

COST CLINIMARK TRAINING SCHOOL
Approaches for Biomarker Discovery and Validation

Eureka: there is something rotten in the biomarker kingdom



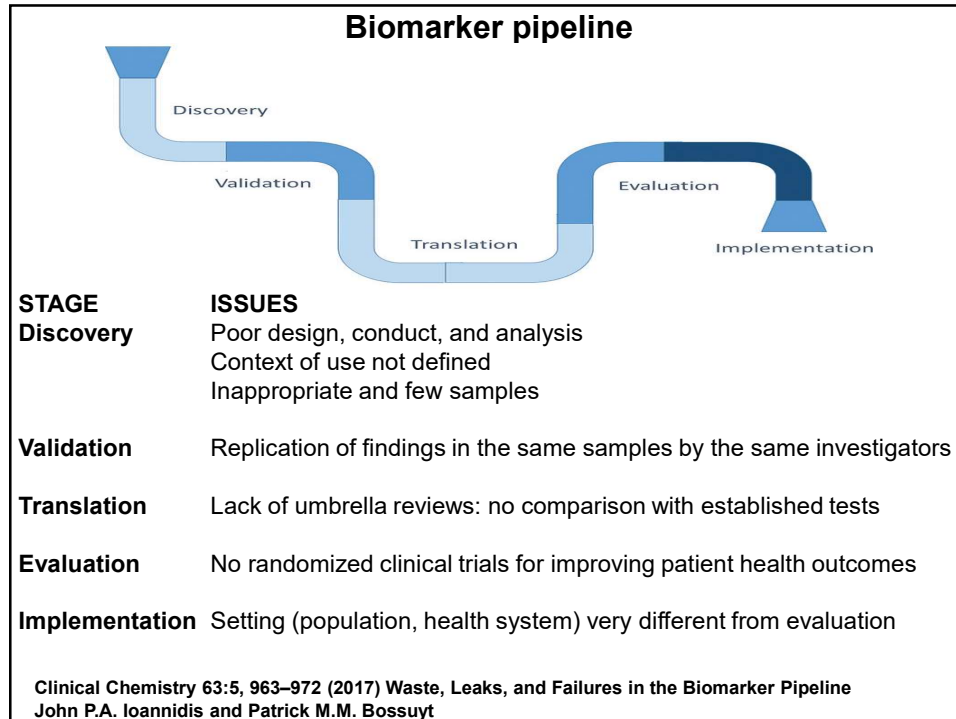
26/09/2019

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Overview

- Introduction:
 - The biomarker pipeline leaks
 - Terrible example of biomarker discovery
 - Terrible example of biomarker implementation
- My greatest scientific failure (so far):
 - Tragic tale of ELISA analytical and diagnostic performance in urine
 - MRM (Multiple Reaction Monitoring) assay performance for urine samples
- There is hope:
 - Proteomics biomarkers in clinical use



Failed application of proteomics in biomarker discovery

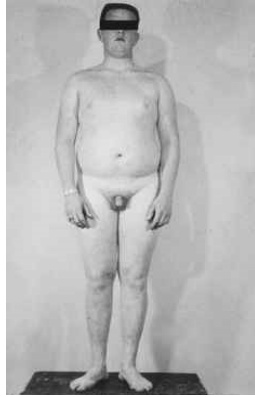
Proteomic analysis of amniotic fluid in pregnancies with Klinefelter syndrome fetuses

JOURNAL OF PROTEOMICS 73 (2010) 943– 950

Klinefelter syndrome

Phenotype: sterility, low IQ?

Karyotype: mainly XXY



Diagnosis

Amniocentesis or chorionic villus sampling (CVS) followed by karyotype determination

AIM OF THE STUDY

Discovery of biomarkers for Klinefelter syndrome diagnosis

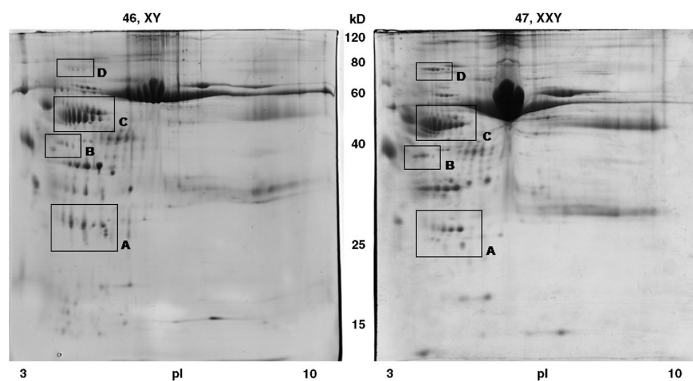
Proteomic Methodology

Analysis of amniotic fluid by 2D Electrophoresis coupled to mass spectrometry from 4 cases and 8 controls

Representative Data of 2D Electrophoresis

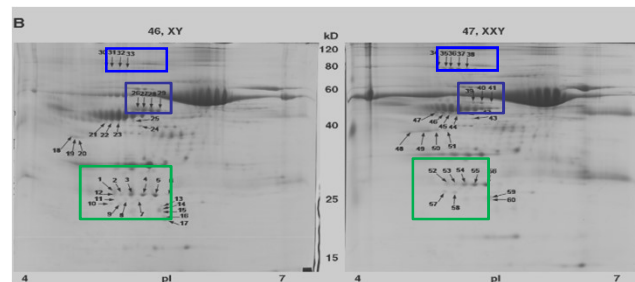
What went wrong in this experiment?

The authors claim that 1mg of protein was loaded on each gel



Data on differentially expressed proteins

Spot no.	Protein entry name/identity	Spot no. %total density ($\times 100$)	Expression level	Case/control
		47, XXY 46, XY		
31-33 34-38	(CERU_HUMAN) Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase)	42 \pm 3 22 \pm 4	1.9**	
18-20 48-51	(ZA2G_HUMAN) Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein) (Zn-alpha-2-GP)	30 \pm 3 20 \pm 1	1.5*	
21-23 44-47	(A1AT_HUMAN) Alpha-1-antitrypsin precursor (Alpha-1-protease inhibitor) (Alpha-1-antiprotease)	34.5 \pm 1.5 17 \pm 1.2	2.02*	
13-17 59, 60	(RETBP_HUMAN) Plasma retinol-binding protein precursor (PRBP) (RBP) [contains: Plasma retinol-binding pr (CELS_HUMAN) Gelsolin precursor (Actindepolymerizing protein)]	29 \pm 2.1 70 \pm 1.3	0.41**	
24, 25 42, 43	(GELS_HUMAN) Gelsolin precursor (Actindepolymerizing protein)	26 \pm 3.2 54.5 \pm 4	0.47**	
26-29 39-41	(VDBP_HUMAN) Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDBP)	29 \pm 2.2 50 \pm 1.31	0.58**	
1-12 52-58	(APOA1_HUMAN) Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) [contains: Apolipoprotein A-I(1-242)]	50 \pm 2 76 \pm 1.1	0.65*	



Results were validated by WB using the same amniotic fluid samples (4 cases and 8 controls)

Regulation trend (case/control) and biological function of differentially expressed proteins

- ApoA1 ↓ lipid metabolism
- RETBP ↓ Vitamin A transport
- VDBP ↓ Vitamin D transport
- A1AT, CERU ↑ Inflammation
- GELS ↓ Inflammation
- ZA2G ↑ lipid metabolism

All of these candidate biomarkers are highly abundant plasma proteins with a high concentration in blood

They are not involved in the molecular processes relevant for Klinefelter syndrome

Question: Comment on the following statement from the discussion of the publication

« it is appealing that none of the proteins identified by the analytical methods used in this study can be directly correlated with the Klinefelter phenotype »

Conclusion

The proteomic study did not yield any useful biomarkers

Question: What are the weaknesses of this study?

Failed implementation The Theranos story

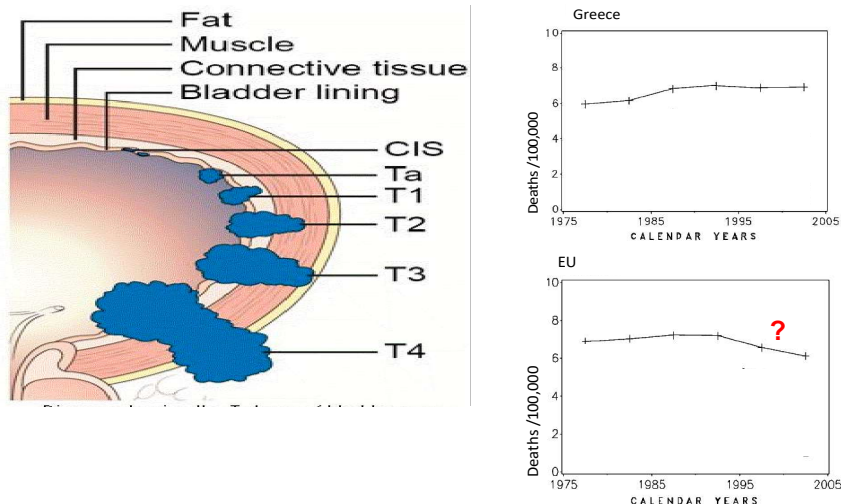


John Carreyrou, 2018 "Bad Blood: Secrets and Lies in a Silicon Valley Startup"

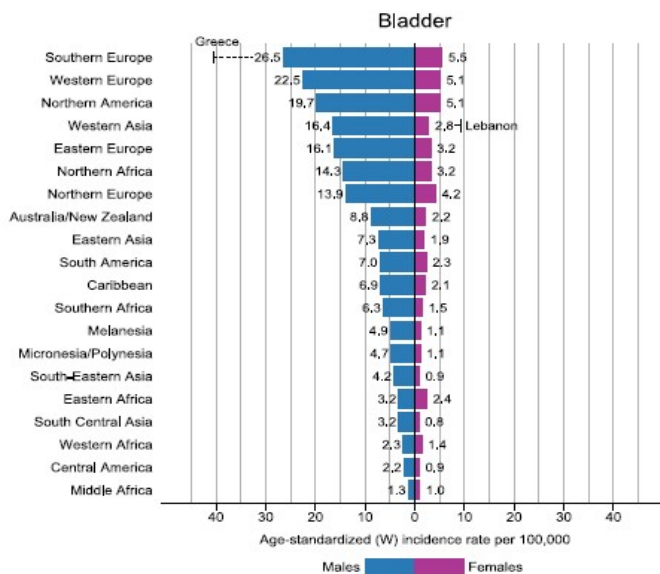
Bladder Cancer

Bladder cancer is a clear and present danger with serious economic and social consequences

Bladder cancer is characterized by a high recurrence rate and patients need to be monitored for their entire life



GLOBOCAN DATA 2018

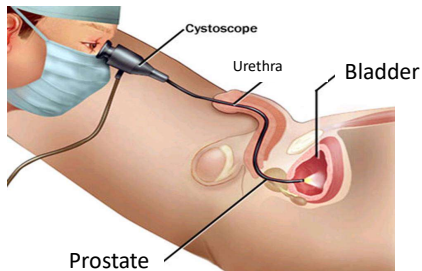


Bladder Cancer Diagnosis

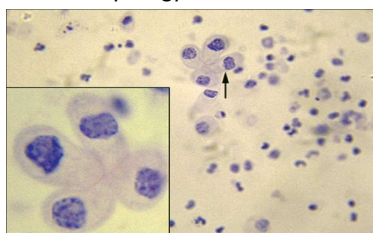
Cystoscopy



Biomarkers



Urine Cytology



There is an unmet clinical need for non-invasive accurate diagnosis of BC
The obvious choice is a urine based test

Biomarker Discovery by proteomics/peptidomics studies of our lab

- 1) IMAC fractionation in combination with LC-MS reveals H2B and NIF-1 peptides as potential bladder cancer biomarkers.

J Proteome Res, 2013. 12(9): p. 3969-79.

- 2) Profilin 1 is a potential biomarker for bladder cancer aggressiveness.

Mol Cell Proteomics, 2012. 11(4): p. M111 009449.

- 3) Analysis of secreted proteins for the study of bladder cancer cell aggressiveness.

J Proteome Res, 2010. 9(6): p. 3243-59.

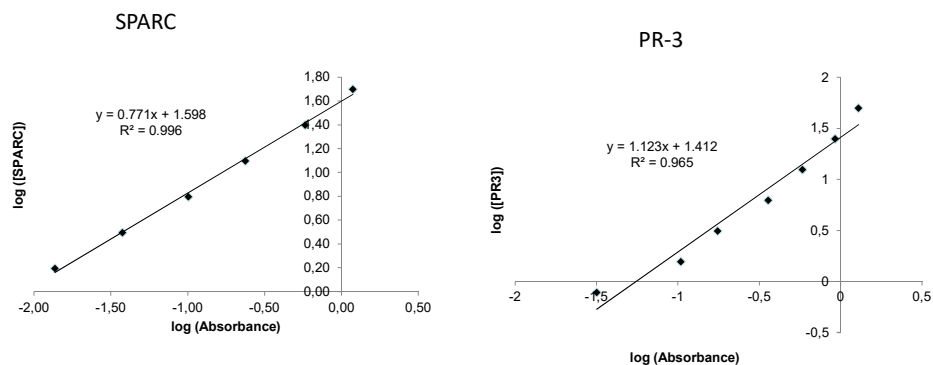
List of candidate biomarkers for detection by ELISA in urine

Myeloblastin (PR-3)
SPARC
Survivin
Profilin 1 (PFN1)
NIF-1
H2B
SLIT-2

Evaluation of ELISA kits analytical performance in urine (FDA guidelines)

- STANDARD CURVE EVALUATION (3 different curves minimum)
- SPIKING RECOVERY(HIGH, MEDIUM, LOW standard concentration spiked in urine that did not contain the biomarker, 8 replicates measured)
- REPRODUCIBILITY (HIGH, MEDIUM, LOW biomarker concentration urine samples, 8 replicates measured)
- LINEARITY (DILUTION OF HIGH concentration urine sample from 1:2 to 1:32, each dilution measured 8 times)

STANDARD CURVE EVALUATION



The range of ELISA assay standard curves spans 1 to 2 orders of magnitude

RECOVERY

SPARC

LOW	mean [SPARC] (ng/ml)	1.81
	expected [SPARC] (ng/ml)	1.57
	% Recovery	115%
MEDIUM	mean [SPARC] (ng/ml)	5.80
	expected [SPARC] (ng/ml)	6.25
	% Recovery	93%
HIGH	mean [SPARC] (ng/ml)	24.13
	expected [SPARC] (ng/ml)	25.00
	% Recovery	97%

PR-3

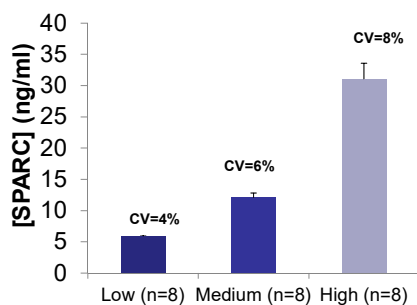
LOW	mean [PR3] (ng/ml)	1.96
	expected [PR3] (ng/ml)	0.78
	% Recovery	251%
MEDIUM	mean [PR3] (ng/ml)	4.35
	expected [PR3] (ng/ml)	3.13
	% Recovery	139%
HIGH	mean [PR3] (ng/ml)	14.89
	expected [PR3] (ng/ml)	12.50
	% Recovery	119%

SPARC recovery was satisfactory

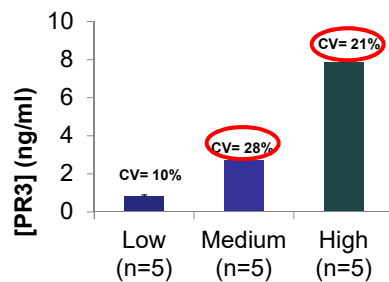
PR-3 recovery was not acceptable at low and medium concentrations

REPRODUCIBILITY

SPARC

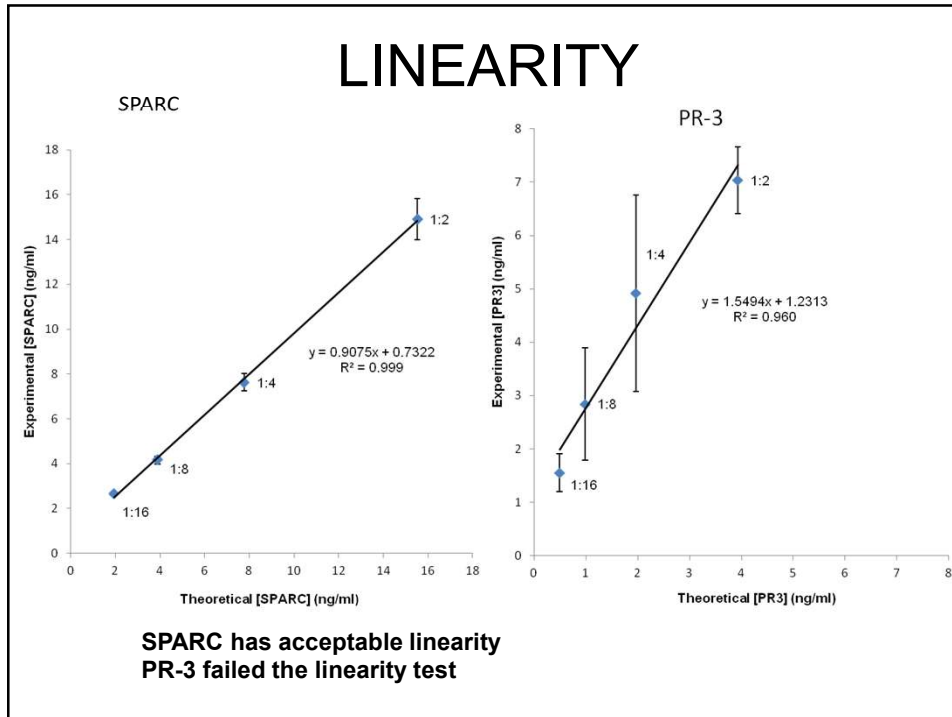


PR-3



SPARC reproducibility was excellent

PR-3 reproducibility was not acceptable for medium and high concentrations



ELISA analytical performance in urine

Biomarker	Company	Catalogue number	Analytical Performance Issues
SPARC	R&D Systems	DSP00	successful
SLIT-2	Cloud-Clone Corp	SEA672Hu	successful
H2B	US Biological Life Sciences	25705	recovery, linearity
	Cloud-Clone Corp	SEA356Hu	recovery, reproducibility, linearity
SURVIVIN	R&D Systems	DSV00	successful
	Enzo Life Sciences	ADI-900-111	linearity
PFN-1	USCN LIFE	E2122h	standard curve, recovery, reproducibility, linearity
	US Biological Life Sciences	27613	recovery, linearity
	Cloud Clone Corp	SEC233Hu	reproducibility, linearity
NIF-1	CUSABIO	EL026683HU	recovery, reproducibility, linearity
	USCN LIFE	E1019h	recovery, linearity
Proteinase 3	CUSABIO	E13058h	reproducibility, linearity

Urine samples are not compatible with most ELISA assays
R&D systems ELISA kits are a notable exception

Analytical Performance of ELISA Assays in Urine: One More Bottleneck towards Biomarker Validation and Clinical Implementation.

Chatziharalambous D, Lygiriou V, Latosinska A, Stravodimos K, Vlahou A, Jankowski V, Zoidakis J.
PLoS One. 2016;11(2):e0149471

Problems with urinary ELISA assays

- **Specificity:** proteins are mainly present as fragments

Crit Rev Clin Lab Sci. 2011 Mar-Apr;48(2):87-96

Immunochemically unreactive albumin in urine: fiction or reality?

- **Interference from hematuria**

World J Urol. 2012 Dec;30(6):869-73

Urinary BTA: indicator of bladder cancer or of hematuria?

The presence of hematuria in subjects without malignant disease can result in false-positive BTA assays

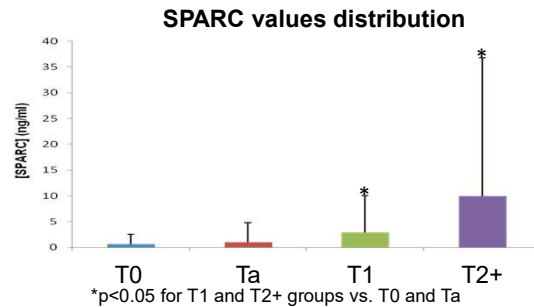
- **Interference from organic and inorganic urine components**

Population Data for Integrated Study of Bladder Cancer (ISBLAC)*

	Study Arm 1: Primary UBC (N=264)		Study Arm 2: Recurrent UBC (N=307)	
	Case Group (n=179) n (%)	Control Group (n=85) n (%)	Case Group (n=56) n (%)	Control Group (n=251) n (%)
Age, years	69.7 ± 12.2	66.4 ± 14.1	72.9 ± 9.6	70.1 ± 11.8
Gender				
Male	155 (86.6)	64 (74.1)	48 (85.7)	208 (82.9)
Female	24 (13.4)	22 (25.9)	8 (14.3)	43 (17.1)
Tumor stage				
T0	0 (0.0)	85 (100.0)	0 (0.0)	251 (100.0)
Ta	97 (54.2)	0 (0.0)	35 (62.5)	0 (0.0)
T1	53 (29.6)	0 (0.0)	12 (21.4)	0 (0.0)
T2	29 (16.2)	0 (0.0)	9 (16.1)	0 (0.0)

*urine samples obtained from CNIO: Centro Nacional de Investigaciones Oncológicas

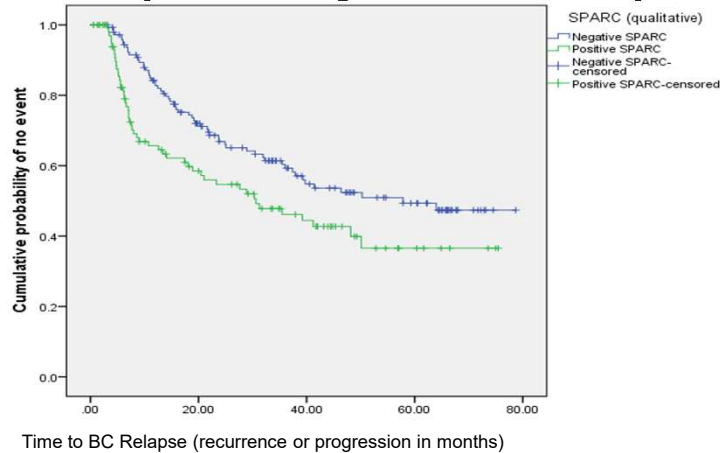
SPARC ELISA results in ISBLAC urine samples



Performance of SPARC for detecting primary and recurrent BC

BC vs. controls	Primary BC	Recurrent BC
Sensitivity (%)	43.0	39.3
Specificity (%)	70.6	78.9
AUC	0.593	0.592

ISBLAC Study: Kaplan-Meier Curves for the probability of tumor relapse



Positive SPARC findings are associated with higher rates of UBC relapse
Log rank p -value = 0.013

Effect of Hematuria on SPARC ELISA

	Study Arm 1: Primary BC	
	Case Group (n=176) n (%)	Control Group (n=81) n (%)
Hematuria Absent		
SPARC	70 (100.0)	49 (100.0)
Negative	58 (82.9)	39 (79.6)
Positive	12 (17.1)	10 (20.4)
Hematuria Present		
SPARC	106 (100.0)	32 (100.0)
Negative	41 (38.7)	18 (56.3)
Positive	65 (61.3)	14 (43.8)

- The majority of UBC patients without hematuria were found to have false negative findings
- The false positive rate of SPARC in detecting incident and recurrent UBC was more than two fold greater in controls with hematuria compared to controls without hematuria

Greek movie: Όλα είναι δρόμος

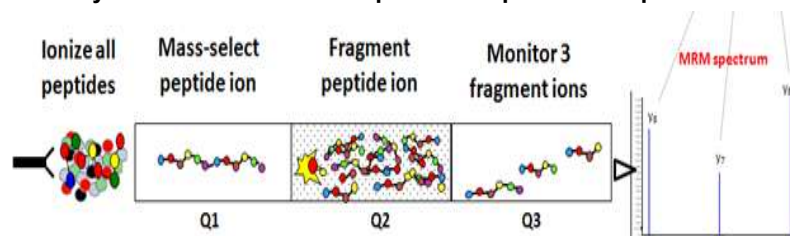
<https://www.youtube.com/watch?v=5nwgjluGjI4>

Development of MRM assays

Urine sample preparation

1. Protein concentration measurement and precipitation
2. Resuspension in Urea buffer, reduction and alkylation
3. Trypsin digestion (overnight) and peptide desalting
4. Drying of peptide solution and resuspension in HPLC mobile phase

MRM analysis in ABSCIEX 4000 Triple Quadrupole Mass Spectrometer



Excellent Review

Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions.
Nature methods 9(6), 555-566 (2012)

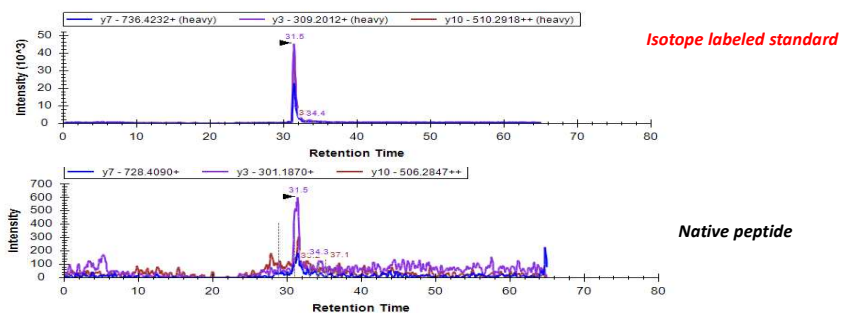
MRM application in urine

MRM targeted proteomics as a tool for disease protein biomarker validation and absolute quantification in human urine

Expert Review in Molecular Diagnostics 15(11):1441-54 (2015)

MRM assays in urine: Specificity and Reproducibility

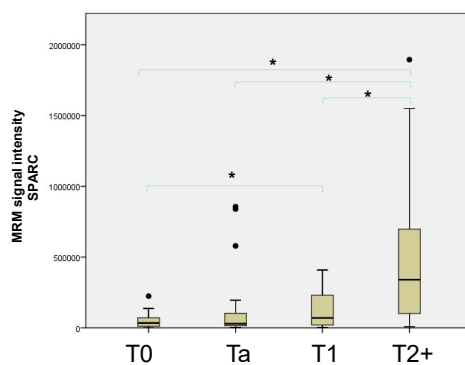
Specificity proven by analysis of the native and isotope labeled standard SPARC peptide



Reproducibility

	Inter-assay CV (%)
Profilin-1	8.5
SLIT-2	8.9
SPARC	8.0

Representative Data SPARC MRM signal intensity for Benign controls and BC patient groups



(*p<0.05, dots represent outliers)

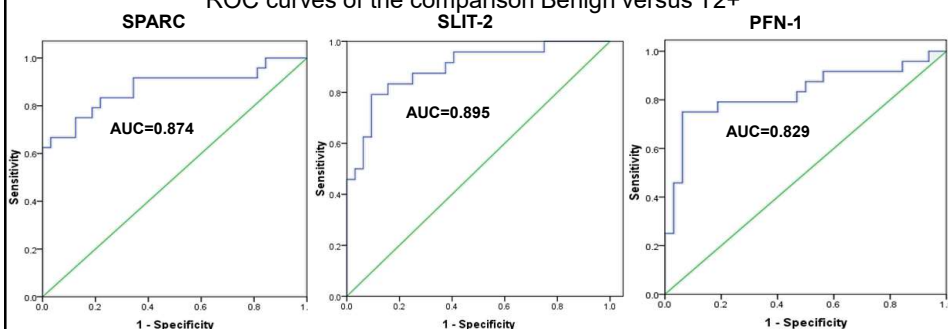
SPARC levels increase with stage

The T2+ group has significantly higher SPARC levels compared to Benign controls

Initial experiments in 97 urine samples (primary BC cases and benign controls) allowed the detection of native peptides and their fragments for 3 biomarkers: SPARC, SLIT2, Profilin-1

Evaluation of the diagnostic potential

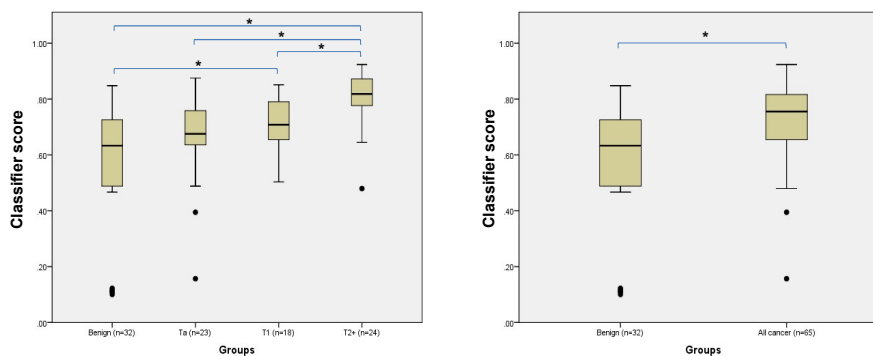
ROC curves of the comparison Benign versus T2+



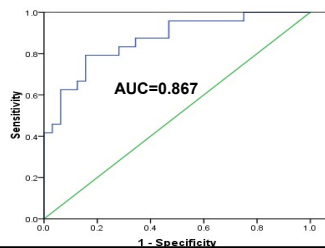
AUC for comparisons

	Area under the curve (AUC)		
	SPARC	SLIT-2	PFN-1
Benign vs Ta	0.516	0.614	0.556
Benign vs T1	0.658	0.752	0.552
Benign vs T2+	0.874	0.895	0.829
Benign vs Cancer (Ta,T1, T2+)	0.688	0.756	0.656

Diagnostic performance of a panel from the 3 biomarkers



ROC curve of the comparison Benign versus T2+



- The values of the 3 biomarkers have high correlation
- The information obtained is redundant
- No added value by combining them

Conclusions

- MRM assays in urine are specific unlike ELISA
- The 3 biomarkers perform better for T2+ BC tumors diagnosis
- Combining these 3 highly correlated biomarkers does not improve diagnostic performance

Greek song: βρέχει φωτιά στη στράτα μου

<https://www.youtube.com/watch?v=GQSYqh1eMF4>

Is it possible to apply MRM assays in the clinical setting? Blood-based proteomic classifier for discrimination of benign from malignant lung nodules

Clinical need

80% of lung nodules detected by imaging methods (mainly CT) are benign but a biopsy has to be performed. It is necessary to develop a non invasive diagnostic test with high negative predictive value (>90%).

Financial Aspect

The test also has to be cheaper than lung biopsy.

The solution relies on a classifier score derived from individual biomarker measurements by MRM assays.

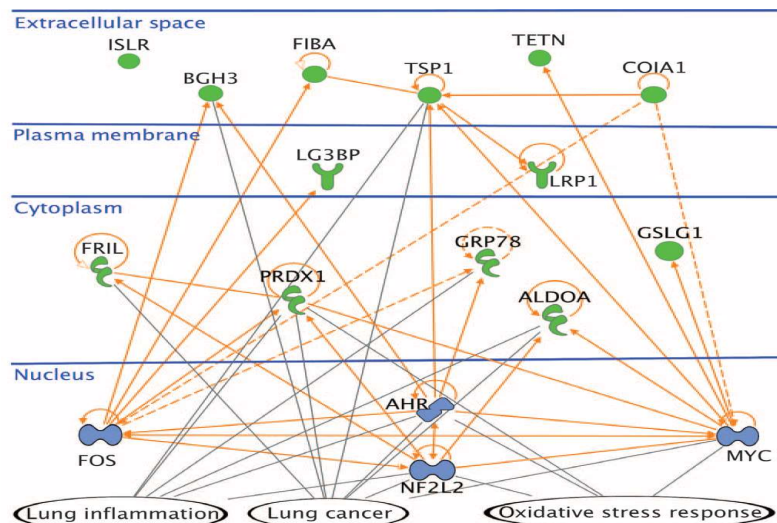
How were these biomarkers selected?

Li XJ et al. Sci Transl Med. 2013;5(207):207ra142

Biomarker Selection

Number of proteins	Selection criteria
388	Lung cancer-associated protein candidates sourced from tissue and literature
371	Number of the 388 protein candidates successfully developed into an MRM assay
190	Number of the 371 MRM protein assays detected in plasma
125	Number of the 190 MRM protein assays detected in at least 50% of cancer or 50% of benign discovery samples
36	Number of the 125 detected proteins that were cooperative
21	Number of the 36 cooperative proteins with robust MRM assays (no interfering signals, good signal-to-noise ratios, etc.)
11	Number of the 21 robust and cooperative proteins with stable logistic regression coefficients

Pathway analysis indicates that the selected biomarkers are implicated in biological processes relevant to lung cancer



Classifier refinement and validation

The main challenge was to discover proteins for **normalization** (present in all samples with low variability between samples and robust MRM assays)

Proteins		
Diagnostic	Name	
ALDOA_Human	Fructose-1,6-bisphosphatealdolase	In multi-center validation study
COIA1_Human	Collagen alpha-1(XVIII) chain	
FRIL_Human	Ferritin light chain	Sensitivity 92% Specificity 20%
LG3BP_Human	Galectin-3 binding protein	
TSP1_Human	Thrombospondin-1	
Normalization		
	Name	
C163A_Human	Scavenger receptor cysteine-rich type 1 protein M130	
GELS_Human	Gelsolin	
LUM_Human	Lumican	
MASP1_Human	Mannan-binding lectin serine protease 1	
PEDF_Human	Pigment epithelium-derived factor	
PTPRJ_Human	Receptor-type tyrosine-protein phosphatase	Vachani A et al. J Thorac Oncol. 2015, 10(4):629-37 Vachani A et al. Lung. 2015, 193(6):1023-7

Classifier clinical implementation

A modified classifier based on 2 plasma proteins (LG3BP_Human, C163A_Human) measured by MRM and clinical data was validated in a large clinical trial
PANOPTIC: Pulmonary Nodule Plasma Proteomic Classifier

It is used in the clinical setting in order to avoid unnecessary lung biopsies for benign nodules!

The clinical test is covered by Medicare and Medicaid in the US

Silvestri et al. Chest, 2018, Volume 154, Issue 3, Pages 491–500
<https://www.biodesix.com/products/nodify-xl2/>

Advice based on
Greek tragedies and football

ΥΒΡΙΣ Hubris



ΑΤΙΣ Confusion



NEMESIS / ΤΙΣΙΣ Divine Revenge / Obliterating Punishment







ΚΑΘΑΡΣΙΣ Redemption



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But the Wise Perceive Things about to Happen

Ordinary people know what's happening now,
the gods know future things
because they alone are totally enlightened.

**Of what's to come the wise perceive
things about to happen.**

Sometimes during moments of intense study
their hearing's troubled: **the hidden sound
of things approaching** reaches them,
and they listen reverently, while in the street outside
the people hear nothing whatsoever.

C. Kavafis (1863-1933)

