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36P

Multiplexed RNA-FISH-guided Laser Capture Microdissection RNA Sequencing Improves Breast Cancer Molecular Subtyping and Prognostic Classification

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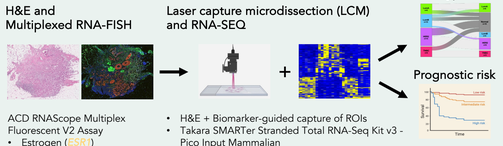
Background

- Multigene tests provide valuable information about molecular breast cancer (BCa) subtypes (Luminal A, Luminal B, Her2 overexpressed, and basal-like) and prognostic risk groups that differ in terms of prognosis, response to therapy, and clinical outcomes[1,2].
- However, multigene tests show only moderate reproducibility at the single-sample level depending on the array platform, tumor composition, gene list, and thresholds[3-5].
- This raises the following questions: *Did I order the right test? Would multiple tests provide better information?*
- **Objective** – We aim to assess the level of discordance in multigene tests and determine if combining information from multiple tests improves diagnostic and prognostic performance.

Methods

The mFISHseq (Multiplex8+) assay



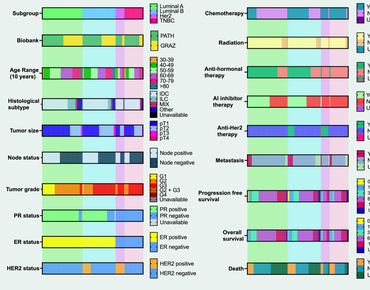
ACD RNAScope Multiplex Fluorescent V2 Assay
- Estrogen (*ESR1*)
- Progesterone (*PGR*)
- Her2 (*ERBB2*)
- Ki67 (*MKI67*)

- H&E + Biomarker-guided capture of ROIs
- Takara SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian
- NovaSeq 6000 –100M reads/sample (2 x 100 PE)
- Signatures implemented via GeneFu[6]
  - PAM50, AIMS
  - OncotypeDX, ROR-S, GGI, and GENE70

- Classifier concordance (Cohen's kappa)
- Kaplan-Meier (log-rank) and Cox regression

Retrospective cohort

- 1,082 FFPE breast tissues
- 2 biobanks (Biobank Graz, PATH Biobank), 1 hospital (Malaga), 2 companies
- Clinicopathological data
  - 18 years follow up
- Signed informed consent
- Approved by the Ethics Committee of the Bratislava Self-Governing Region (Ref. No. 05320/2020/HF) and the Ethics Commission of the Medical University of Graz on behalf of Biobank Graz (No. 34-354 ex 21/21, 1158-2022)



Results – Molecular Subtyping

1 All multigene subtyping tools classify similar proportions of samples into respective subtypes and show equivalent prognostic ability

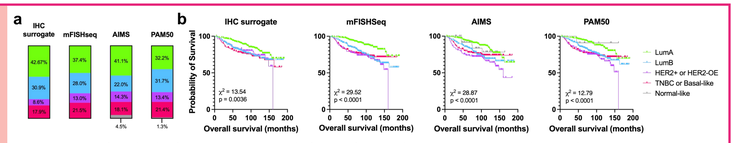


Figure. Proportion of samples classified into each subtype by the four classifiers (**a**) and their association with overall survival (**b**).

2 Discordance in ≥1 multigene molecular subtyping classifier occurs in 45% of patients leading to clinically relevant differences in survival

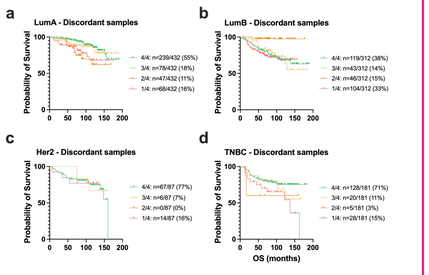


Figure. Overall survival (OS) stratified by IHC surrogate subtype for Luminal A (**a**), Luminal B (**b**), Her2 (**c**), and TNBC (**d**) samples that showed perfect concordance between all four classifiers (4/4) as well as discordant samples (3/4, 2/4, or only the IHC surrogate classifier, 1/4).

3 Assigning subtypes by combining multigene classifiers using a majority rule voting scheme results in better patient stratification

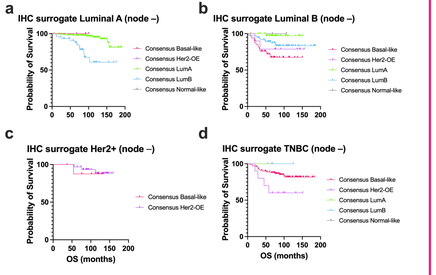


Figure. Overall survival (OS) stratified by IHC surrogate subtype for node negative Luminal A (**a**), Luminal B (**b**), Her2 (**c**), and TNBC (**d**) samples according to their consensus subtype as determined by a majority vote of mFISHseq, AIMS, and PAM50 classifications.

4 As single-sample discordance increases among multigene classifiers, the correlation to PAM50 centroids decreases highlighting the instability

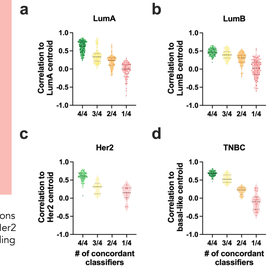


Figure. PAM50 centroid correlations for Luminal A (**a**), Luminal B (**b**), Her2 (**c**), and TNBC (**d**) samples according to the number of concordant classifiers.

5 Consensus subtyping reclassifies 30% of samples into subtypes that better match their tumor biology, resulting in better stratification according to survival

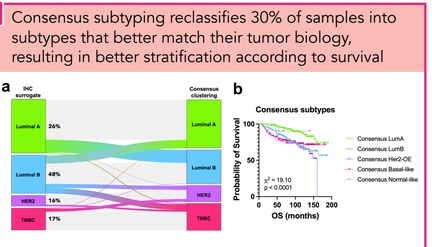


Figure. (**a**) Sankey diagram shows the IHC surrogate subtypes and the proportion (%) of samples reclassified by consensus subtyping. (**b**) Overall survival (OS) of consensus molecular subtypes.

Results – Prognostic risk

6 While multigene risk tools classify different proportions of samples into respective risk groups, they show similar prognostic ability

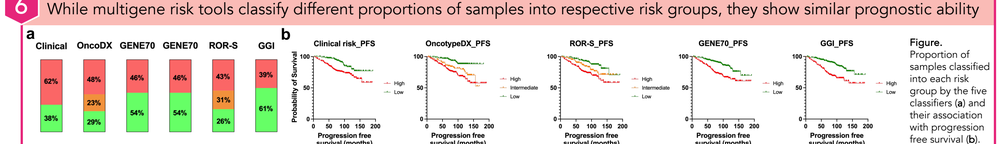


Figure. Proportion of samples classified into each risk group by the five classifiers (**a**) and their association with progression free survival (**b**).

7 Discordance in ≥1 multigene prognostic risk classifiers occurs in 61.8% of patients leading to clinically relevant differences in survival

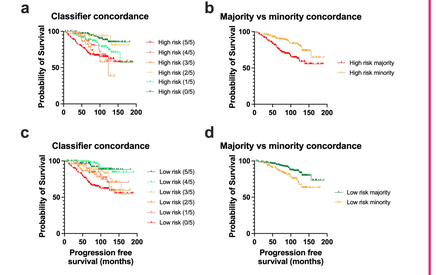


Figure. Concordance of each classifier for high (**a**,**b**) and low (**c**,**d**) risk samples as illustrated by the number of concordant classifiers (**a**,**c**) and after consolidating into majority (agreement in ≥3 prognostic classifiers) and minority (agreement in 1-2 prognostic classifiers) categories (**b**,**d**).

8 Discordance in high-risk classification is associated with increased *PGR* and reduced *MKI67* expression

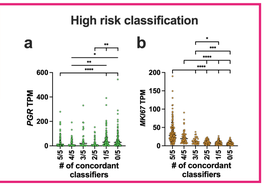


Figure. *PGR* (**a**) and *MKI67* (**b**) gene expression depending on the number of concordant classifiers for high-risk classification. .

9 Combining multigene classifiers using a decision tree may identify patients with early/late relapse and indolent tumors

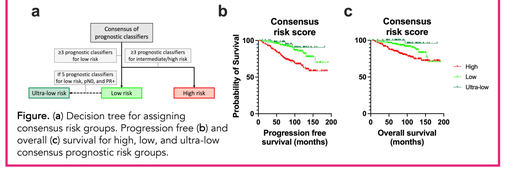


Figure. (**a**) Decision tree for assigning consensus risk groups. Progression free (**b**) and overall (**c**) survival for high, low, and ultra-low consensus prognostic risk groups.

Summary and Conclusions

- Like prior reports[3-5], we observed considerable discordance at the single-sample level, which resulted in **unstable classification and altered prognosis as the level of discordance increased.**
- Approximately **60%** of reclassified patients by consensus molecular subtyping received **suboptimal treatment.**
- By leveraging unbiased transcriptome profiling, the spatially informed mFISHseq (Multiplex8+) assay provides a novel approach to ***combine multigene tests, yielding superior diagnostic and prognostic information.*** This finding is supported by analysis of the TEAM pathology study[7].

References: 1. Litton, J. K., et al. Am Soc Clin Oncol Educ Book 39, e1–e7 (2019). **2**. Kittaneh, M., et al. Biomark Cancer 5, 61–70 (2013). **3**. Mackay, A. et al. JNCI: Journal of the National Cancer Institute 103, 662–673 (2011). **4**. Weigelt, B. et al. The Lancet Oncology 11, 339–349 (2010). **5**. Bartlett, J. M. S. et al. J Natl Cancer Inst 108, djw050 (2016). **6**. Gendoo, D. M. A. et al. Bioinformatics 32, 1097–1099 (2016). **7**. Bartlett, J.M.S. et al. npj Breast Cancer 7, 90 (2021).

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