting early relapse in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**Medium (62%)**	
	Residual_ disease_ ER.Neg	Chemoresistance gene signature predicting residual disease in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**High (68%)**	
	Pathologic_ response_ ER.Neg	Chemosensitivity gene signature predicting pathological complete response in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**High (75%)**	
	Early_Relapse_ER.Pos	Chemoresistance gene signature predicting early relapse in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Medium (50%)**	
	Residual_ disease_ ER.Pos	Chemoresistance gene signature predicting residual disease in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Medium (50%)**	
	Pathologic_ response_ ER.Pos	Chemosensitivity gene signature predicting pathological complete response in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Medium (65%)**	

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
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.	**Medium (50%)**	
	CD274	CD274 codes for the immune checkpoint marker PD-L1. PD-L1 is the target of atezolizumab (Tecentriq), an immunotherapy approved for approved for the first-line treatment of metastatic TNBC.	**Medium (34%)**	
	CTLA4	Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an immune checkpoint marker and the target of several immunotherapies such as durvalumab (Imfinzi).	**Low (32%)**	
	Module5_ TcellBcell	Immune signatures predictive of response to pembrolizumab in TNBC patients enrolled in (I-SPY2 trial) [14]. All signatures, with the exception of Mast_cells, were associated with increased probability of achieving pathological complete response.	**Medium (47%)**	
	Chemokine12		**Low (26%)**	
	STAT1		**Medium (59%)**	
	Dendritic_cells		**High (83%)**	
	Mast_cells		**Low (11%)**	
DNA damage and repair	VCpred_TN	DNA damage repair / immune signature predictive of response to veliparib (PARP inhibitor) and carboplatin (I-SPY2 trial) [14].	**Medium (63%)**	
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).	**High (92%)**	
	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].	**Medium (57%)**	
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].	**High (90%)**	
	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 TNBC [24].	**Medium (60%)**	
	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.	**High (78%)**	
	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.	**Low (32%)**	
	FOLR1	FOLR1 encodes the protein Folate Receptor Alpha, which is an antibody-drug conjugate target under investigation for the treatment of metastatic TNBC in several phase 1 and 2 clinical trials.	**Low (16%)**	
	F3	F3 codes for tissue factor, coagulation factor III a target of several antibody-drug conjugates in phase 1 clinical trials.	**Medium (62%)**	
	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 (0%)**	
	TPBG	The trophoblast glycoprotein (TPBG) is overexpressed in many breast cancers and is the target of at least two antibody-drug conjugates undergoing phase 1 clinical trials.	**Medium (57%)**	

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
	ROR2	A gene that encodes the Receptor Tyrosine Kinase Like Orphan Receptor 2 protein, a target of the antibody-drug conjugate (Ozuriftamab Vedotin (BA3021/CAB-ROR2-ADC) that is under investigation in a phase clinical trial for advanced solid cancers, including TNBC.	**Low (32%)**	
	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.	**High (69%)**	
	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 phase1 clinical trial for advanced solid cancers, including breast cancer.	**Medium (60%)**	
	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 (95%)**	

INTERPRETATION AND RECOMMENDATIONS

- The elevated levels of ERBB2 and related Her2 amplicon gene signatures together with low-moderate expression of resistance markers (MUC4, NRG1 and pSTAT3-GS) suggest Her2-related treatments like trastuzumab may be beneficial.
- High ERBB2 and TOP1, which is the target of the cytotoxic payload for the ADC trastuzumab deruxtecan suggest this may be beneficial.
- The classification of a luminal A subtype, moderate ESR1 expression, and low E2F4 score suggests endocrine therapies like tamoxifen and aromatase inhibitors may be beneficial.
- The tumor has several markers of resistance to chemotherapies such as 5-fluorouracil (low TYMS expression), gemcitabine/ capecitabine (moderate SLC29A1 expression), methotrexate (low SLC19A1 expression), low proliferation, and low (MAPs_Mitotic_kinases_neoadj_chemo17) and moderate (Pathologic_ response_ER.Pos) expression of signatures predictive of anthracycline/taxane-based chemotherapies, suggesting uncertain benefits of chemotherapy.
- This sample shows high expression of antibody-drug conjugate target CEACAM5 (95th percentile), which is under investigation in phase 2 of clinical trial.

REFERENCES

1. Gendoo, D.M.A. et al. Bioinformatics 32(7): 1097–1099 (2016). **2.** Lehmann, B. D. et al. J Clin Invest 121: 2750–2767 (2011). **3.** Lehmann, B. D. et al. PLoS One 11: e0157368 (2016). **4.** Bareche, Y. et al. Ann Oncol 29: 895–902 (2018). **5.** Paik, S. et al. N Engl J Med 351(27): 2817-2826 (2004). **6.** van't Veer, L.J. et al. Nature 415(6871): 530-536 (2002). **7.** Parker, J.S. et al. J Clin Oncol 27(8): 1160-1167 (2009). **8.** Cardoso, F. et al. Ann Oncol 30(8): 1194-1220 (2019). **9.** Guerrero-Zotano, A.L. et al. Clin Cancer Res 24(11): 2517-2529 (2018). **10.** Mercogliano, M.F. et al. Clin Cancer Res 23(3): 636-648 (2017). **11.** Guardia, C. et al., Clin Cancer Res 27(18): 5096-5108 (2021). **12.** Sonnenblick, A. et al. BMC Med 13:177 (2015). **13.** Wolf, D. M. et al. Cancer Cell 40: 609-623.e6 (2022). **14.** Ma, C.X. et al. Clin Cancer Res 23(15): 4055-4065 (2017). **15.** Loi, S. et al. PNAS 107(22): 10208-10213 (2010). **16.** Foekens, J.A. et al. Cancer Res. 61: 1421-1425 (2001). **17.** Mackey, J.R. et al. Clin Cancer Res. 8(1): 110-116 (2002). **18.** Yang, V. et al. RSC Med Chem. 11(6): 646-664 (2020). **19.** Filippone, M.G. et al. Nat Commun. 13(1): 2642 (2022). **20.** Rodrigues-Ferreira, S. et al. Proc Natl Acad Sci USA 116(47): 23691-23697 (2019). **21.** Hatzis, C. et al. JAMA 305(18):1873-81 (2011). **22.** Karn, T. et al. Clin Cancer Res 26: 1896–1904 (2020). **23.** Modi, S. et al. N Engl J Med 387: 9–20 (2022). **24.** Michaleas, S. et al. ESMO Open 7 (2022).