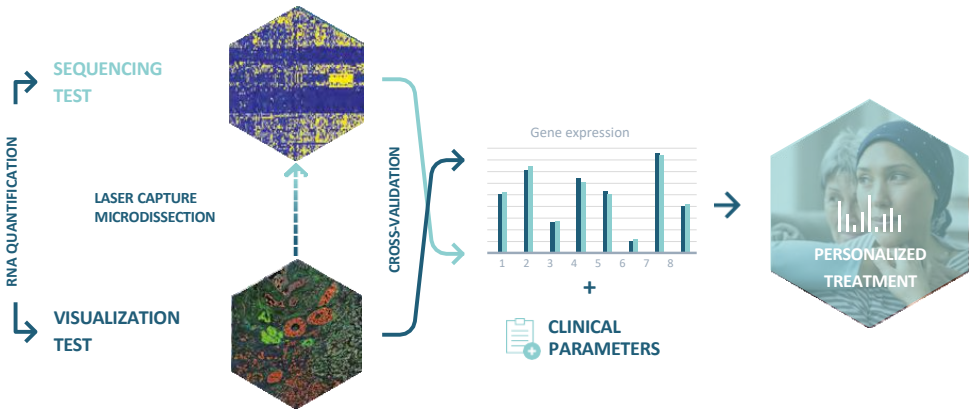
Multiplex8+ RESULTS

PATIENT	SAMPLE	ORDERING PHYSICIAN
Name:	Specimen ID: MDX-PT-1	Name:
ID:	Date of collection:	Address:
Report date:	Type: Metastatic	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	–	–	– low	+
B	–	–	– low	+

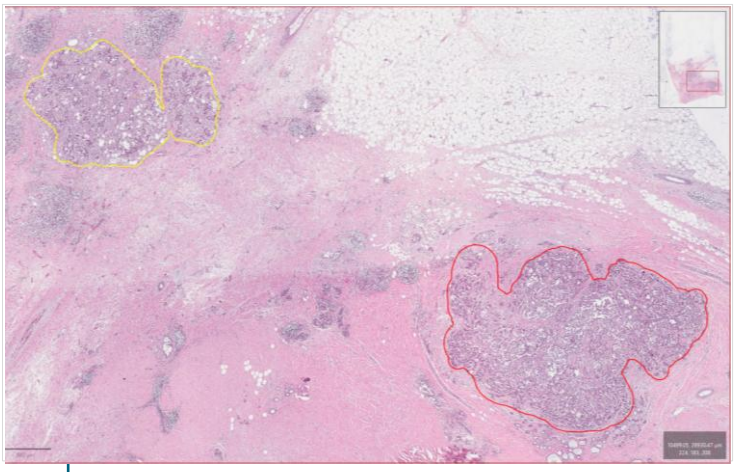
MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype
Basal-like	Mesenchymal (M)
Basal-like	Immuno-modulatory (IM)

RELEVANT TREATMENT

THERAPY	KEY FINDINGS	CLINICAL BENEFIT
Bevacizumab (Avastin)	Molecular subtype, gene expression, gene expression signatures	Predicted benefit
Sacituzumab govitecan (Trodelvy)	Gene expression	Predicted benefit
Trastuzumab deruxtecan (Enhertu)	Gene expression, gene expression signature	Predicted benefit

LASER CAPTURE MICRODISSECTION



Based on histological assessment and RNA-FISH biomarker expression, two samples were laser capture microdissected for further analysis.
Sample A (red outline)
Sample B (yellow outline)

MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype [2-4]
Basal-like	Mesenchymal (M)
Basal-like	Immuno-modulatory (IM)

RECEPTOR STATUS

Sample	ESR1	PGR	ERBB2	MKI67
A	–	–	– low	+
B	–	–	– low	+

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 are concordant with the immunohistochemistry findings.
- According to the SEQUENCING TEST, ERBB2 (HER2) expression is low (HER2-low), therefore this patient may benefit from treatment with trastuzumab deruxtecan (Enhertu).
- *MKI67* (KI67) expression was in the 99th (**Sample A**) and 95th (Sample B) percentile when comparing to results of our retrospective cohort of 1080 breast tumors, suggesting extremely high proliferation.

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 biology of the basal-like tumor type is consistent with the immunohistochemical and clinical designation.
- The Mesenchymal TNBC subtype is characterized by elevated expression of genes involved in epithelial-mesenchymal transition and growth factor pathways, cellular proliferation, an immunosuppressed tumor microenvironment, association with metaplastic histological subtype, poor responses to chemotherapy, and reduced survival [2-4]. M may respond well to treatments targeting the PI3K/mTOR pathways.
- The immunomodulatory TNBC subtype shows enriched immune gene signatures, including checkpoint inhibitor genes, association with high grade, and shows favorable prognosis [2-4].

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	Sample B 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**	**N/A**

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B Percentil
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 (26%)**	**Low (27%)**
	PGR		**Low (13%)**	**Low (24%)**
	ESR1_PGR average	The average gene expression of ESR1 and PGR. Higher levels of hormone receptors are predictive markers for endocrine therapies.	**Low (25%)**	**Low (27%)**
	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].	**High (100%)**	**High (99%)**
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].	**Medium (56%)**	**Low (27%)**
	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 (96%)**	**High (90%)**
	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].	**Low (6%)**	**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].	**High (96%)**	**High (97%)**
	Her2 amplicon_ MDX	Proprietary MDX 43-gene signature used to assess Her2 status.	**High (82%)**	**High (77%)**
	Module7_ ERBB2	Her2-signaling signature predictive of response to multiple anti-Her2 treatments in the I-SPY2 trial [13].	**Medium (49%)**	**Low (27%)**
Proliferation	AURKA	Aurora Kinase A (AURKA) is a protein coding gene involved in cell proliferation and is an independent prognostic marker in breast cancer.	**High (99%)**	**High (98%)**
	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 (99%)**	**High (95%)**
	Module11_ proliferation	Proliferation index used in I-SPY2 trial broadly predictive of pathological complete response in hormone receptor positive patients [4].	**High (84%)**	**Medium (59%)**
	Proliferation_ MDX	Proprietary MDX 7-gene signature used to assess cellular proliferation and cross-validate MKI67 expression levels.	**High (91%)**	**High (68%)**
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 (99%)**	**High (99%)**
	CDK6		**High (98%)**	**High (98%)**
	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].	**High (99%)**	**High (99%)**
	CCND3		**High (96%)**	**High (85%)**
	CDKN2D		**High (96%)**	**High (95%)**
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 (93%)**	**Medium (66%)**

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B Percentile
Chemotherapy	TOP1	The gene encoding DNA topoisomerase I, an enzyme critical for DNA transcription, is a target for anticancer drugs.	**High (100%)**	**High (100%)**
	TOP2A	The gene encoding DNA topoisomerase IIa, an enzyme critical for DNA transcription, is a target for anticancer drugs.	**High (96%)**	**High (89%)**
	RAD51	The DNA repair protein RAD51 homolog 1 (RAD51) is involved in the repair of damaged DNA and is associated with resistance to chemotherapy.	**High (100%)**	**High (99%)**
	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 (91%)**	**High (91%)**
	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 (98%)**	**High (96%)**
	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].	**High (88%)**	**High (70%)**
	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 (99%)**	**High (95%)**
	SLC19A1	SLC19A1 codes for the reduced folate carrier 1 (RFC1) protein, which transports methotrexate into the cell [18].	**High (97%)**	**High (97%)**
	CDK12	The protein product of the Cyclin Dependent Kinase 12 (CDK12) gene regulates transcription, DNA repair pathways, and cell cycle [19].	**High (67%)**	**High (75%)**
	MAPs_Mitotic_kinases_neoadj_chemo118	A 118-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**High (100%)**	**High (100%)**
	MAPs_Mitotic_kinases_neoadj_chemo17	A 17-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**High (100%)**	**High (98%)**
	Early_Relapse_ER.Neg	Chemoresistance gene signature predicting early relapse in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**High (97%)**	**High (92%)**
	Residual_ disease_ ER.Neg	Chemoresistance gene signature predicting residual disease in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**High (99%)**	**High (94%)**
	Pathologic_ response_ ER.Neg	Chemosensitivity gene signature predicting pathological complete response in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**High (100%)**	**High (99%)**
	Early_Relapse_ER.Pos	Chemoresistance gene signature predicting early relapse in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**High (88%)**	**High (85%)**
	Residual_ disease_ ER.Pos	Chemoresistance gene signature predicting residual disease in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**High (100%)**	**High (98%)**
	Pathologic_ response_ ER.Pos	Chemosensitivity gene signature predicting pathological complete response in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**High (99%)**	**High (98%)**

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B 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.	Low (15%)	High (85%)
	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 (56%)	High (83%)
	CTLA4	Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an immune checkpoint marker and the target of several immunotherapies such as durvalumab (Imfinzi).	Medium (37%)	High (83%)
	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.	Low (22%)	Medium (66%)
	Chemokine12		Low (22%)	Medium (61%)
	STAT1		Low (18%)	Medium (54%)
	Dendritic_cells		Low (4%)	Medium (40%)
	Mast_cells		Low (9%)	High (78%)
DNA damage and repair	VCpred_TN	DNA damage repair / immune signature predictive of response to veliparib (PARP inhibitor) and carboplatin (I-SPY2 trial) [14].	Low (14%)	Medium (48%)
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 (75%)	Low (32%)
	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].	High (84%)	High (81%)
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].	Medium (56%)	Low (27%)
	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].	High (100%)	High (100%)
	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 (99%)	High (98%)
	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 (65%)	Medium (48%)
	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.	High (99%)	High (94%)
	F3	F3 codes for tissue factor, coagulation factor III a target of several antibody-drug conjugates in phase 1 clinical trials.	High (99%)	High (98%)
	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.	Medium (43%)	Medium (42%)
	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 (59%)	Medium (51%)

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B 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 (33%)**	**High (93%)**
	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 (96%)**	**High (95%)**
	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.	**High (99%)**	**High (99%)**
	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.	**Low (18%)**	**Medium (40%)**

INTERPRETATION AND RECOMMENDATIONS

- While Sample A and Sample B showed high to low expression of VEGFA, respectively, both samples scored high in a gene signature that assesses hypoxia, angiogenesis, and inflammation. The signature contains genes that are known to be predictive of pathological complete response to bevacizumab (Avastin) [22], suggesting this may be a suitable treatment for this patient.
- Both samples show extremely high TACSTD2 (Trop-2) expression (100th percentile). High levels of Trop-2 are associated with poor survival and the Ascent III trial showed patients with high to medium expression of Trop-2 improved response to sacituzumab govitecan [24]. If the patient is eligible, it is recommended to explore treatment options with sacituzumab govitecan (Trodelvy).
- ERBB2 expression for both samples is in the Her2-low range based on our retrospective cohort of 1080 breast tumors, which was also confirmed by high expression of our proprietary Her2 amplicon signature. If the patient is eligible, it is recommended to explore treatment options with trastuzumab deruxtecan (Enhertu), which has showed efficacy in patients with Her2–low expression in the DESTINY-Breast04 trial [23]. TOP1, the target of deruxtecan, is also very high (100th percentile in both samples) further supporting trastuzumab deruxtecan as a predicted beneficial therapy. However, it is important to note high pSTAT3-GS and MUC4 signatures, that may indicate resistance to trastuzumab.
- Sample B, which is classified as the immunomodulatory (IM) TNBC subtype, showed high to moderate gene expression of immune checkpoint markers (PD-1 and PD-L1) and medium scores for immune signatures predictive of response to pembrolizumab (Keytruda). If the patient is eligible, it is recommended to conduct IHC testing for PD-1 and PD-L1 to check eligibility for treatment with pembrolizumab (Keytruda).

REFERENCES

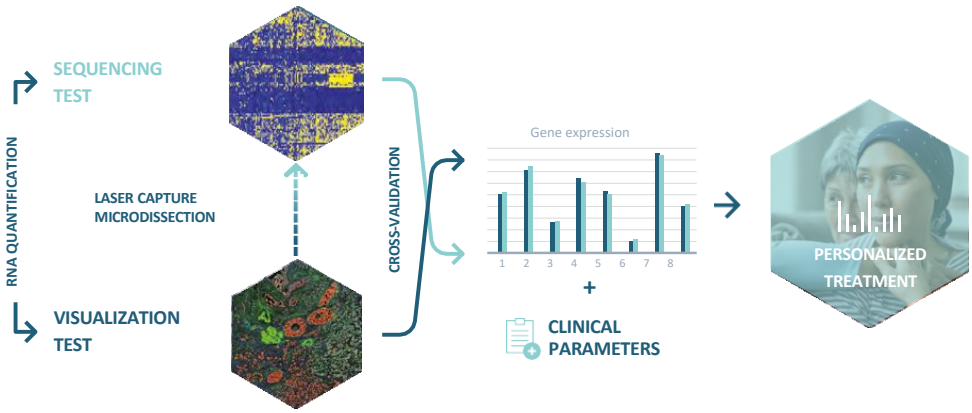
1. Gendoo, D.M.A. et al. Bioinformatics 32(7): 1097–1099 (2016). **2.** Lehmann, B. D. et al. J Clin Invest 121: 2750–2767 (2011). **3.** Lehmann, B. D. et al. PLoS One 11: e0157368 (2016). **4.** Bareche, Y. et al. Ann Oncol 29: 895–902 (2018). **5.** Paik, S. et al. N Engl J Med 351(27): 2817-2826 (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).

Multiplex8+ RESULTS

PATIENT		SAMPLE		ORDERING PHYSICIAN
Name:		Specimen ID:	MDX-PT-1 (primary)	Name:
ID:		Date of collection:		Address:
Report date:	21 December 2023	Type:	Metastatic	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:

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2. **MOLECULAR SUBTYPE:** based on RNA gene expression tumor biology.
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A SUMMARY IS PROVIDED BELOW AND ADDITIONAL DETAILS ARE PROVIDED IN THE FOLLOWING PAGES.

RESULTS SUMMARY

RECEPTOR STATUS

Sample	ESR1	PGR	ERBB2	MKI67
A (primary)	–	–	–	–

MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype
Basal-like	Mesenchymal (M)

RELEVANT TREATMENT

THERAPY	KEY FINDINGS	CLINICAL BENEFIT
Sacituzumab govitecan (Trodelvy)	Gene expression	Predicted benefit
Trastuzumab deruxtecan (Enhertu)	Gene expression	Predicted benefit
ADC clinical studies	Gene expression	Predicted benefit

LASER CAPTURE MICRODISSECTION

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

RECEPTOR STATUS

Sample	ESR1	PGR	ERBB2	MKI67
A (primary)	–	–	–	–

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 are concordant with the immunohistochemistry findings.

MOLECULAR SUBTYPE

Intrinsic subtype	TNBC subtype [2-4]
Basal-like	Mesenchymal (M)

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 biology of the basal-like tumor type is consistent with the immunohistochemical and clinical designation.
- The Mesenchymal TNBC subtype is characterized by elevated expression of genes involved in epithelial-mesenchymal transition and growth factor pathways, cellular proliferation, an immunosuppressed tumor microenvironment, association with metaplastic histological subtype, poor responses to chemotherapy, and reduced survival [2-4].
- M may respond well to treatments targeting the PI3K/mTOR pathways.

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	Sample B 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	Sample B Percentil
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 (14%)**	
	PGR		**Low (14%)**	
	ESR1_PGR average	The average gene expression of ESR1 and PGR. Higher levels of hormone receptors are predictive markers for endocrine therapies.	**Low (13%)**	
	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 (29%)**	
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 (16%)**	
	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 (92%)**	
	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 (64%)**	
	pSTAT3-GS	A signature that predicts phosphorylation of STAT3 and was found to be predictive of trastuzumab resistance in the FinHer study [12].	**Medium (44%)**	
	Her2 amplicon_ MDX	Proprietary MDX 43-gene signature used to assess Her2 status.	**Low (29%)**	
	Module7_ ERBB2	Her2-signaling signature predictive of response to multiple anti-Her2 treatments in the I-SPY2 trial [13].	**Medium (41%)**	
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 (29%)**	
	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].	**Medium (60%)**	
	Module11_ proliferation	Proliferation index used in I-SPY2 trial broadly predictive of pathological complete response in hormone receptor positive patients [4].	**Low (26%)**	
	Proliferation_ MDX	Proprietary MDX 7-gene signature used to assess cellular proliferation and cross-validate MKI67 expression levels.	**Low (30%)**	
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 (28%)**	
	CDK6		**High (67%)**	
	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 (62%)**	
	CCND3		**Low (33%)**	
	CDKN2D		**Medium (36%)**	
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].	**Medium (45%)**	

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B Percentile
Chemotherapy	TOP1	The gene encoding DNA topoisomerase I, an enzyme critical for DNA transcription, is a target for anticancer drugs.	**Medium (51%)**	
	TOP2A	The gene encoding DNA topoisomerase IIa, an enzyme critical for DNA transcription, is a target for anticancer drugs.	**Low (20%)**	
	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 (53%)**	
	ERCC1	The DNA excision repair protein ERCC-1 (ERCC1) is involved in the repair of DNA damage and is associated with resistance to chemotherapy.	**Medium (36%)**	
	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 (25%)**	
	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].	**High (83%)**	
	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].	**Medium (53%)**	
	SLC19A1	SLC19A1 codes for the reduced folate carrier 1 (RFC1) protein, which transports methotrexate into the cell [18].	**High (72%)**	
	CDK12	The protein product of the Cyclin Dependent Kinase 12 (CDK12) gene regulates transcription, DNA repair pathways, and cell cycle [19].	**Low (29%)**	
	MAPs_Mitotic_kinases_neoadj_chemo118	A 118-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**Low (31%)**	
	MAPs_Mitotic_kinases_neoadj_chemo17	A 17-gene signature predicting response to neoadjuvant taxane chemotherapy [20].	**Low (26%)**	
	Early_Relapse_ER.Neg	Chemoresistance gene signature predicting early relapse in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**Medium (33%)**	
	Residual_ disease_ ER.Neg	Chemoresistance gene signature predicting residual disease in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**Medium (53%)**	
	Pathologic_ response_ ER.Neg	Chemosensitivity gene signature predicting pathological complete response in ER-negative (ER-) patients after taxane-anthracycline chemotherapy [21].	**Medium (54%)**	
	Early_Relapse_ER.Pos	Chemoresistance gene signature predicting early relapse in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Medium (35%)**	
	Residual_ disease_ ER.Pos	Chemoresistance gene signature predicting residual disease in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Medium (44%)**	
	Pathologic_ response_ ER.Pos	Chemosensitivity gene signature predicting pathological complete response in ER-positive (ER+) patients after taxane-anthracycline chemotherapy [21].	**Low (30%)**	

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B 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 (39%)	
	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.	Low (20%)	
	CTLA4	Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an immune checkpoint marker and the target of several immunotherapies such as durvalumab (Imfinzi).	Low (15%)	
	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.	Low (20%)	
	Chemokine12		Low (27%)	
	STAT1		Low (24%)	
	Dendritic_cells		Low (7%)	
	Mast_cells		Medium (46%)	
DNA damage and repair	VCpred_TN	DNA damage repair / immune signature predictive of response to veliparib (PARP inhibitor) and carboplatin (I-SPY2 trial) [14].	Low (19%)	
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 (64%)	
	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 (39%)	
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 (16%)	
	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].	High (96%)	
	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 (80%)	
	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 (61%)	
	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.	Medium (41%)	
	F3	F3 codes for tissue factor, coagulation factor III a target of several antibody-drug conjugates in phase 1 clinical trials.	High (98%)	
	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.	Medium (50%)	
	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.	High (81%)	

GENE SIGNATURE

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	Sample B 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.	**Medium (66%)**	
	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.	**High (94%)**	
	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.	**Low (8%)**	

INTERPRETATION AND RECOMMENDATIONS

- The sample has high TACSTD2 (Trop-2) expression (96th percentile). High levels of Trop-2 are associated with poor survival and the Ascent III trial showed patients with high to medium expression of Trop-2 improved response to sacituzumab govitecan (Trodelvy) [24]. Moderate levels of TOP1 (51st percentile), the target of the SN-38 payload of Trodelvy, support the predicted benefit. If the patient is eligible, it is recommended to explore treatment options with sacituzumab govitecan (Trodelvy).
- While the sample is negative for ERBB2 mRNA and protein, there is evidence that Her2 IHC 0 (negative) patients may respond to trastuzumab deruxtecan (Enhertu). In the phase 2 DAISY trial (NCT04132960, EudraCT 2018-004868-57), 33.3% of patients with Her2 IHC 0 had a confirmed objective response to Enhertu. The efficacy of Enhertu in Her2 ultra-low patients is also being investigated in the DESTINY-Breast06 trial (NCT04494425, EudraCT 2019-004493-26). Moderate levels of TOP1 (51st percentile), the target of the deruxtecan payload of Enhertu, support the predicted benefit.
- The patient also has high expression of several antibody drug-conjugate targets that are currently under investigation in clinical trials, including the following: F3 (98th percentile), VTCN1 (94th percentile), TPBG (81st percentile), NECTIN4 (80th percentile), and CD276 (69th percentile).
- High expression of SLC29A1, the transporter for capecitabine, may predict response to this therapy; however, low expression of TYMS, the target of the 5-FU metabolite of capecitabine, suggests an uncertain benefit of this chemotherapy drug.

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