# Differently expressed immune related genes in metastatic vs. non-metastatic LUMA, LUMB1 and TNBC primary breast carcinoma cases

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# Conflict of interest

- Biomedica Ltd. sponsored my travel to the ECP Nice.

Biomedica Ltd. is the Hungarian distributor of Nanostring.

# Introduction

**The contribution of the immune micro-environment of breast cancer to metastatic progression is less clear and has been less extensively studied.**

Why? Small number of metastatic specimens available? High number of very contradictory results?

**Current clinical efforts are focused on:**

- identifying biomarkers that can predict the response to single-agent immunotherapy
- identify the best immunotherapy combinations for a particular patient
- developing immunotherapy combinations that convert nonresponder patients to responders
- deepen those responses that do occur

# Aim of the study

To define the „immune gene signature” associated with metastatic potential of LUMA, LUMB1 and TNBC cases by using Nanostring technology.

# Patients

- 35 FFPE breast carcinoma cases presenting ≥1% stromal TIL and with a minimum of 6 year available follow up data.

  -6 non-metastatic LUMA (KA), 6 metastatic LUMA (A)

  -5 non-metastatic LUMB1(KB), 6 metastatic LUMB1(B)

  -6 non-metastatic TNBC (KT), 6 metastatic TNBC (T)

| *Molecular subtype* | *Surrogate subtype* | *ER* | *PgR* | *HER2* | *PI (Ki-67)* |
|---|---|---|---|---|---|
| Luminal A | Luminal A-like | + | ≥20% | - | <20% |
| Luminal B | Luminal B-like (HER2-negative) | + | <20%* | - | ≥20%* |
| | Luminal B-like (HER2-positive) | + | any | + | Any |
| HER2-overexpression | HER2-positive | - | - | + | Any |
| Basal-like | Triple negative | - | - | - | Any |

ER: Estrogen receptor; PgR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; PI: Proliferation index; +: Positive; -: Negative; *: Only one of these criteria must be met to define luminal B-like breast cancer

**Surrogate definitions of molecular subtypes of breast cancer according to St Gallen 2013 consensus**

# Evaluation of tumor-infiltrating lymphocytes (TILs)

TIL was assessed on HE stained slides following the scoring guidelines of the International Immuno-Oncology Biomarker Working Group on Breast Cancer.

Seminars in Cancer Biology Volume 52, Part 2, October 2018, Pages 16-25

The ***highest*** TIL was observed in non-metastatic TNBC cases (mean 9, 66%) and
the ***lowest*** in metastatic LUMA cases (mean 1,16%).

# Methods

**We compared mRNA expression level using a 730 immune-related gene panel by using Nanostring technology**

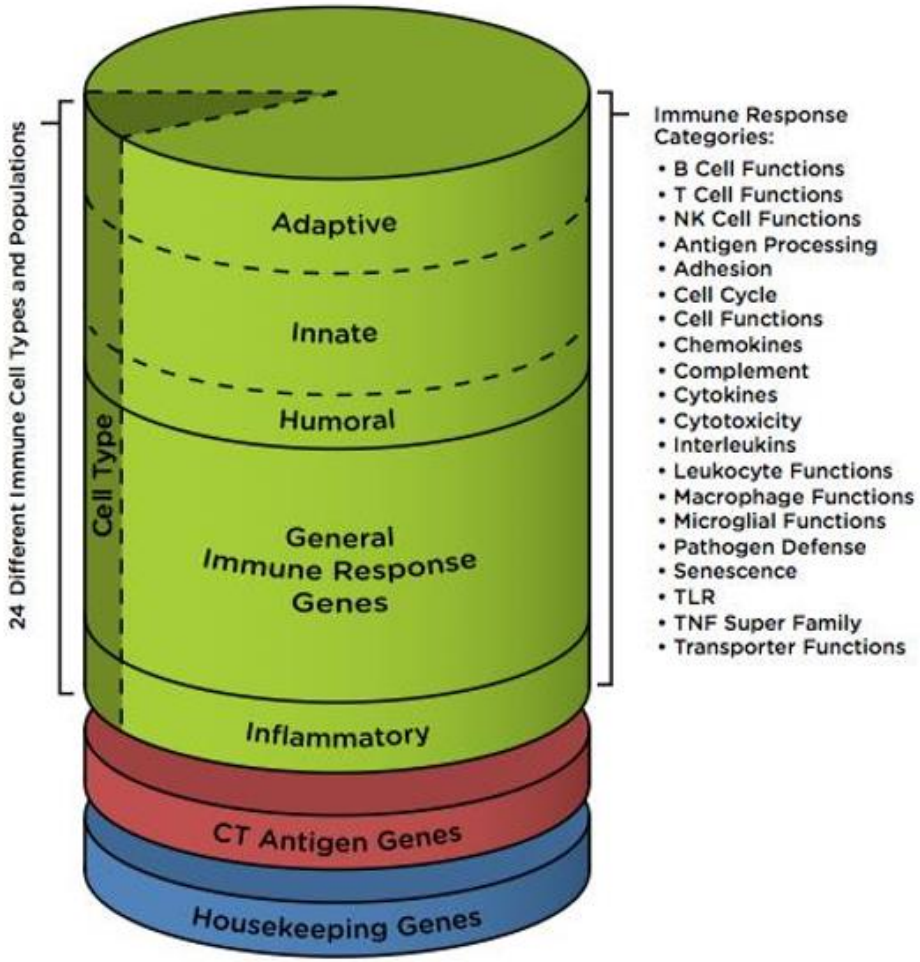
- Total RNA was isolated from 4x5 µm FFPE sections using the Qiagen RNeasy FFPE kit

- RNA was measured with Qubit system

- 250 ng RNA was hybridized to the NanoString nCounter® PanCancer Immune Profiling Panel code set and read on the nCounter platform

- The nSolver 4.0 software was used to normalize expression values.

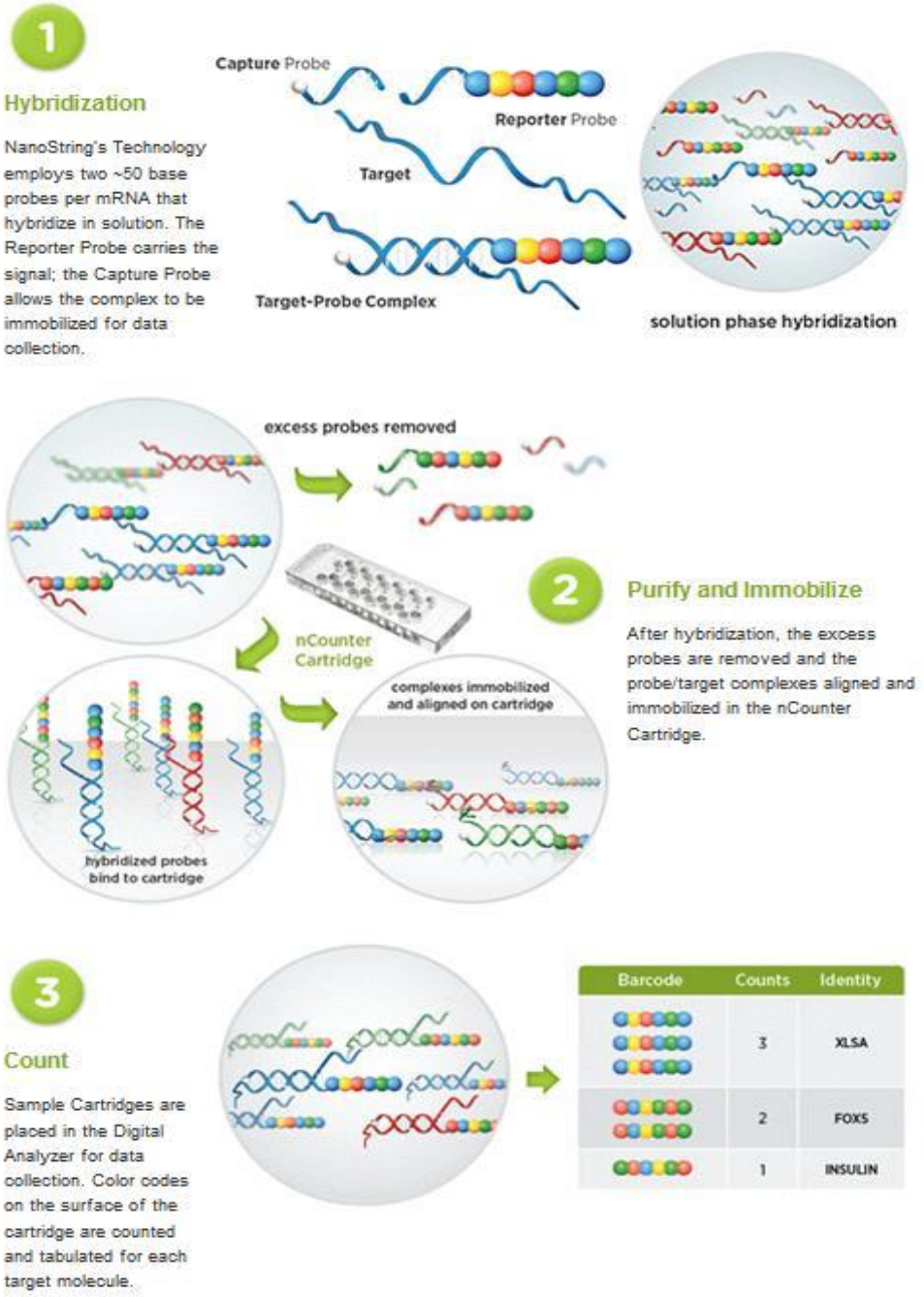
# nCounter® PanCancer Immune Profiling Panel- immune cell type gene coverage




| Annotation | # Genes |
|---|---|
| Adaptive Immune System | 141 |
| Apoptosis | 54 |
| Autophagy | 11 |
| B cell Receptor Signaling | 35 |
| Cell Adhesion | 60 |
| Chemokine Signaling | 63 |
| Complement System | 39 |
| Cytokine Signaling | 259 |
| Hemostasis | 73 |
| Host-pathogen Interaction | 252 |
| Immunometabolism | 32 |
| Inflammasomes | 8 |
| Innate Immune System | 201 |
| Lymphocyte Activation | 245 |
| Lymphocyte Trafficking | 21 |
| MHC Class I Antigen Presentation | 39 |
| | |
| MHC Class II Antigen Presentation | 14 |
| NF-kB Signaling | 62 |
| NLR signaling | 64 |
| Oxidative Stress | 36 |
| Phagocytosis and Degradation | 48 |
| T Cell Receptor Signaling | 61 |
| TGF-b Signaling | 9 |
| Th1 Differentiation | 14 |
| Th17 Differentiation | 31 |
| Th2 Differentiation | 17 |
| TNF Family Signaling | 49 |
| TLR Signaling | 73 |
| Transcriptional Regulation | 53 |
| Treg Differentiation | 10 |
| Type I Interferon Signaling | 28 |
| Type II Interferon Signaling | 36 |

https://www.nanostring.com/scientific-content/technology-overview/ncounter-technology

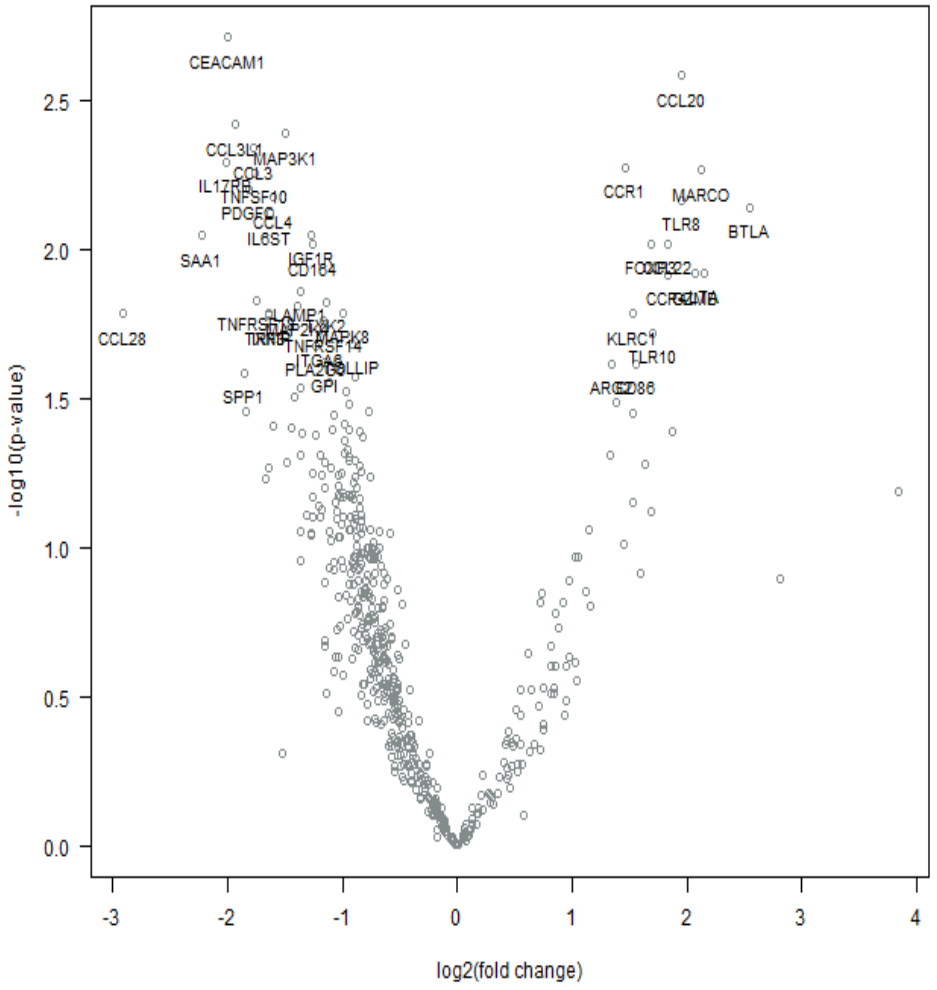
# Nanostring technology




https://www.nanostring.com/scientific-content/technology-overview/ncounter-technology

# Results

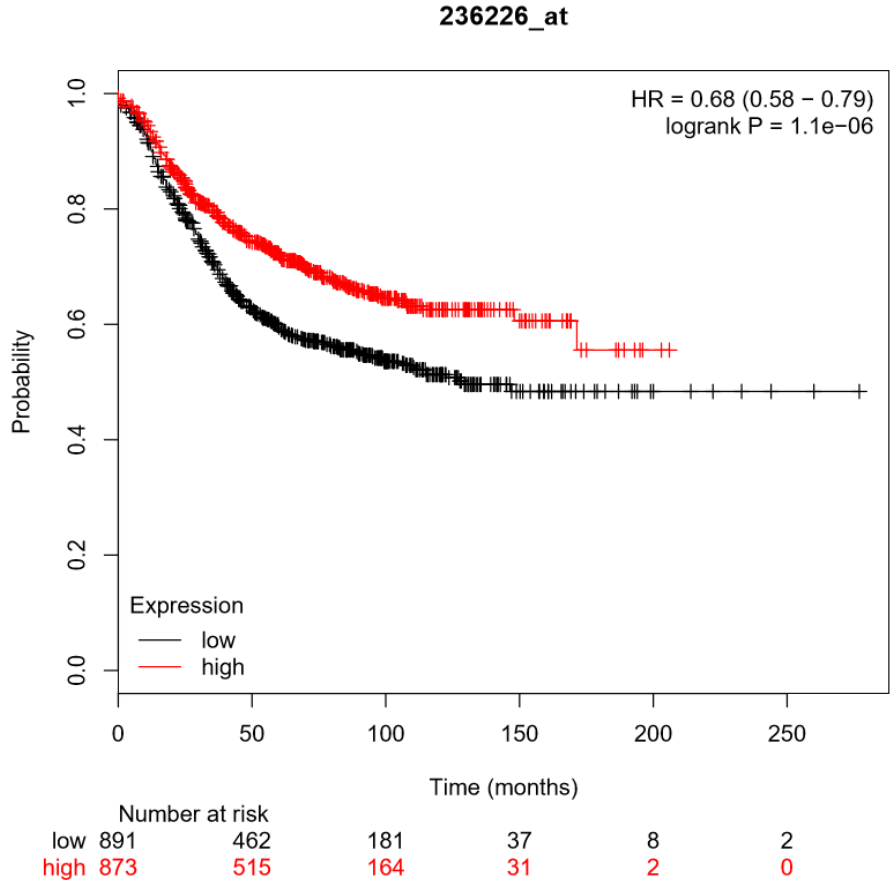
# Differentially expressed genes in non-metastatic (KA) vs. metastatic (A) LUMA breast carcinoma cases



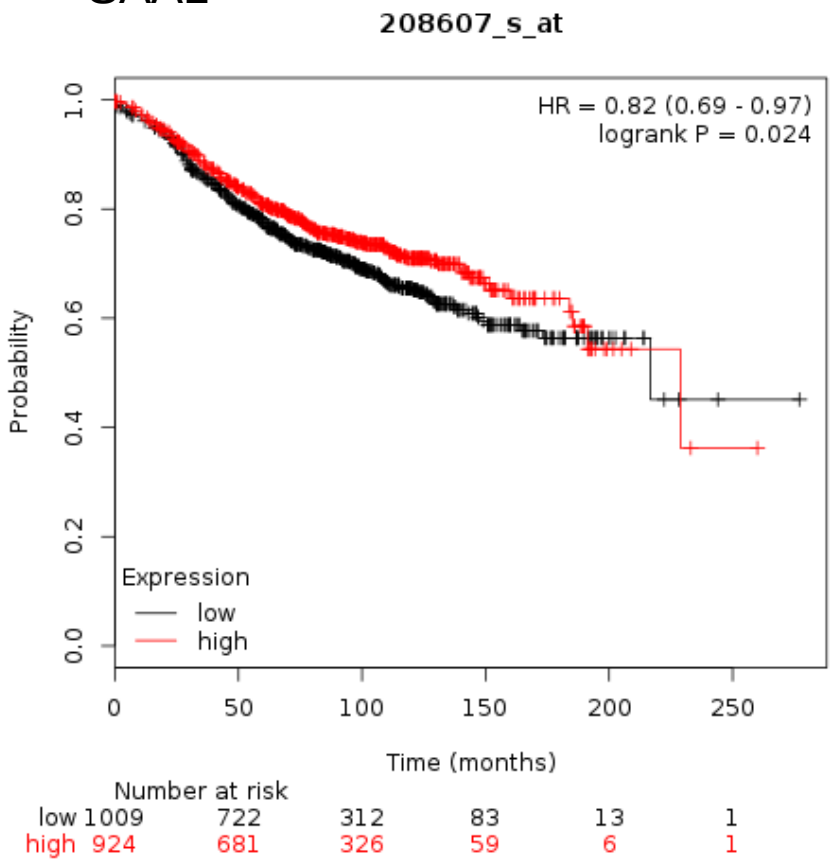

| gene | Log2 fold change | P-value |
|---|---|---|
| SAA1-mRNA | -2.23 | 0.00895 |
| IL17RB-mRNA | -2.02 | 0.00507 |
| CEACAM1-mRNA | -2 | 0.00193 |
| **CCL3L1-mRNA** | **-1.94** | **0.00379** |
| PDGFC-mRNA | -1.82 | 0.00625 |
| **CCL3-mRNA** | **-1.78** | **0.00456** |
| TNFSF10-mRNA | -1.76 | 0.00551 |
| IL6ST-mRNA | -1.63 | 0.00757 |
| **CCL4-mRNA** | **-1.61** | **0.00665** |
| MAP3K1-mRNA | -1.5 | 0.00407 |
| **CCR1-mRNA** | **1.46** | **0.00529** |
| FOXP3-mRNA | 1.69 | 0.00958 |
| **CCL22-mRNA** | **1.83** | **0.00949** |
| **CCL20-mRNA** | **1.95** | **0.0026** |
| TLR8-mRNA | 1.95 | 0.0068 |
| MARCO-mRNA | 2.130 | 0.00539 |
| LTA-mRNA | 2.150 | 0.0119 |
| BTLA-mRNA | 2.55 | 0.00723 |

# Expression of BTLA and SAA1 in correlation with survival data (Kaplan Meier-plotter) in LUMA cases

BTLA




SAA1




BTLA⁺ TIL were present in 15 of the 660 breast cancer cases (2.3 %, range of BTLA⁺ TIL 1–452) suggesting that this co-inhibitory receptor does not play a biologically relevant role in breast cancer immunosurveillance. Breast Cancer Res Treat. 2013 Jun; 139(3): 10.1007/s10549-013-2581-3.

Gyorffy B, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, **Breast Cancer Res Treatment,** 2010 Oct;123(3):725-31

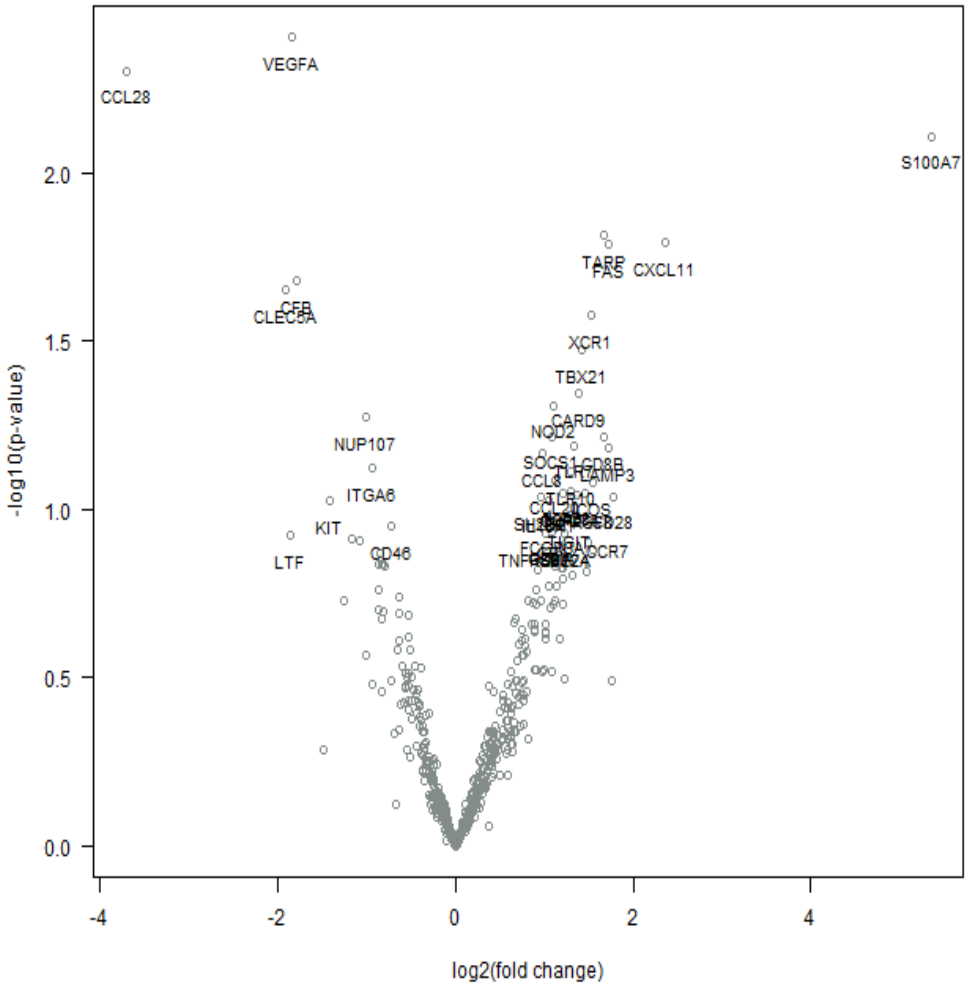
# CC- Chemokines associated with breast cancer progression

Univariate Cox analysis of prognostic association of all 24 CC-Chemokine's expression on metastatic relapse in BrCa based on the bc-GenExMiner v4.1 DNA gene chip database.

| Chemokine | p value | HR | 95% CI |
|---|---|---|---|
| CCL1 | 0.4894 | 0.98 | 0.92–1.04 |
| CCL2 | 0.6384 | 1.01 | 0.96–1.08 |
| CCL3 | 0.0785 | 0.91 | 0.83–1.01 |
| **CCL4** | 0.0038 | 0.91 | 0.86–0.97 |
| **CCL5** | 0.0335 | 0.94 | 0.88–0.99 |
| CCL7 | 0.173 | 1.05 | 0.98–1.11 |
| **CCL8** | 0.0017 | 1.1 | 1.04–1.17 |
| CCL11 | 0.4707 | 0.98 | 0.92–1.04 |
| CCL13 | 0.9444 | 1 | 0.93–1.07 |
| CCL14 | 0.5541 | 0.94 | 0.76–1.15 |
| CCL15 | 0.1379 | 1.2 | 0.94–1.52 |
| CCL16 | 0.6384 | 1.01 | 0.96–1.08 |
| CCL17 | 0.8037 | 0.99 | 0.93–1.06 |
| CCL18 | 0.6961 | 1.01 | 0.95–1.08 |
| **CCL19** | 0.001 | 0.9 | 0.85–0.96 |
| CCL20 | 0.8989 | 1 | 0.94–1.07 |
| **CCL21** | <0.0001 | 0.88 | 0.82–0.93 |
| **CCL22** | <0.0001 | 0.87 | 0.82–0.93 |
| **CCL23** | 0.0014 | 0.88 | 0.81–0.95 |
| CCL24 | 0.9997 | 1 | 0.94–1.07 |
| CCL25 | 0.1064 | 1.05 | 0.99–1.12 |

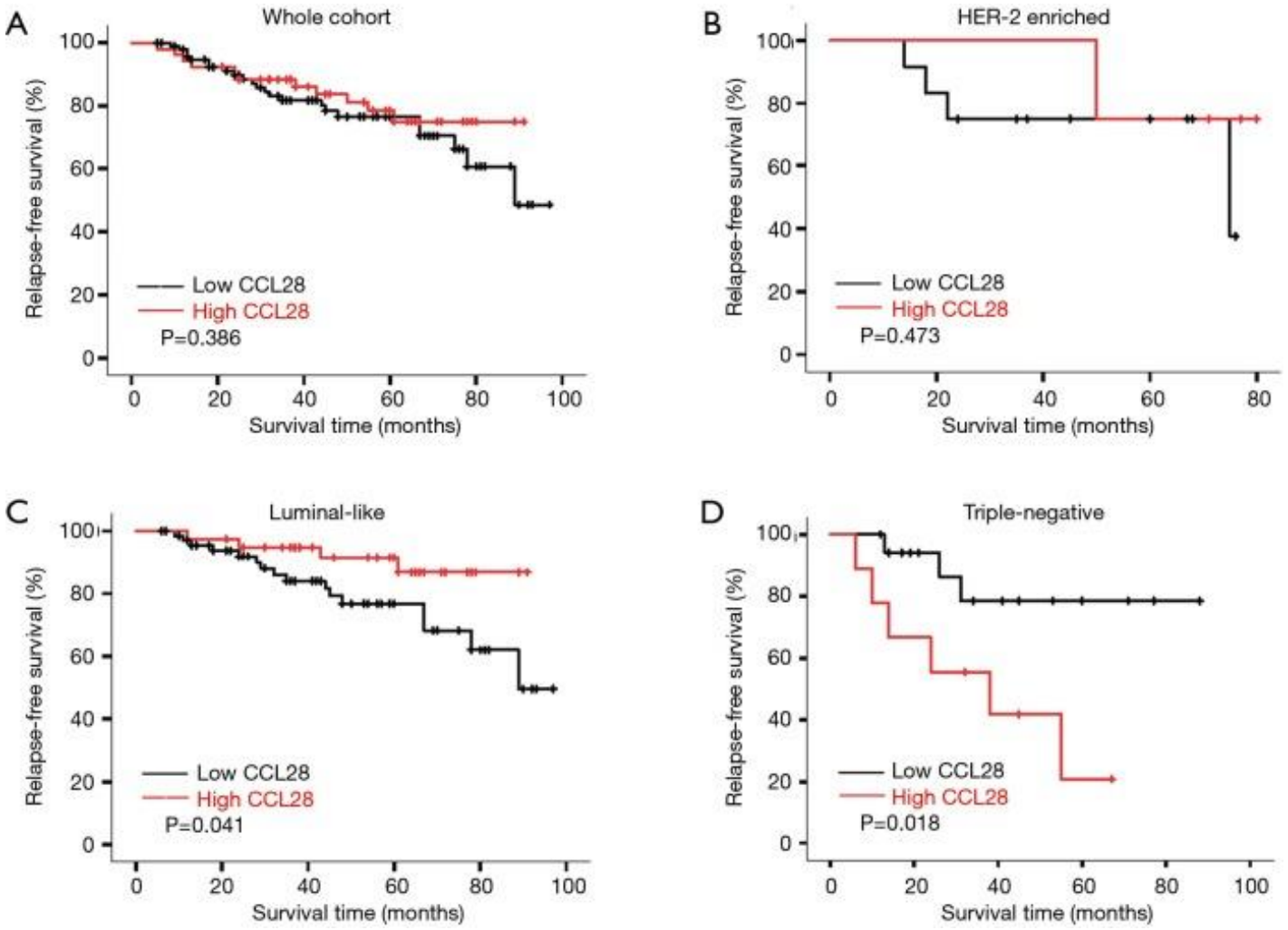
Sci Rep. 2019 Mar 8;9(1):4014..
**CC chemokines are differentially expressed in** Breast **Cancer and are associated with disparity in overall survival.**

# Differentially expressed genes in non-metastatic (KB) vs. metastatic (B) LUMB1 breast carcinoma cases



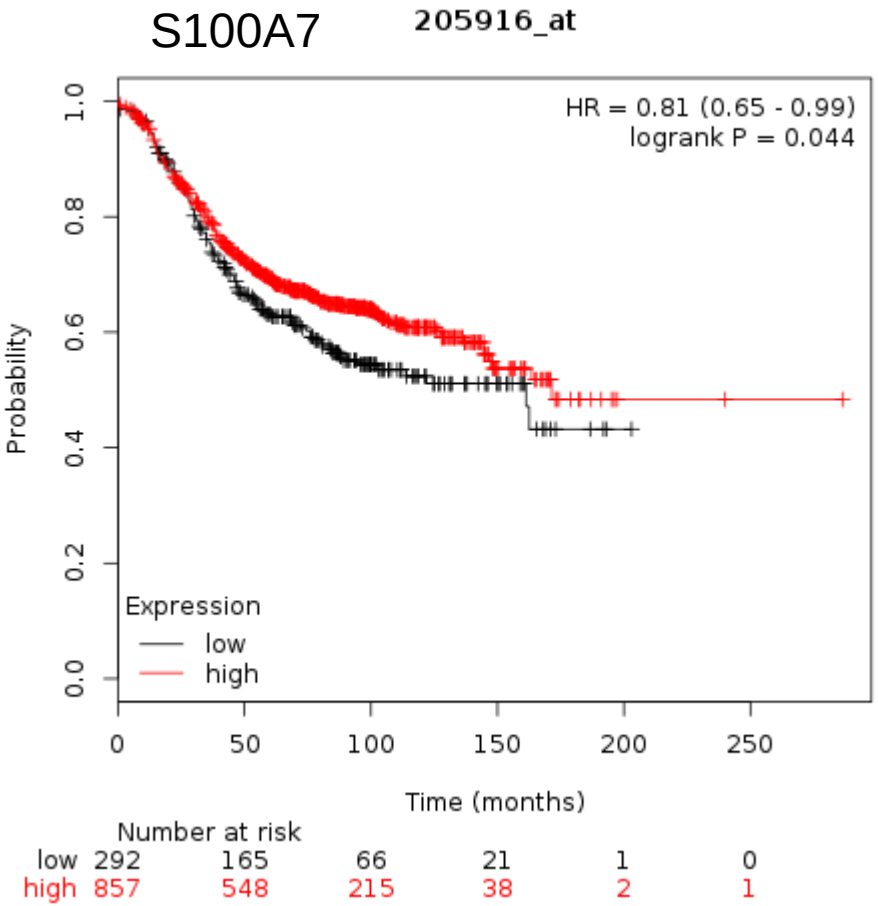

| gene | Log2 fold change | P-value |
|---|---|---|
| **CCL28-mRNA** | **-3.71** | **0.00497** |
| CLEC5A-mRNA | -1.92 | 0.0223 |
| VEGFA-mRNA | -1.84 | 0.00396 |
| CFB-mRNA | -1.78 | 0.021 |
| **XCR1-mRNA** | **1.52** | **0.0266** |
| TARP-mRNA | 1.67 | 0.0154 |
| FAS-mRNA | 1.73 | 0.0163 |
| **CXCL11-mRNA** | **2.36** | **0.0161** |
| S100A7-mRNA | 5.35 | 0.00778 |

# CCL28 association with survival



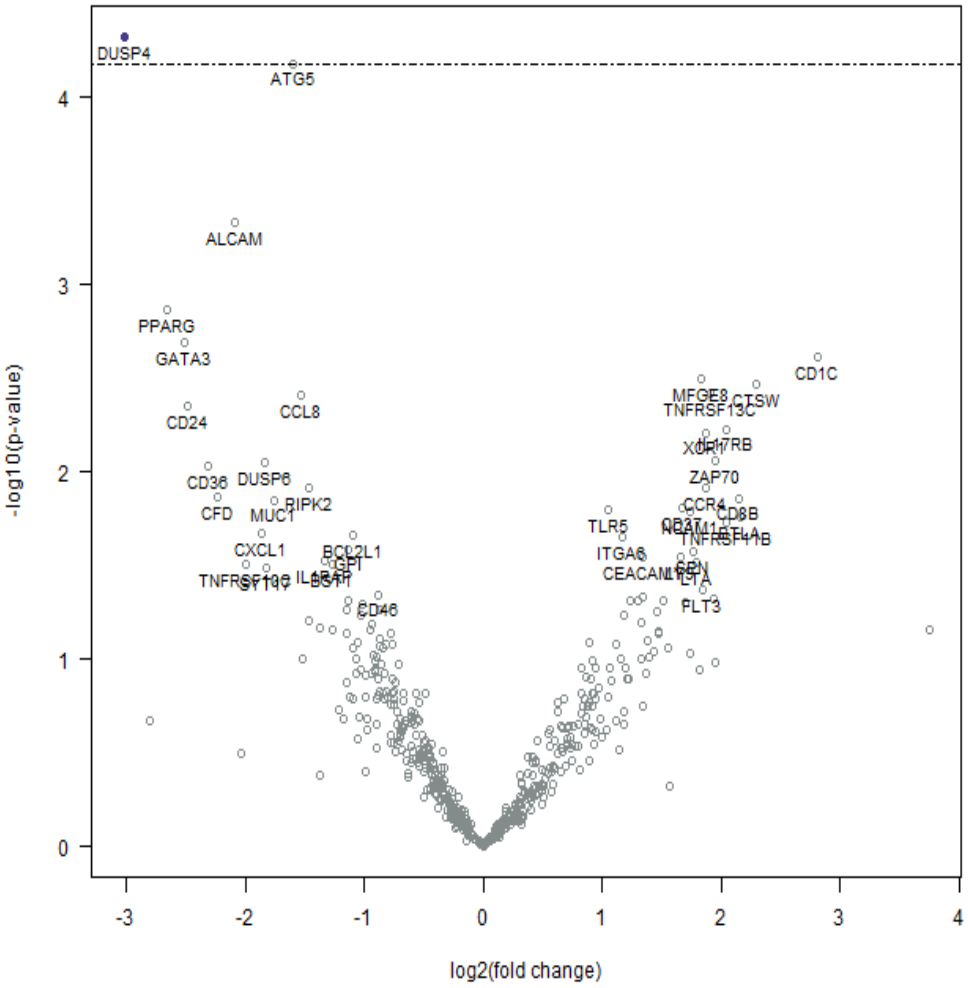

CCL28 was a favorable prognostic factor for luminal-like cases and detrimental for triple-negative subtype, indicating that the same chemokine may play different or even opposite roles in the recurrence and metastasis of different molecular subtypes of breast cancer. J Thorac Dis. 2019 Mar;11(3):777-787. doi: 10.21037/jtd.2019.02.26.

# Expression of S100A7 in correlation with survival data (Kaplan Meier-plotter) in LUMB1 cases




Gyorffy B, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, **Breast Cancer Res Treatment,** 2010 Oct;123(3):725-31

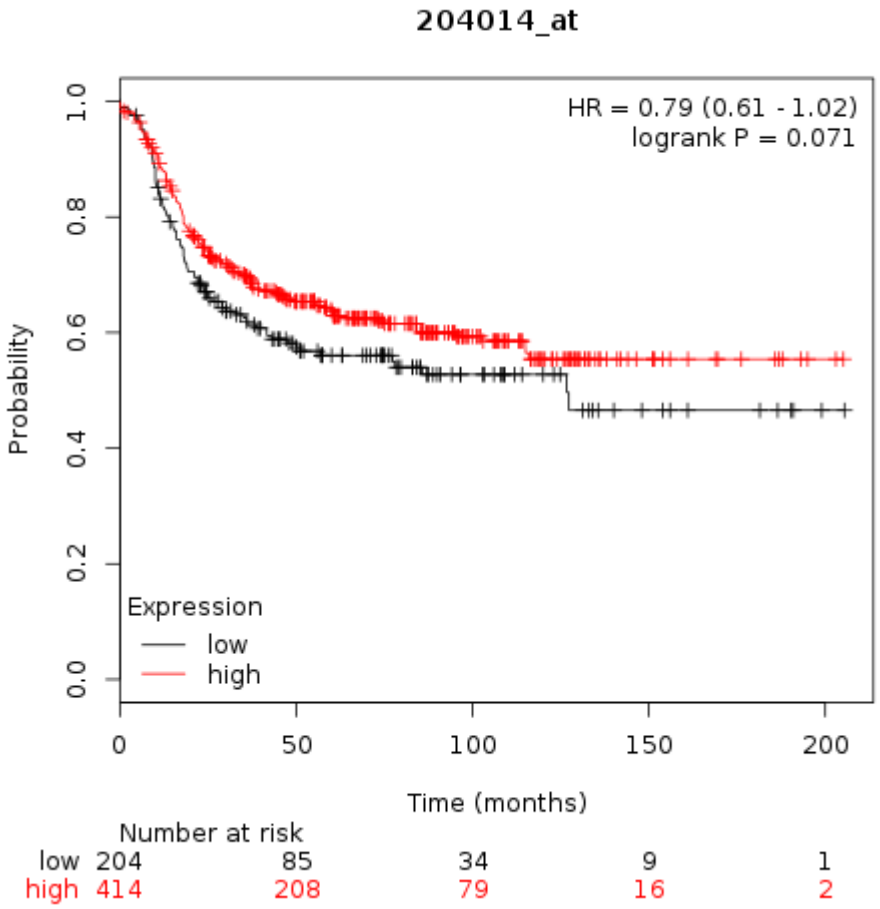
# Differentially expressed genes in non-metastatic (KT) vs. metastatic (T)TNBC breast carcinoma cases



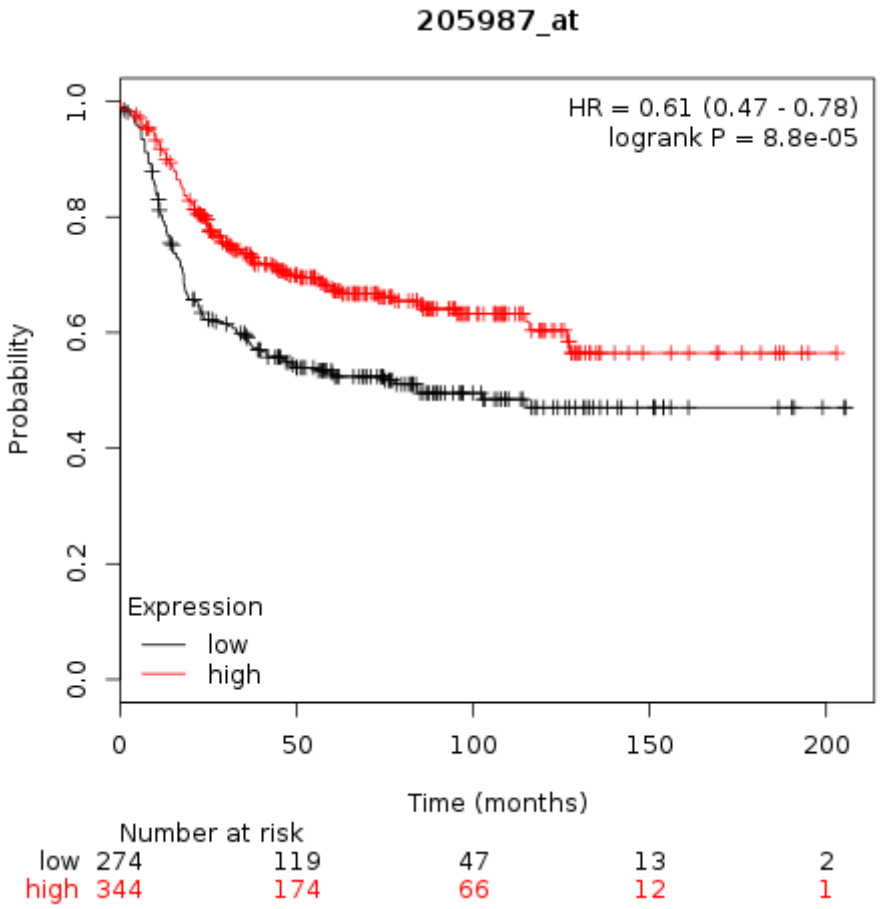

| Gene | Log2 fold change | P-value |
|---|---|---|
| DUSP4-mRNA | -3.02 | 4.77e-05 |
| PPARG-mRNA | -2.66 | 0.00137 |
| GATA3-mRNA | -2.51 | 0.00205 |
| CD24-mRNA | -2.48 | 0.00451 |
| CD36-mRNA | -2.31 | 0.00934 |
| CFD-mRNA | -2.23 | 0.0138 |
| ALCAM-mRNA | -2.09 | 0.00047 |
| DUSP6-mRNA | -1.83 | 0.00895 |
| ATG5-mRNA | -1.6 | 6.57e-05 |
| CCL8-mRNA | -1.53 | 0.00391 |
| RIPK2-mRNA | -1.47 | 0.0123 |
| MFGE8-mRNA | 1.84 | 0.00321 |
| CCR4-mRNA | 1.87 | 0.0122 |
| XCR1-mRNA | 1.88 | 0.00621 |
| TNFRSF13C-mRNA | 1.92 | 0.00385 |
| ZAP70-mRNA | 1.95 | 0.0088 |
| IL17RB-mRNA | 2.050 | 0.00594 |
| CD8B-mRNA | 2.150 | 0.0139 |
| CTSW-mRNA | 2.300 | 0.00344 |
| CD1C-mRNA | 2.81 | 0.00244 |

# Expression of DUSP4 and CD1C in correlation with survival data (Kaplan Meier-plotter) in TNBC cases

DUSP4




CD1C

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# Conclusions

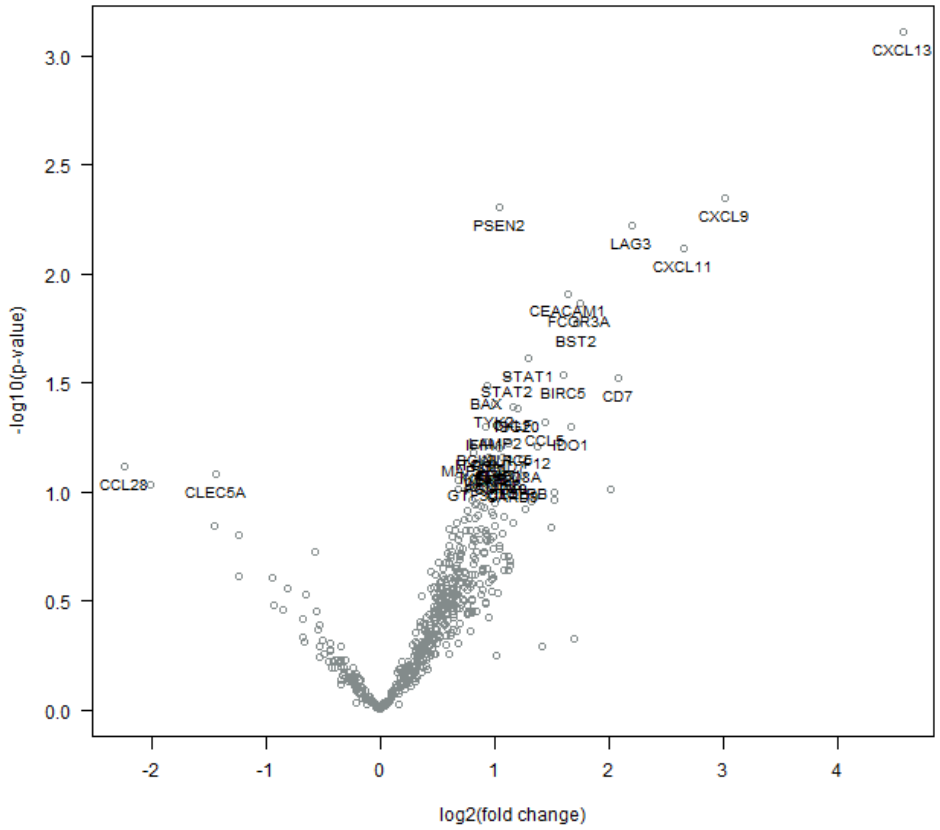
- Cancer immunity genes involved in tumor progression vary considerably among breast carcinoma (surrogate) subtypes.

- Chemokines and their receptors might be involved in metastatic progression of LUMA breast carcinoma subtypes.

# Thank you for your attention!

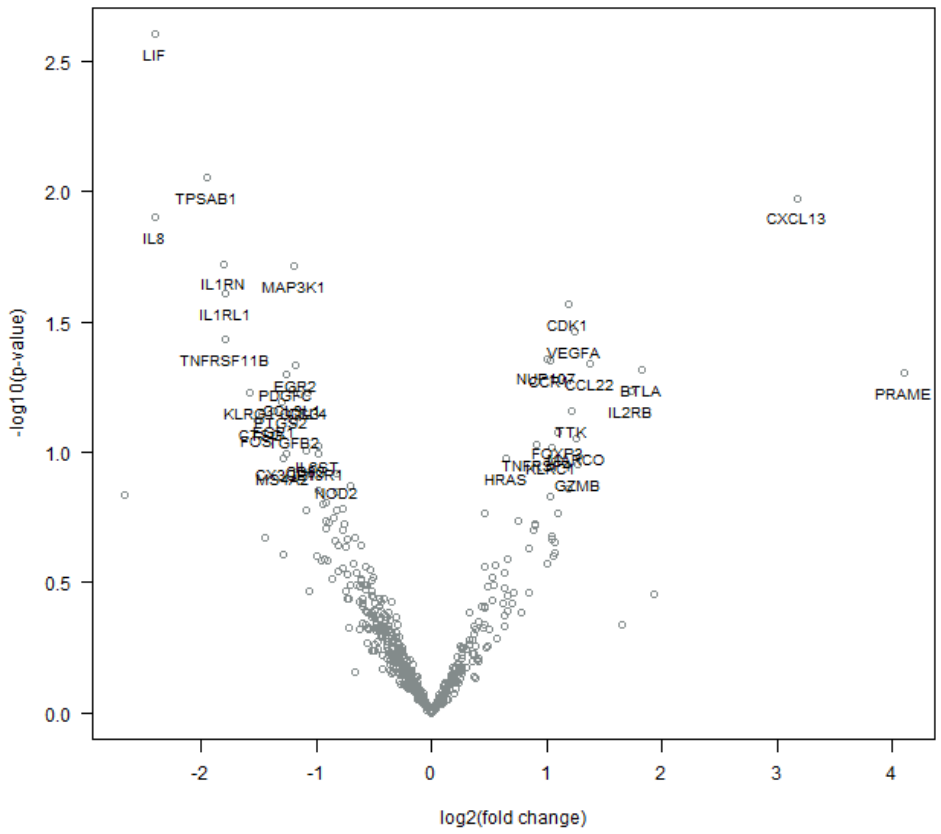
**The work was supported by NVKP_16-1-2016-0004 grant and the congress participation by Biomedica Ltd. Hungary**

# Differentially expressed genes in non-metastatic (KB) vs. non-metastatic (KA) breast carcinoma cases




| genes | Log2 fold change | P-value |
|---|---|---|
| IFIH1-mRNA | 0.929 | 0.0502 |
| BAX-mRNA | 0.941 | 0.0325 |
| TYK2-mRNA | 0.998 | 0.0396 |
| LAMP2-mRNA | 1.020 | 0.0496 |
| PSEN2-mRNA | 1.050 | 0.00493 |
| STAT2-mRNA | 1.110 | 0.0289 |
| CKLF-mRNA | 1.1170 | 0.0409 |
| ISG20-mRNA | 1.210 | 0.0414 |
| STAT1-mRNA | 1.300 | 0.0244 |
| CCL5-mRNA | 1.45 | 0.0478 |
| BIRC5-mRNA | 1.600 | 0.029 |
| CEACAM1-mRNA | 1.64 | 0.0123 |
| IDO1-mRNA | 1.67 | 0.0501 |
| BST2-mRNA | 1.72 | 0.0169 |
| FCGR3A-mRNA | 1.75 | 0.0136 |
| CD7-mRNA | 2.080 | 0.0302 |
| LAG3-mRNA | 2.220 | 0.00598 |
| CXCL11-mRNA | 2.65 | 0.00764 |
| CXCL9-mRNA | 3.020 | 0.00451 |
| CXCL13-mRNA | 4.57 | 0.000779 |

# Differentially expressed genes in metastatic (B) vs. metastatic (A) breast carcinoma cases




| genes | Log2 fold change | P-value |
|---|---|---|
| LIF-mRNA | -2.4 | 0.00249 |
| IL8-mRNA | -2.4 | 0.0125 |
| TPSAB1-mRNA | -1.95 | 0.00887 |
| IL1RN-mRNA | -1.8 | 0.0189 |
| IL1RL1-mRNA | -1.79 | 0.0247 |
| TNFRSF11B-mRNA | -1.79 | 0.0369 |
| KLRG1-mRNA | -1.58 | 0.0591 |
| PDGFC-mRNA | -1.26 | 0.0503 |
| CCL3L1-mRNA | -1.21 | 0.0578 |
| MAP3K1-mRNA | -1.19 | 0.0194 |
| EGR2-mRNA | -1.18 | 0.0464 |
| NUP107-mRNA | 1.010 | 0.0437 |
| CCR1-mRNA | 1.030 | 0.0444 |
| CDK1-mRNA | 1.190 | 0.027 |
| VEGFA-mRNA | 1.250 | 0.0343 |
| CCL22-mRNA | 1.38 | 0.0459 |
| IL2RB-mRNA | 1.73 | 0.0585 |
| BTLA-mRNA | 1.83 | 0.0482 |
| CXCL13-mRNA | 3.180 | 0.0106 |
| PRAME-mRNA | 4.100 | 0.0497 |

The checkpoint receptors HVEM, LIGHT, CD160, and BTLA are part of a complex network of overlapping receptor interactions that function in both immune stimulation and suppression[1, 2] . This regulatory function has made them therapeutic targets for treatment of cancer, autoimmune diseases and allergies, and for improved methods of organ transplants. While presenting opportunities for therapeutic development, this system creates challenges because potential therapies may impact interactions beyond their intended targets resulting in unintended consequences.

csapat

# Nanostring technology 2

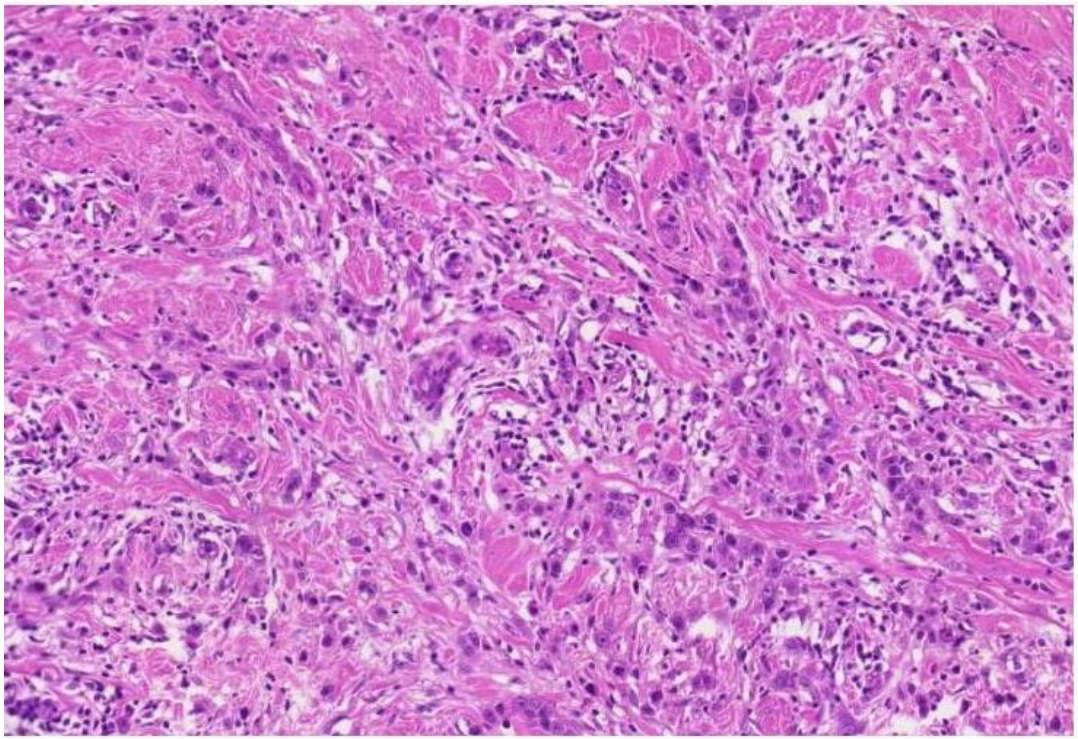
| | 1 | 2 | 3 |
|---|---|---|---|
| | Hybridization | Sample Processing | Digital Data Acquisition |
| Only 15 Minutes of Total Hands-on Time |  |  |  |
| Process | **Set Up Hybridization**<br>Add buffer, CodeSet, and sample into a strip tube and hybridize overnight. | **Set Up Prep Station**<br>Place the strip tube into the automated **nCounter Prep Station** with reagents and consumables from the nCounter Master Kit. | **Set Up Digital Analyzer**<br>Take the cartridge from the nCounter Prep Station and place it into the **nCounter Digital Analyzer** for direct digital counting. |
| Hands-on Time | 5 minutes | 5 minutes | 5 minutes |
| | Day 1 | Day 2 (automated) | Day 2 (automated) |

https://www.nanostring.com/scientific-content/technology-overview/ncounter-technology

- Volcano Plot: New.Annotation: KA vs.A
- Volcano plot displaying each gene's -log10(p-value) and log2 fold change with the selected covariate. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various False Discovery Rate (FDR) thresholds or p-value thresholds if there is no adjustment to the p-values. Genes are colored if the

# High TIL in a non-metastatic TNBC

The highest TIL was observed in non-metastatic TNBC cases (mean 9, 66%) and the lowest in metastatic LUMA cases (mean 1,16%).



NM TNBC
TIL 25%