SWATH®-MS based classification of diffuse large B-cell lymphoma (DLBCL)

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Introduction

- **Lymphomas** are solid tumors of the lymphatic system
- They are classified into Hodgkin and non-Hodgkin-lymphomas
- **Diffuse large-B-cell lymphoma** (DLBCL) constitute the most common type of non-Hodgkin lymphoma in adults, accounting for 30-40% of newly diagnosed lymphomas
- Combination therapy with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) has transformed DLBCL into a curable disease in about 50% of patients affected
- This suggests that DLBCL actually comprises several subgroups that differ in responsiveness to chemotherapy

Prognostication

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- Clinically, the International Prognostic Index (IPI) has been the primary tool to predict outcome
- Based on the number of negative prognostic factors present, **four** discrete outcome groups were originally identified with 5-year overall survival ranging from 26% to 73%.

Negative prognostic factors
Age >60 years
Elevated serum LDH
ECOG performance status ≥ 2 (bedridden)
Ann Arbor stage ≥ 3 (Involvement of lymph node regions on both sides of the diaphragm)
Two or more extranodal sites

Negative risk factors (n)	Risk category	5-yr overall Survival
0 or 1	Low	73%
2	Low intermediate	51%
3	High intermediate	43%
4 or 5	High	26%

Shipp et al., *New Engl J Med* 1993;329:987-994

Prognostication

- With the addition of **rituximab** to CHOP chemotherapy, which led to a marked improvement in survival, it became necessary to revise the IPI
- The revised IPI (R-IPI) identifies 3 distinct prognostic groups

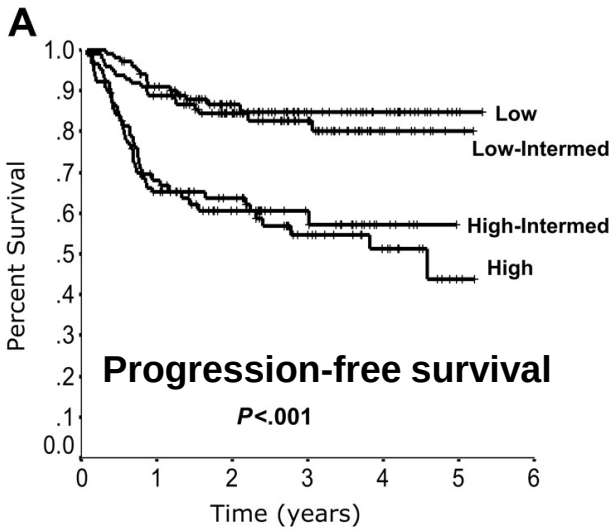
Table 2. Outcome according to International Prognostic Index (IPI) factors in 365 patients treated with R-CHOP in British Columbia

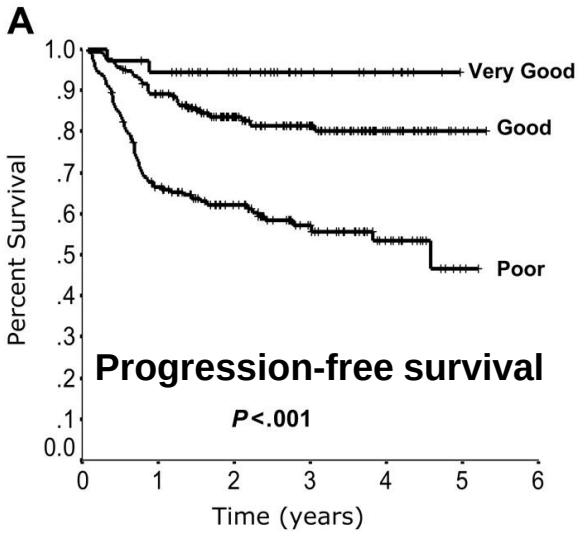
Risk group	No. of IPI factors	% Patients	4-year PFS, %	4-year OS, %
Standard IPI				
Low	0, 1	28	85	82
Low-intermediate	2	27	80	81
High-intermediate	3	21	57	49
High	4, 5	24	51	59
Revised IPI				
Very good	0	10	94	94
Good	1, 2	45	80	79
Poor	3, 4, 5	45	53	55

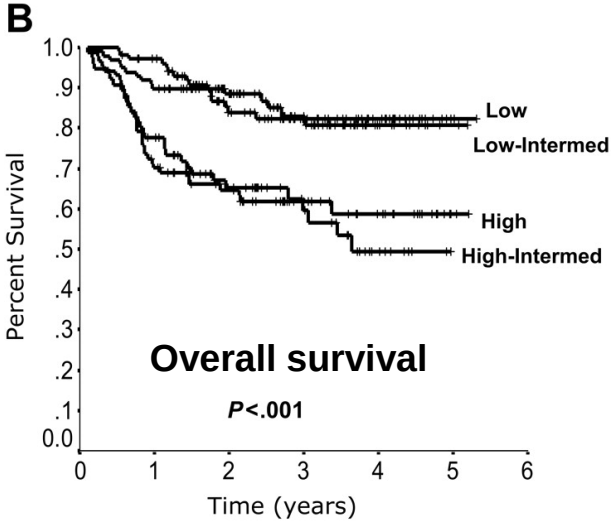
Sehn et al., *Blood* 2007;109:1857-1861

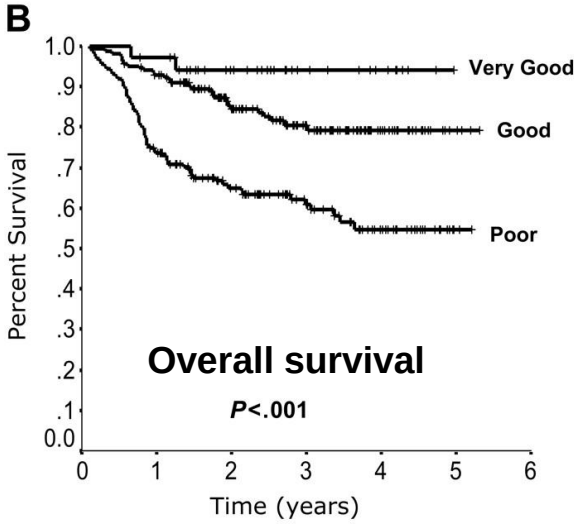
Prognostication

Outcome according to the standard and revised (IPI)









Sehn et al., *Blood* 2007;109:1857-1861

Gene expression based classification of DLBCL

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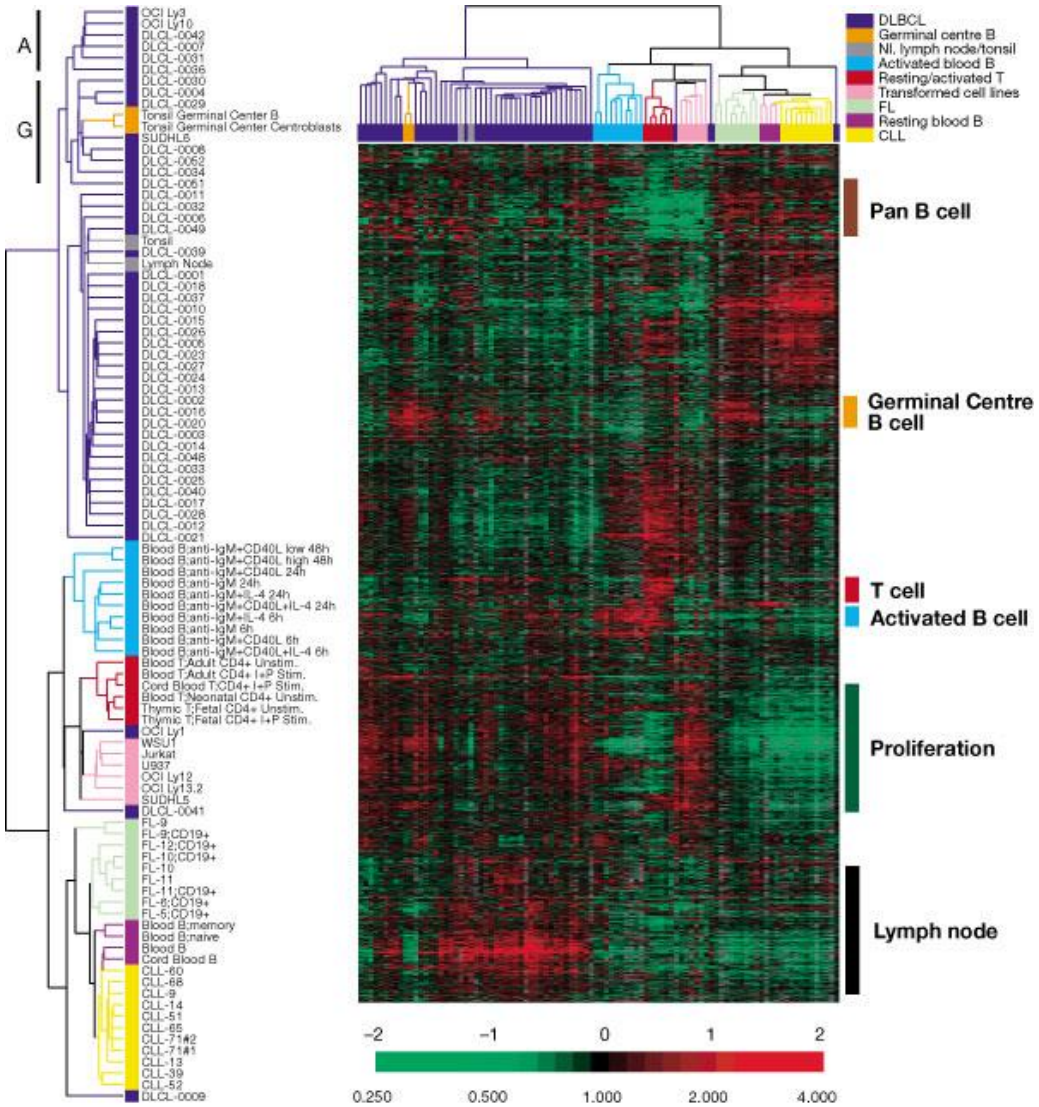
With advances in microarray-based gene expression analysis it bcame possible to define molecular subtypes of DLBCL



Depicted are the 1.8 million measurements of gene expression from 128 microarray analyses of **96 samples of normal and malignant lymphocytes**. The dendrogram at the left lists the samples studied and provides a measure of the relatedness of gene expression in each sample. The dendrogram is colour coded according to the category of mRNA sample studied (see upper right key). Each row represents a separate cDNA clone on the microarray and each column a separate mRNA sample. The results presented represent the ratio of hybridization of fluorescent cDNA probes prepared from each experimental mRNA samples to a reference mRNA sample. These ratios are a measure of relative gene expression in each experimental sample and were depicted according to the colour scale shown at the bottom. As indicated, the scale extends from fluorescence ratios of 0.25 to 4 (-2 to +2 in log base 2 units). Grey indicates missing or excluded data.

Alizadeh et al., *Nature* 2000;403:503-511

Gene expression based classification of DLBCL

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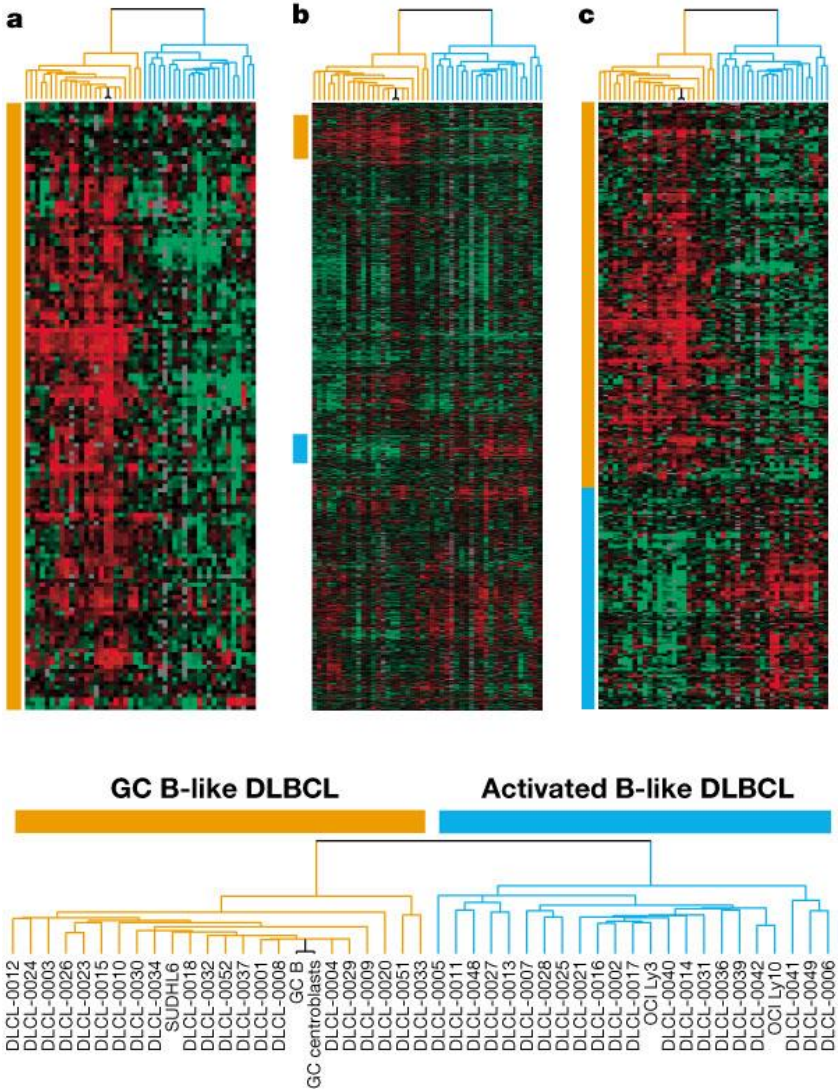
With advances in microarray-based gene expression analysis it bcame possible to define molecular subtypes of DLBCL



a) Hierarchical clustering of DLBCL cases (blue and orange) and germinal centre B cells (black) based on the genes of the germinal centre B-cell gene expression signature led to the definition of two DLBCL subgroups, **GC B-like DLBCL (orange)** and **activated B-like DLBCL (blue)**.

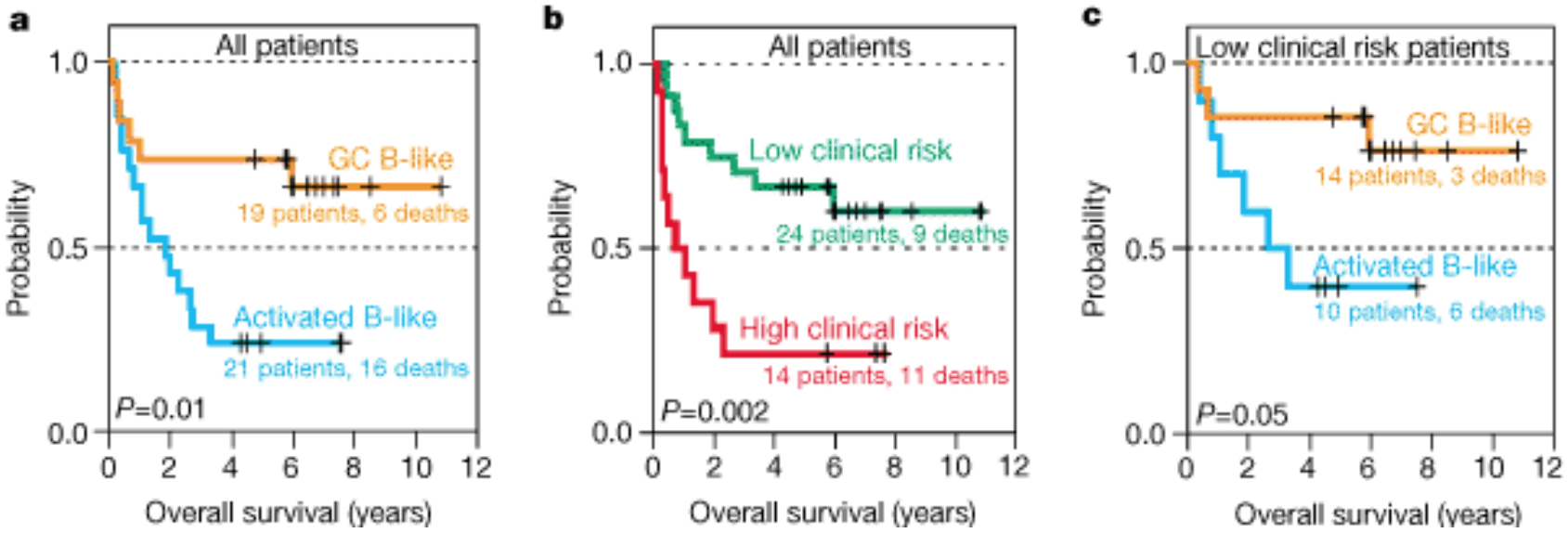
b) Discovery of genes that are selectively expressed in **GCB-** and **ABC-**DLBCL.

c) Hierarchical clustering the genes selectively expressed in **GCB-DLBCL** and **ABC-DLBCL**.

Alizadeh et al., *Nature* 2000;403:503-511

Gene expression based classification of DLBCL

More importantly, the gene expression based subgroups defined prognostic categories



a) Kaplan–Meier plot of overall survival of DLBCL patients grouped on the basis of gene expression profiling.

b) Kaplan–Meier plot of overall survival of DLBCL patients grouped according to the International Prognostic Index (IPI). Low clinical risk patients (IPI score 0–2) and high clinical risk patients (IPI score 3–5) are plotted separately.

c) Kaplan–Meier plot of overall survival of low clinical risk DLBCL patients (IPI score 0–2) grouped on the basis of their gene expression profiles.

Alizadeh et al., *Nature* 2000;403:503-511

Gene expression based classification of DLBCL

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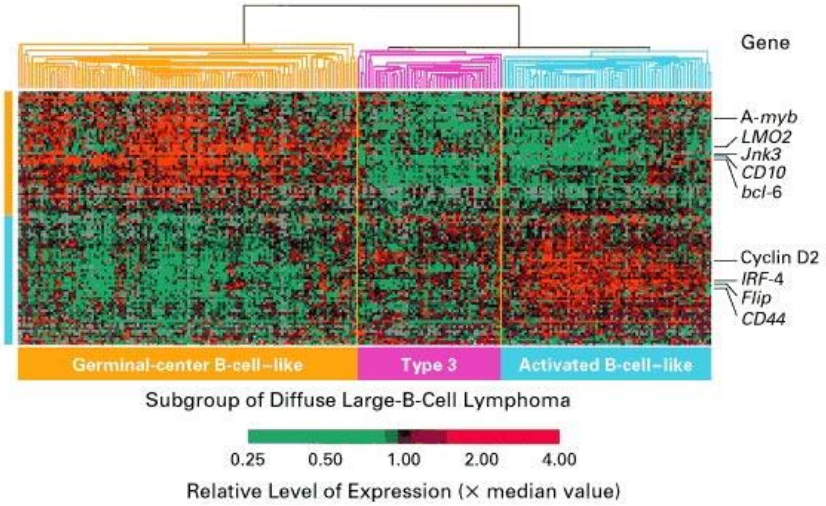
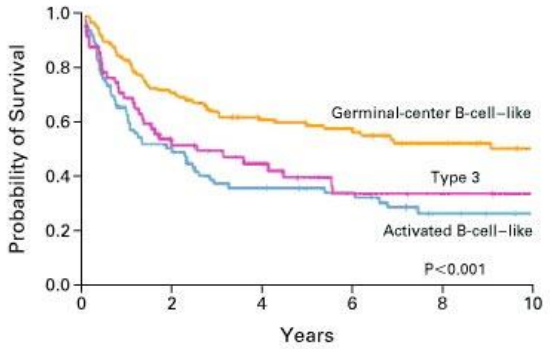
A



B

Oncogenic Abnormality	Germinal-center B-cell–like	Type 3	Activated B-cell–like
	no. of samples		
c-rel amplification	17	0	0
bcl-2 t(14;18)	26	0	0

C



No. at Risk

Germinal-center B-cell–like	115	81	60	46	32	19
Type 3	52	24	18	10	8	5
Activated B-cell–like	73	35	23	19	8	5

Rosenwald et al., *New Engl J Med* 2002;346:1937-1947

DLBCLs are heterogeneous tumors varying in stroma

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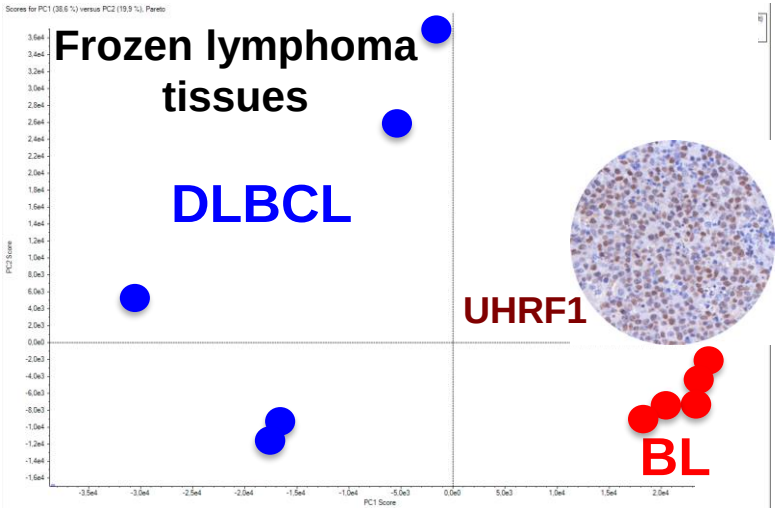
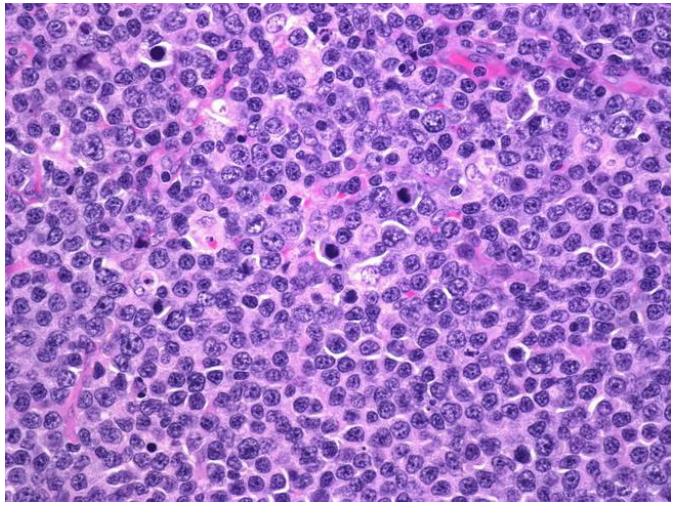
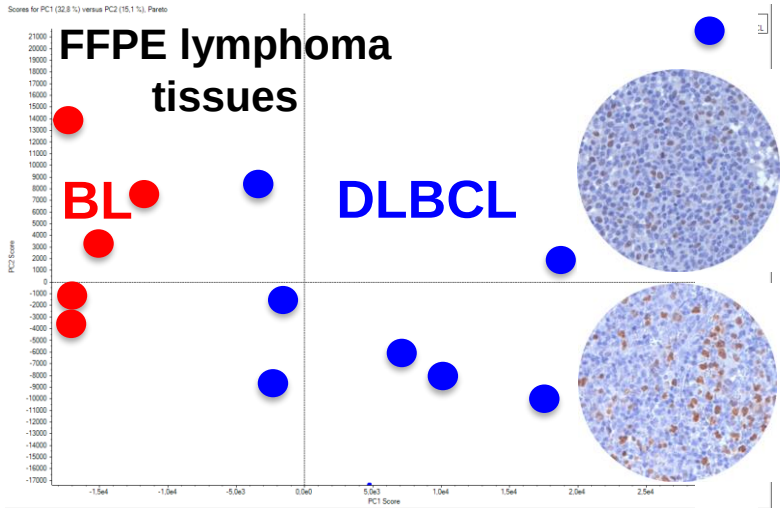
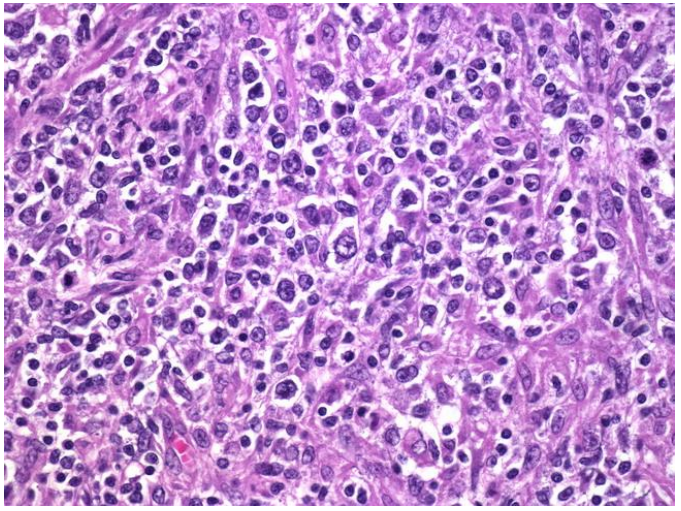
Burkitt‘s lymphoma are homogeneous, rapidly proliferating tumors, whereas DLBCLs are heterogeneous tumors embedded in tumor stroma



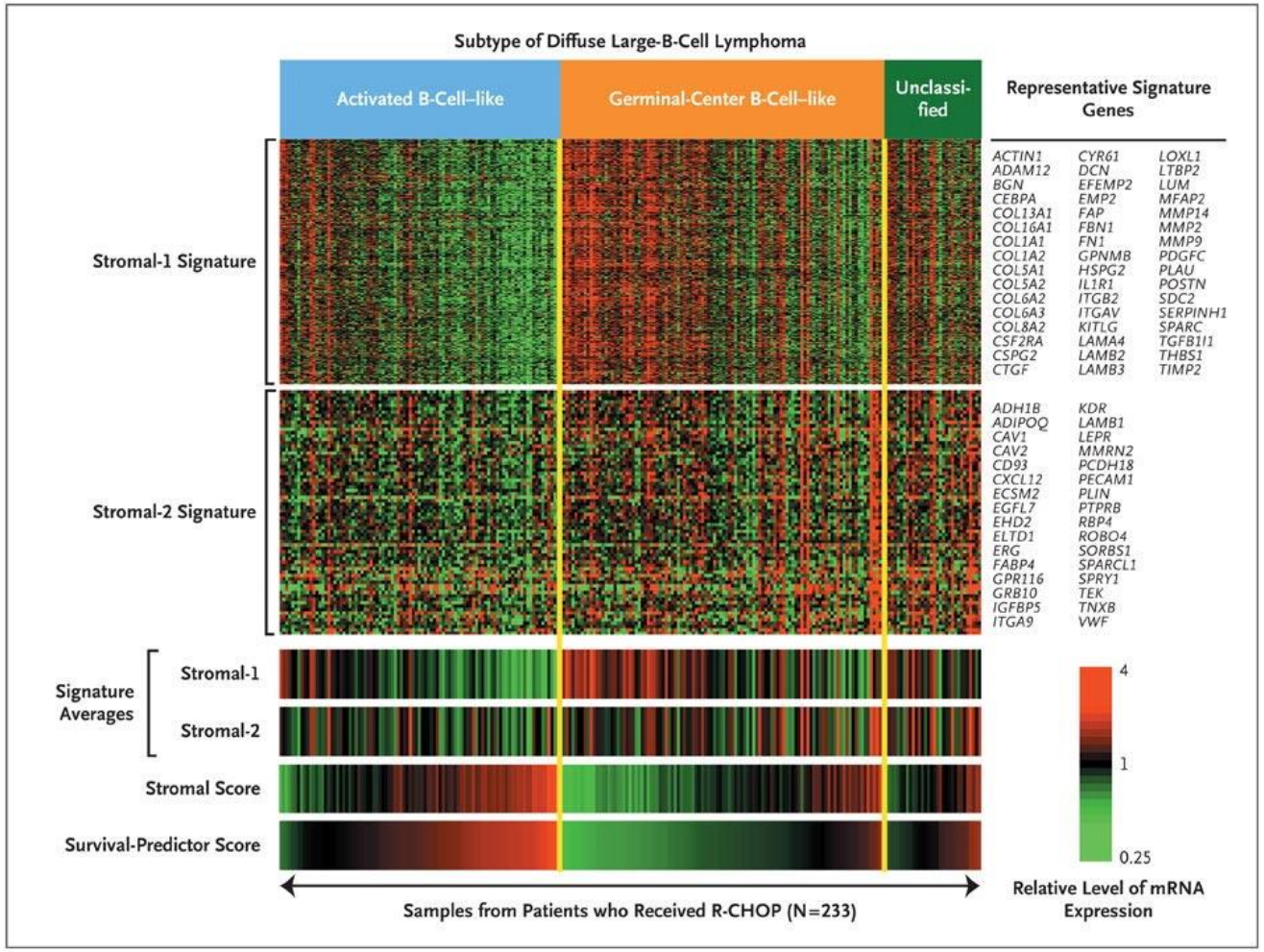






DLBCLs are heterogeneous tumors varying in stroma

The **favorable stromal-1 signature** reflects extracellular matrix deposition and histiocytic infiltration, while the prognostically **unfavorable stromal-2 signature** reflects tumor blood-vessel density („angiogenic switch“). **A high stromal score is associated with adverse outcome.**



Lenz et al., *New Engl J Med* 2008;359:2313-2323

Immunohistochemistry-based classification of DLBCL

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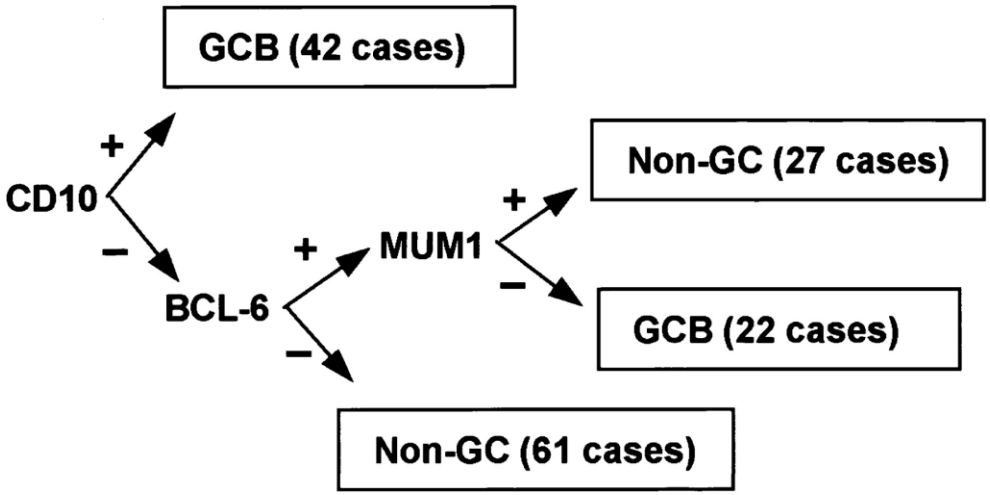

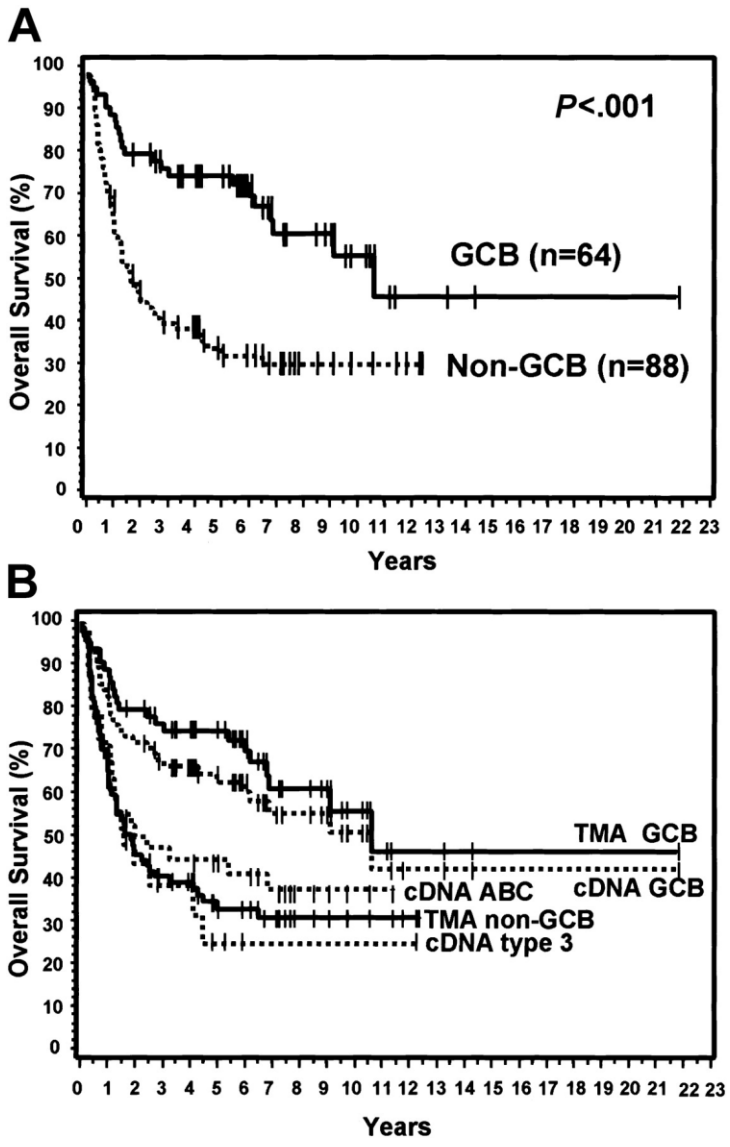

Hans et al., *Blood* 2004;103:275-282

Immunohistochemistry-based classification of DLBCL

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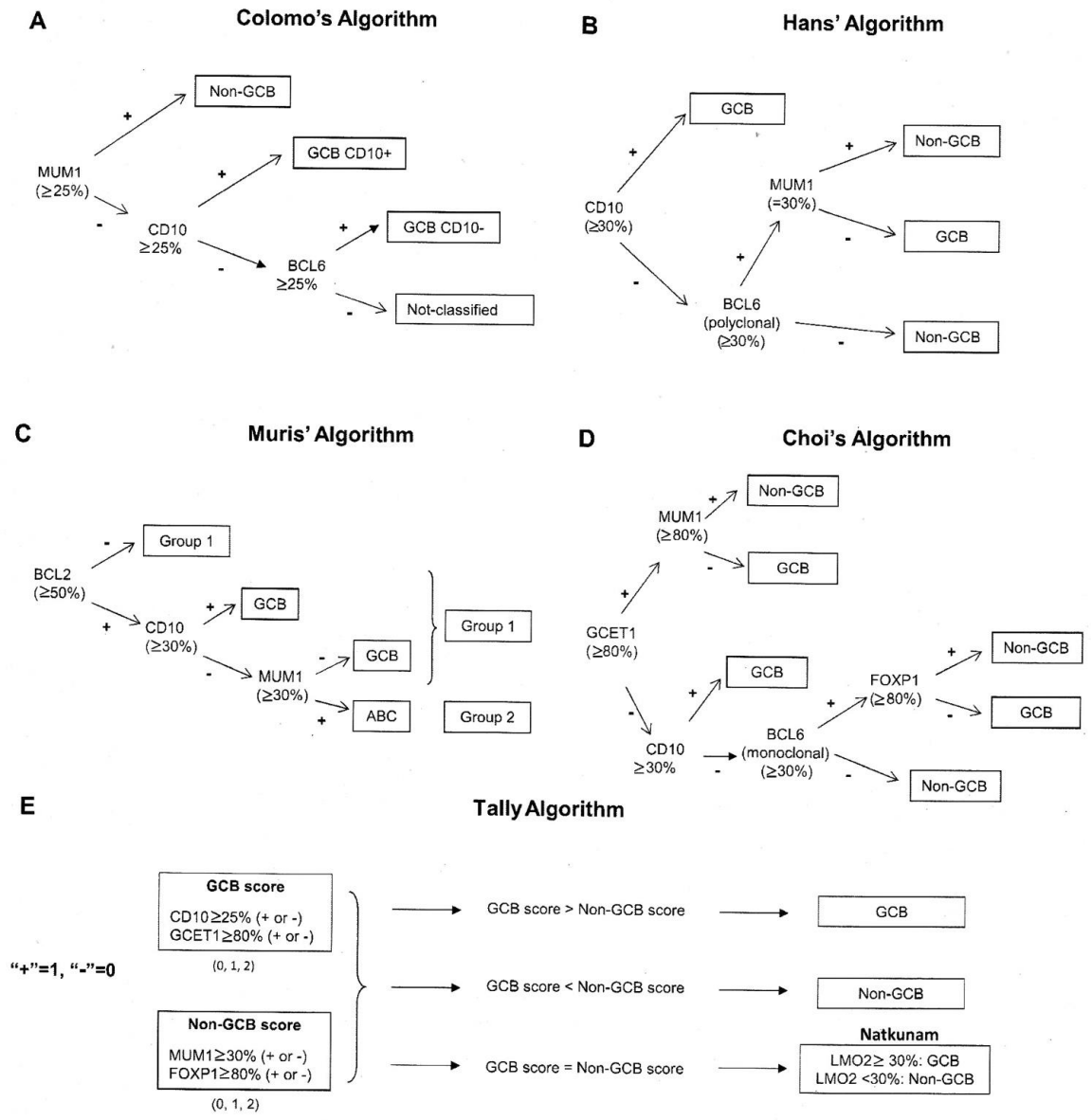

Figure 2. Five immunostaining algorithms used to assess differentiation profile (GCB vs non-GCB) in patients with DLBCL.

Gutierrez-Garcia et al., Blood 2011, 117(18);4836-4843

Immunohistochemistry-based classification of DLBCL

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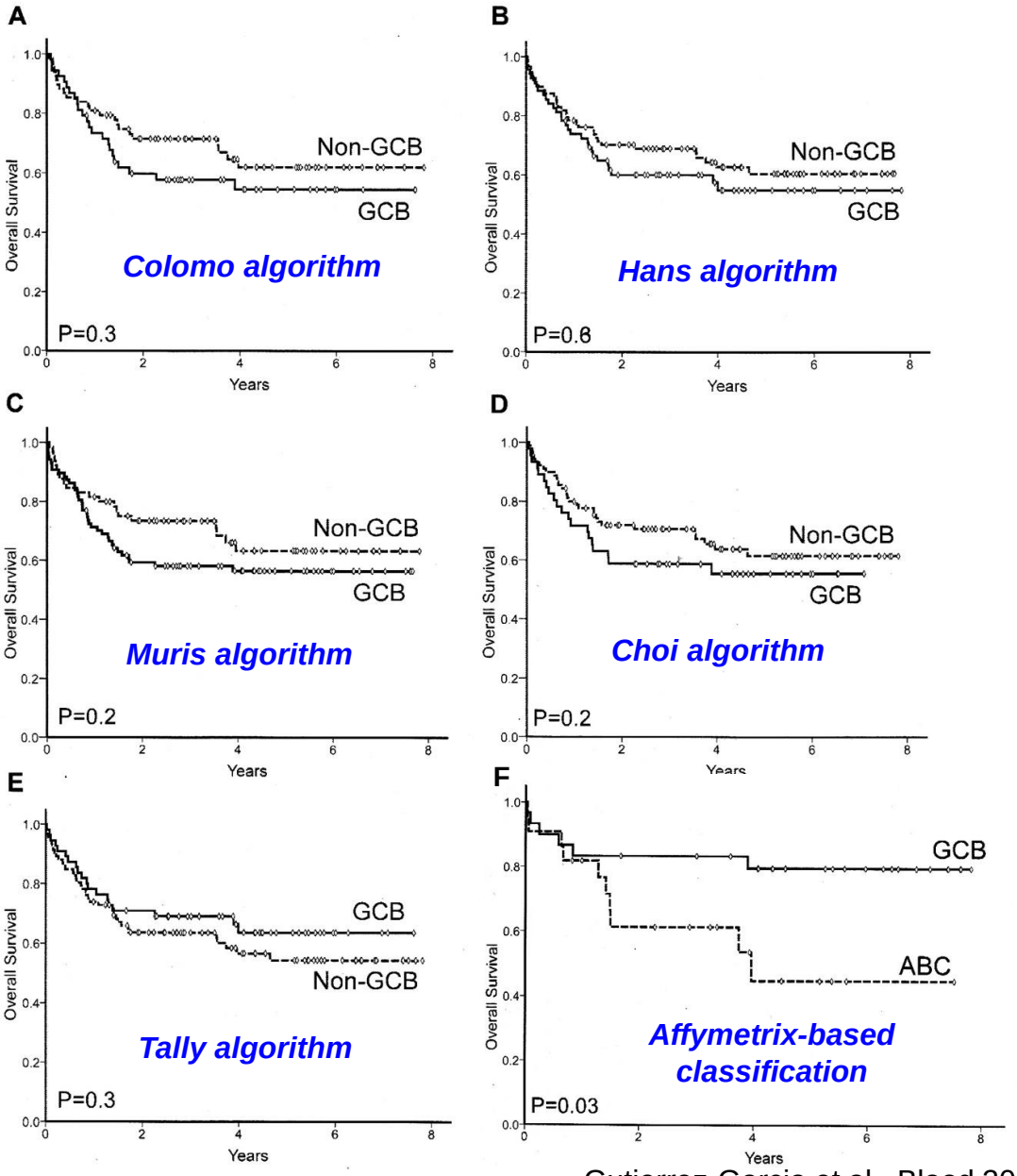

Gutierrez-Garcia et al., Blood 2011, 117(18);4836-4843

Nanostring-based classification of DLBCL (20 genes)

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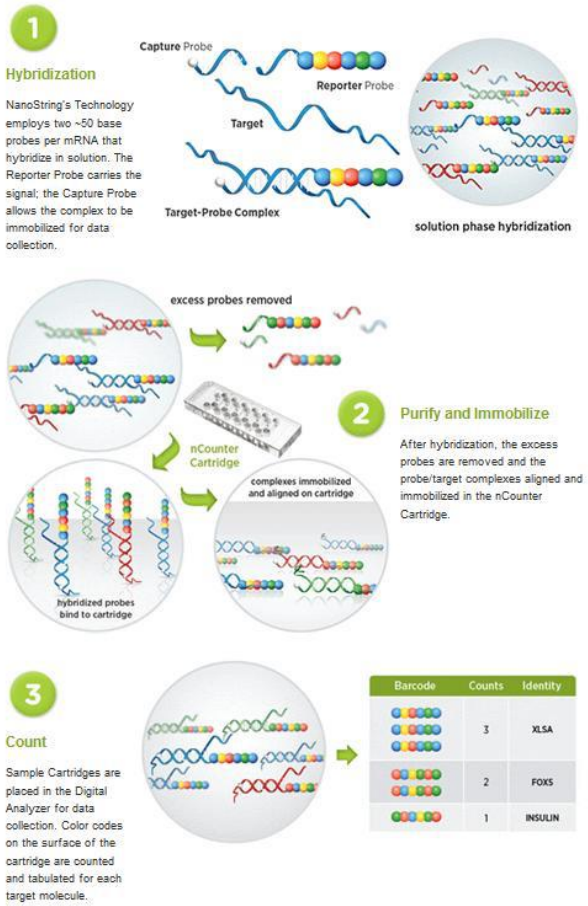
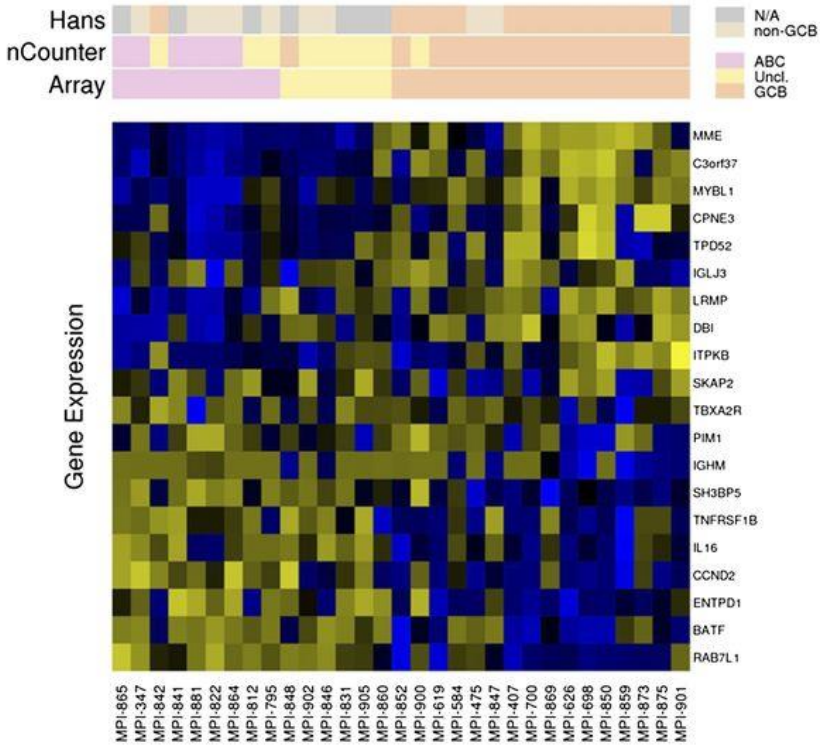
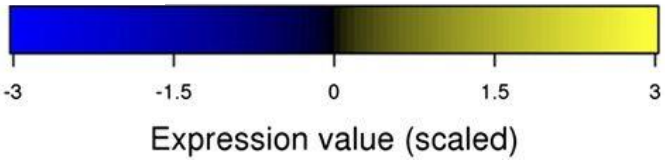


Scheme of NanoString's technology





Masqué-Soler,…., Spang, Klapper *Blood* 2013;122:1985-1986

Nanostring-based classification of DLBCL (20 genes)

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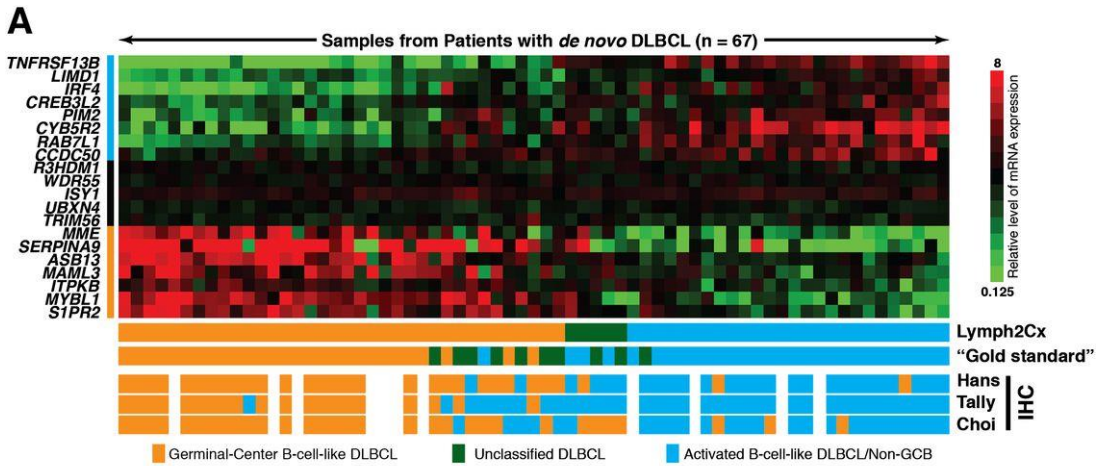

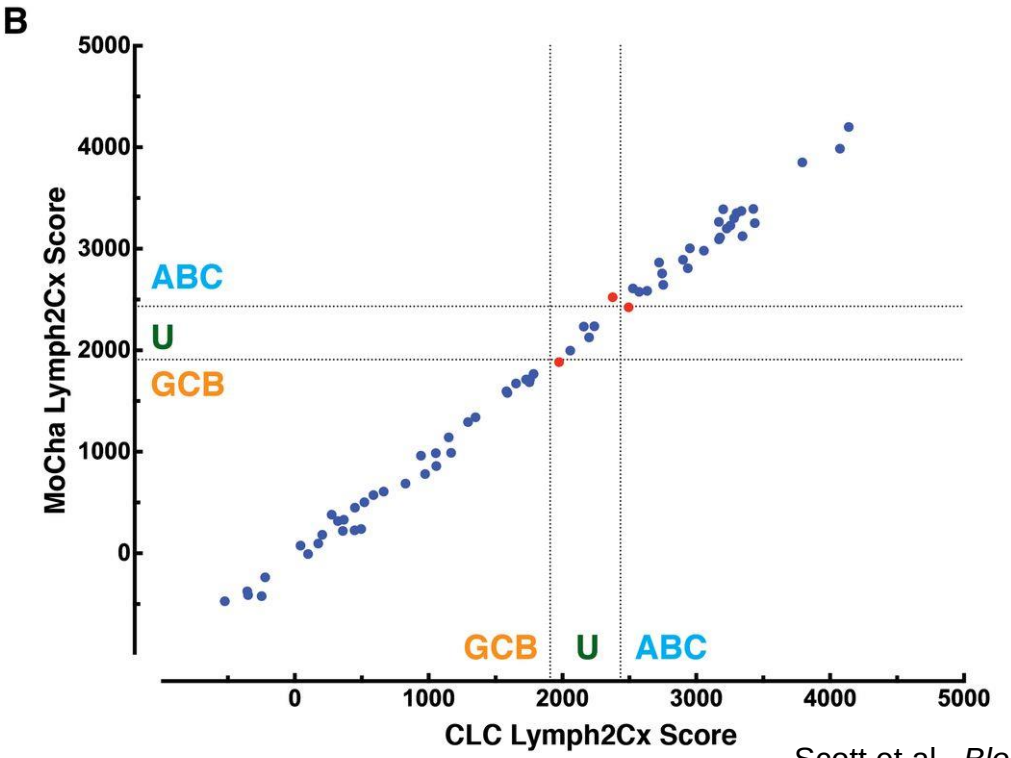

Scott et al., *Blood* 2014;123(8):1214-1217

SWATH-MS-based classification of DLBCL

Material and Methods

- 23 FFPE tissue sections previously characterized as ABC or GCB subtype using Affymetrix-based gene expression profiling
- FFPE sections were first incubated in xylene for 30 min at 56° C
- Next, FFPE sections were subjected to a graded ethanol series (100%, 85%, 5%)
- A 20-fold excess of lysis buffer (20 mM Tris-HCl, pH 8.8, 2% SDS, 200 mM DTT, 200 mM glycine was added to 9.1±2.0 mg rehydrated tissue
- Samples were incubated at 100° C for 30 min and 80° C for 60 min under continuous agitation, then centrfuged at 12,000 g for 10 min
- Protein content of the pellet was determined by the FluoroProfile® Kit (Sigma-Aldrich) and 30 µg of protein were used for subsequent gel-aided sample preparation (GASP) (Fischer & Kessler, *Proteomics* 2015; 15:1224-1229)
- Trypsin/LysC was used for in-gel protein digestion

SWATH-MS-based classification of DLBCL

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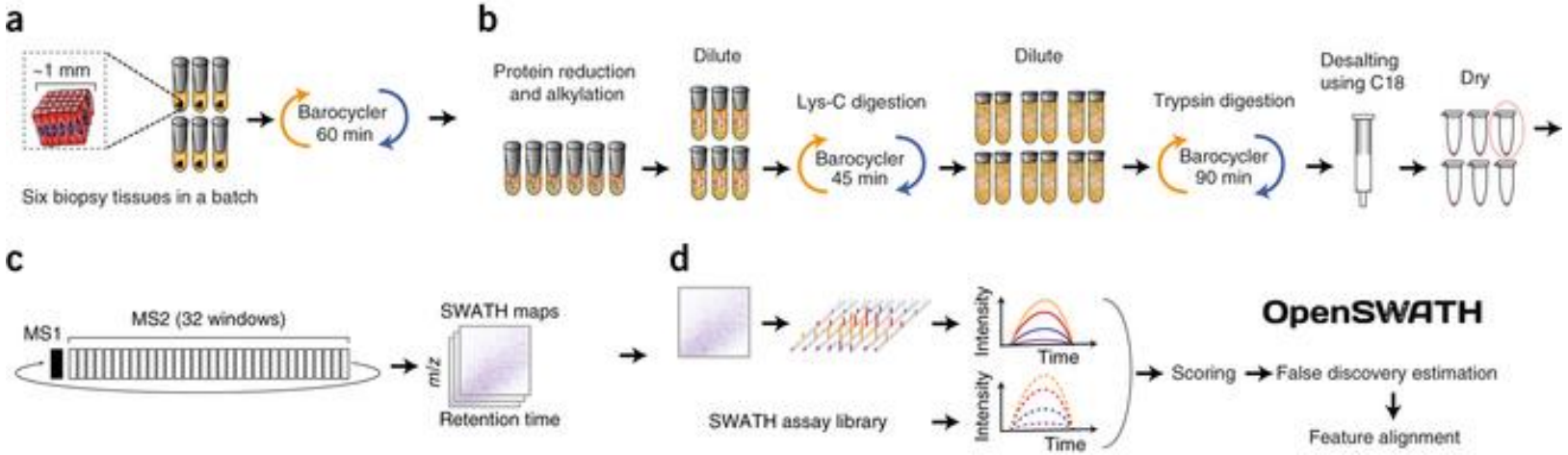
Material and Methods

- For generation of libraries, pools of ABC, GCB, and unclassified DLBCL digests, respectively, were generated and subjected separately to information-dependent acquisition (IDA) runs on a TripleTOF 5600+ MS (400-1000 *m/z* for 250 ms followed by MS/MS spectra from 230-1500 *m/z* of the 30 most intensive precurcors for 100 ms each.
- Nano-HPLC of peptides was performed on a Dionex Ultimate3000 using peptide trapping (Acclaim PepMap, 2 cm x 200 µm ID) and a 88-min binary acetonitrile gradient on a 25-cm Acclaim PepMap column (75 µm ID, 3-µm particles, 250 nL/min at 35˚ C)
- In general terms, we followed the protocol originally described by Guo *et al.* (2015)



Guo et al., *Neture Medicine* 2015, doi: 10.1038/nm.

SWATH-MS-based classification of DLBCL

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Material and Methods

- The data were searched using ProteinPilot4.5 against the Uniprot-database
- The thus obtained result file comprised 1,402 proteins with an FDR of 1% by PeakView 2.1 and using spectra only with a confidence of ≥95% of unique, unmodified peptides with 6 peptides per protein and 6 transitions per peptide
- SWATH-MS analyses were performed using the same chromaographic conditions as for the IDA-runs.
- Given a typical peak width of 30 sec, a fixed duty cycle of 3.3 sec was used to obtain at least 9 data points per LC peak.
- After a 50-ms TOF-MS scan, the entire *m/z* range of 400-1250 was covered using 60 SWATH-windows of varying quadrupole isolation widths (Simbürger *et al.*, *J Proteomics* 2016;145:137-140).
- For targeted analyses, MRM-like measurements were performed on the TripleTOF 5600+

SWATH-MS-based classification of DLBCL

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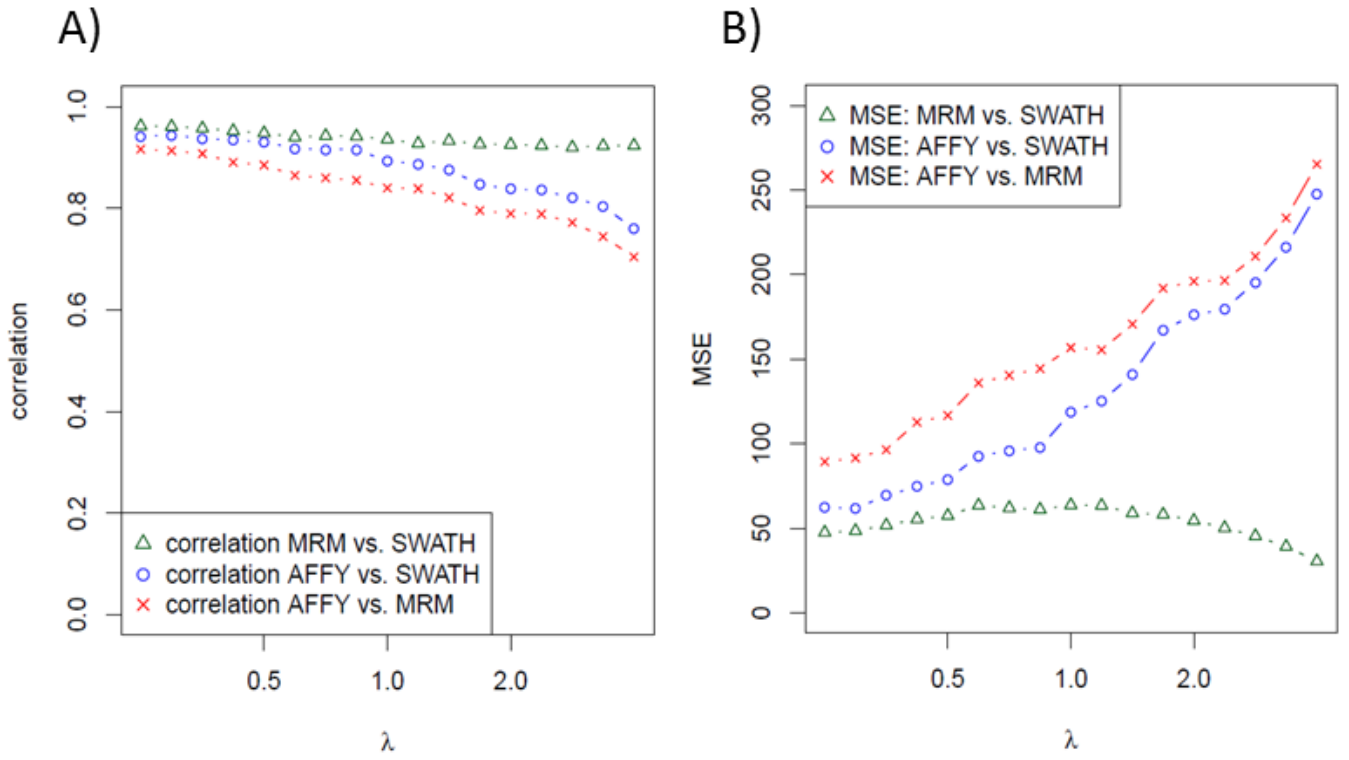
- SWATH-MS yielded protein intensities for 1,237 proteins in all DLBCL specimens
- Of the1,237 proteins only **235 proteins** that had yielded at least 6 different quantified peptides with 6 transitions each were considered for building a sparse classificator by zero-sum regression analysis (Altenbuchinger *et al.*, *Bioinformatics* 2017;33(2):219-226).
- The results were tested via a leave-one-out cross-validation using fixed shrinkage parameters λ.



Zero-sum regression

- Zero-sum regression is a penalized linear regression model for the extraction of biomarker signatures from high-dimensional data
- In contrast to LASSO (Tibshirani 1996) feature weigths sum up to zero, thus making the model invariant to reference point and scale, facilitating transfer of biomarker signature across different analytical platforms and molecular levels

$$y_i = \beta_0 + \sum_{j=1}^{p} \beta_j (x_{ij} + \gamma_i) + \epsilon_i, \qquad (1)$$

$$\sum_{j=1}^{p} \beta_j = 0$$

- Altenbuchinger et al., Reference point insensitive molecular data analysis. *Bioinformatics* 2017, 33(2), 219-226.
- Altenbuchinger et al., Molecular signatures that can be transferred across different omics platforms. *Bioinformatics*, in press.
- Zacharias et al., Scale invariant bio-marker discovery in metabolic fingerprints of body fluids. *In preparation*.

Zero-sum regression

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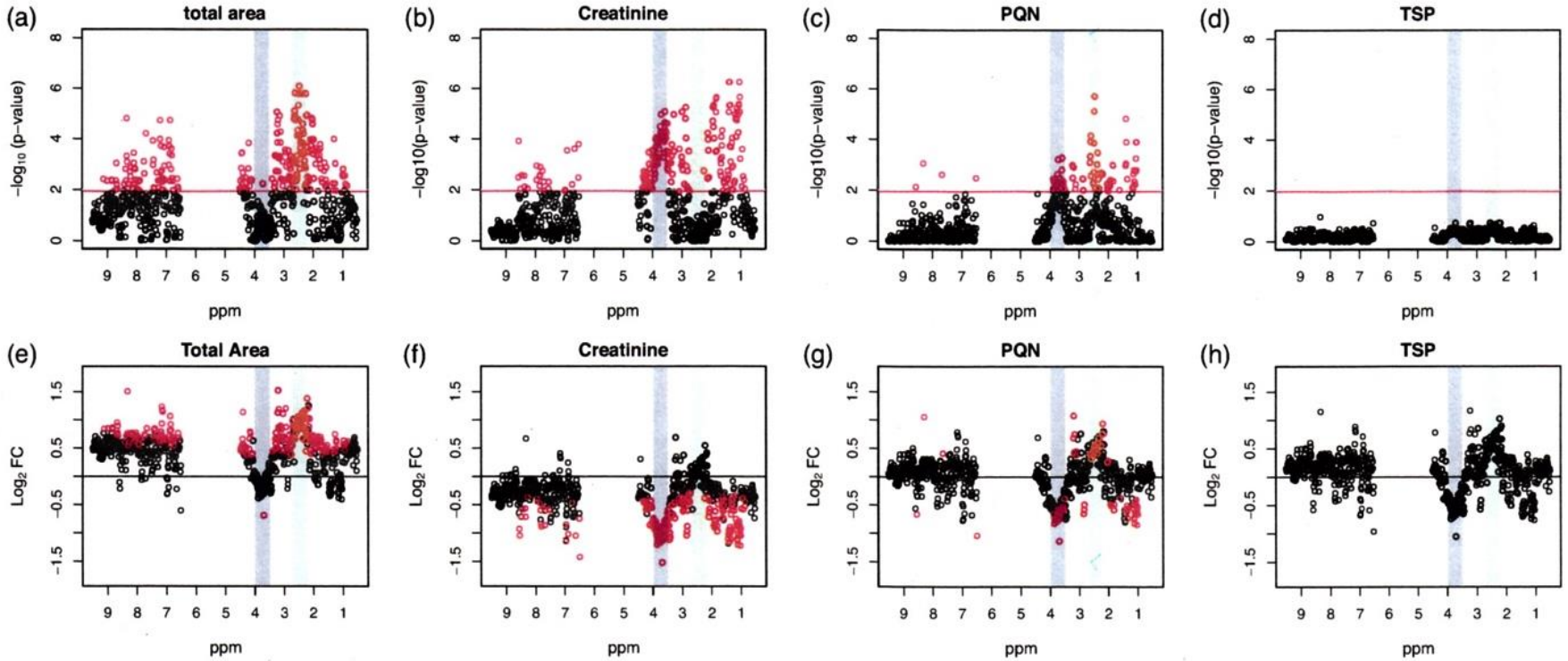
Normalization of data, in this case of urinary 1H NMR metabolite fingerprints, has a significant influence on number of significant features and magnitude of fold changes.



Zacharias et al., arXiv:1703.07724.

Zero-sum regression

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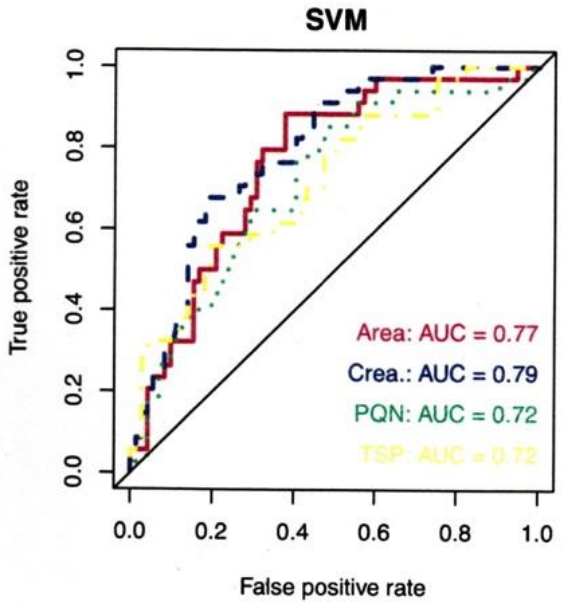

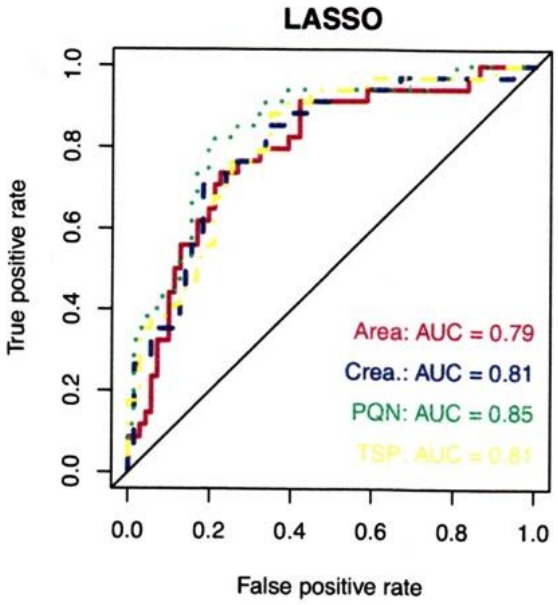

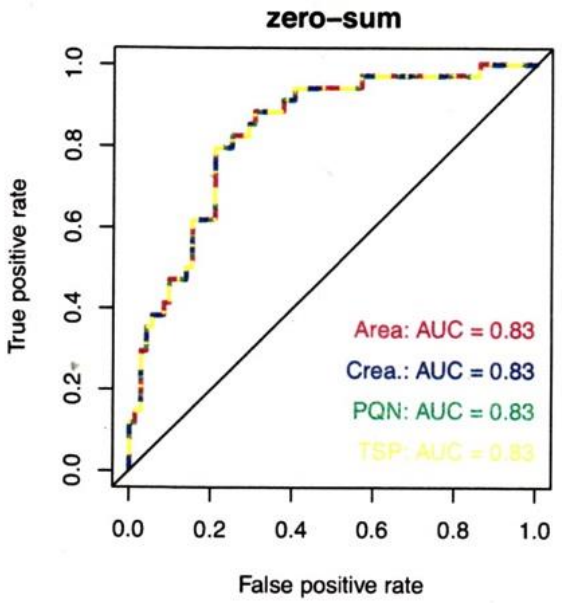

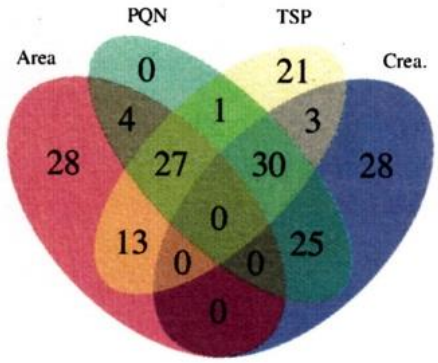

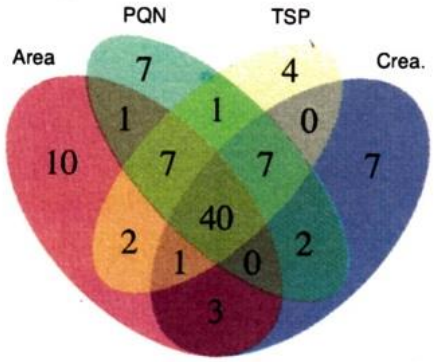

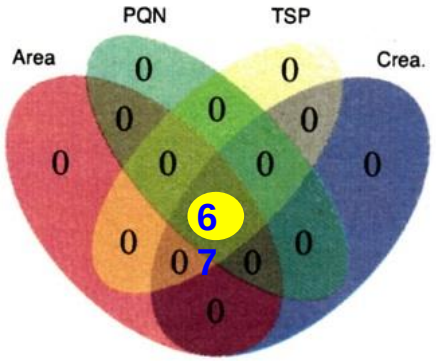

Zacharias et al., arXiv:1703.07724.

SWATH-MS-based classification of DLBCL

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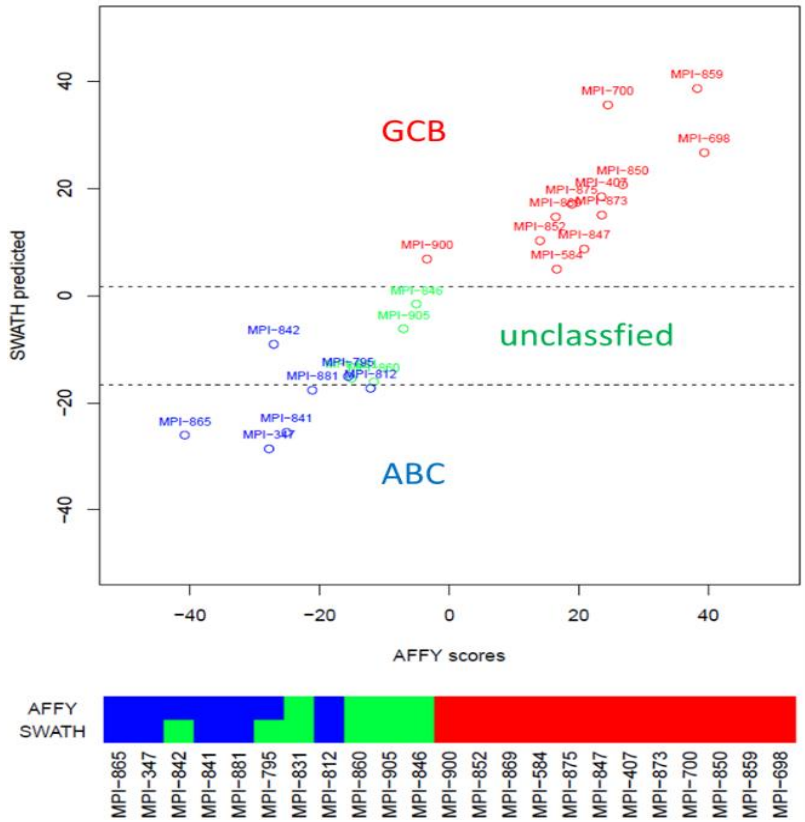
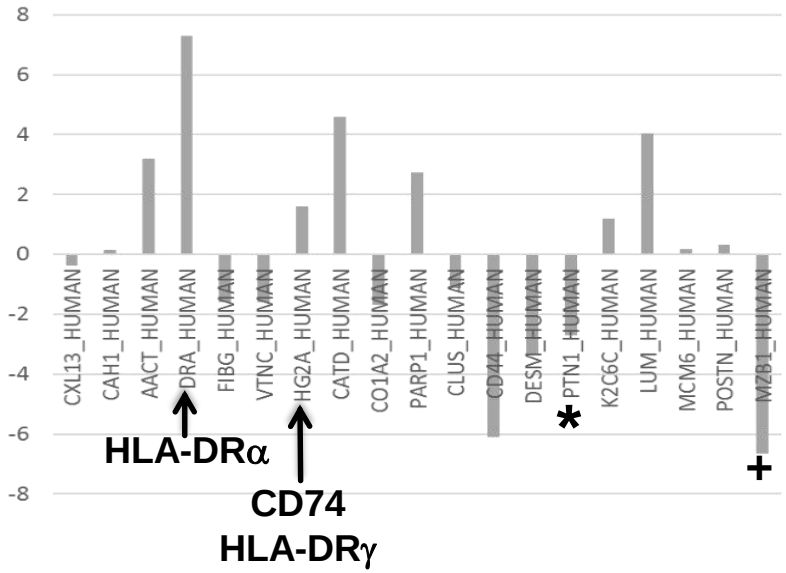
A)



The SWATH-MS based classificator comprised **18 proteins** (*versus* 12 transcripts for the Affymetrix-based classificator).

B)



Both **HLA-DRα** and **HLA-DRγ** are involved in antigen presentation on B lymphocytes, dendritic cells and macrophages. Reduced expression in DLBCL facilitates tumor immune evasion and results in significantly reduced overall (P=0.0003) and progression-free survival (P=0.0012). Brown et al. *Leukemia* 2016;30:605-16.

***PTN1/PTP1B**: tyrosine phosphatase, plays a role in insulin signaling and tumor metabolism; significantly higher expressed in ABC-like DLBCL. Lu et al. *Blood* 2005;105:2924-32.

+MZB1: high expression associated with poor prognosis. Herold et al. Leuk Lymphoma 2013;54:1652-7.

Zacharias et al., arXiv:1703.07724.

SWATH-MS-based classification of DLBCL

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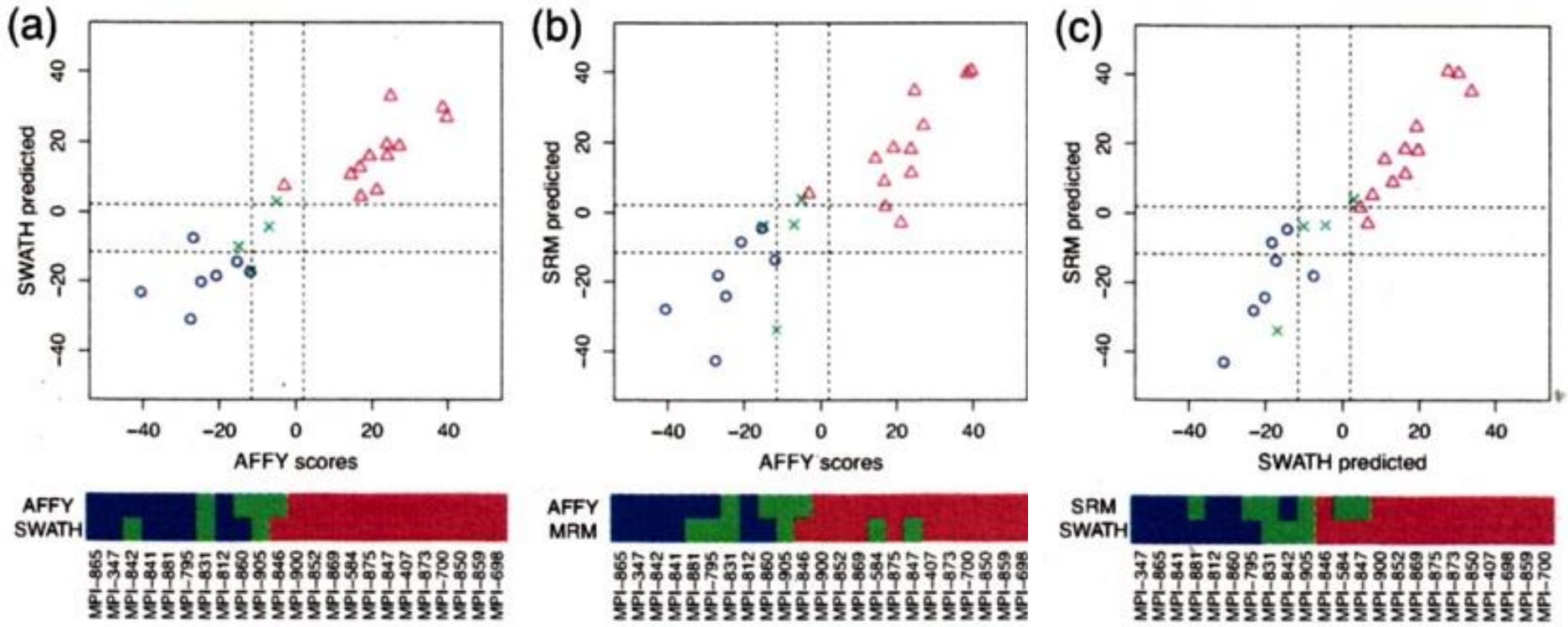
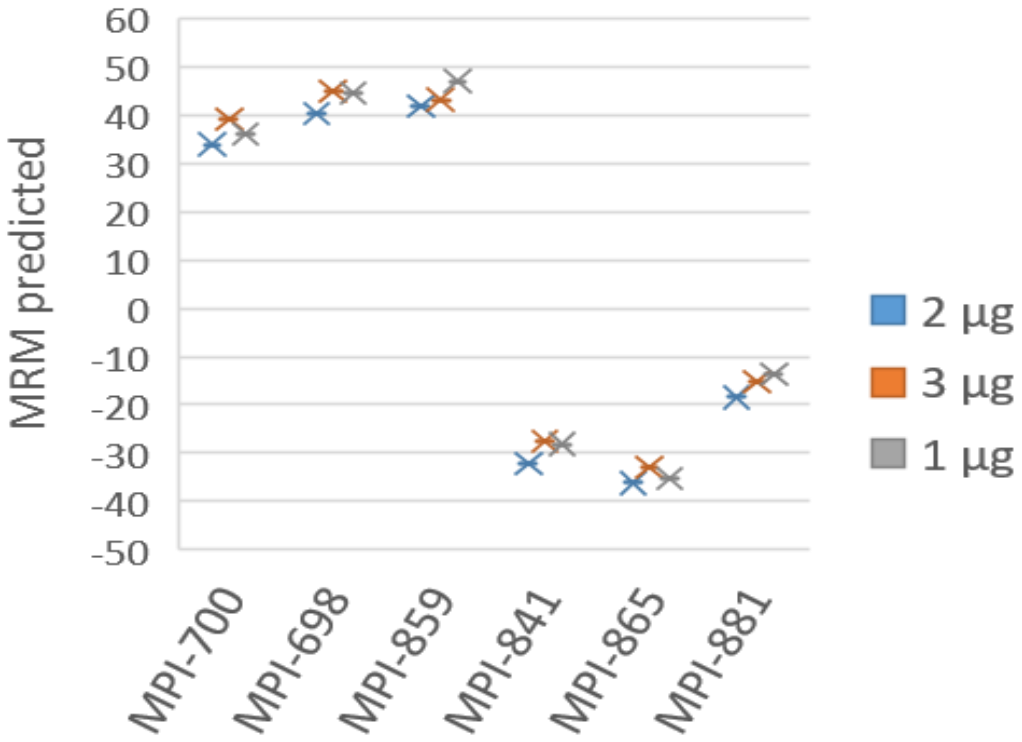
Zero-sum regression based classificator transferable across platforms (SWATH, MRM) without losing discriminatory power, as it is reference point insensitive.



Altenbuchinger et al., *Bioinformatics*, in press.

SWATH-MS-based classification of DLBCL

The classificator obtained is also insensitive to the amount of protein loaded



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Dr. Monika Szczepanowski
Elke Perthen
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Thank you for your attention!