Detection of candidate biomarkers of prostate cancer progression in serum: a depletion-free 3D LC/MS quantitative proteomics pilot study

Nina Fanaropoulou

Maria Peleli

Yorgos Polychronidis

Introduction – Prostate cancer (Pca) screening

- „Prostate cancer has been described as the par excellence example of overdiagnosis... „

- „The test's popularity has led to a hugely expensive public health disaster. It's an issue I am painfully familiar with – I discovered PSA in 1970. . . .„

Chapman S, Barratt A, Stockler M. Let sleeping dogs lie? What men should know before getting tested for prostate cancer. Sydney: Sydney University Press, 2010: p25
Ablin RJ. The great prostate mistake. New York Times, 10 March 2010.

Introduction – Prostate cancer (Pca) screening

- PSA levels <1.0 ng/ml virtually rule out a prostate cancer (for a patient< 50yrs)
- 1068 men had to be screened and 48 men had to have curative treatment in order to save one man's life
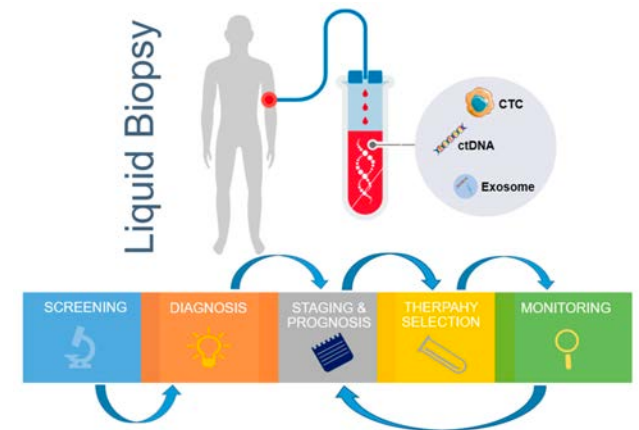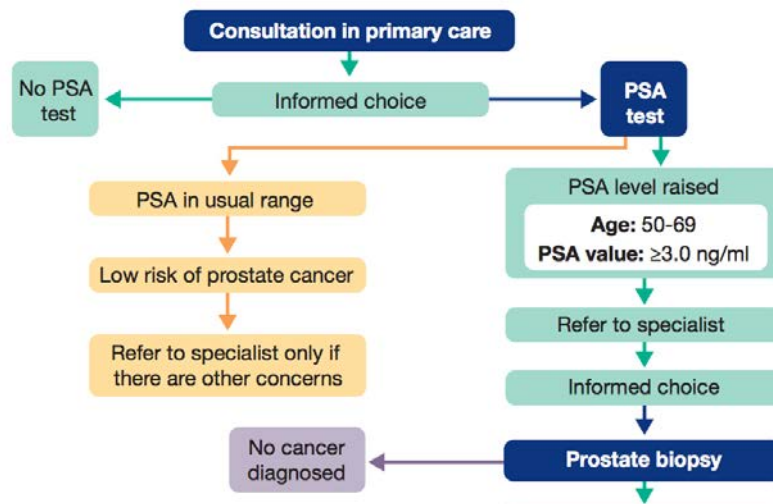
Holmström et al, BMJ. 2009; 339: b3537 , Schröder et al, N Engl J Med 2009; 360:1320-1328
Prostate Cancer Risk Management Programme (PCRMP) sheet – courtesy of the NHS, Micromachines 2018, 9(8), 397

What else is there to utilize and how?
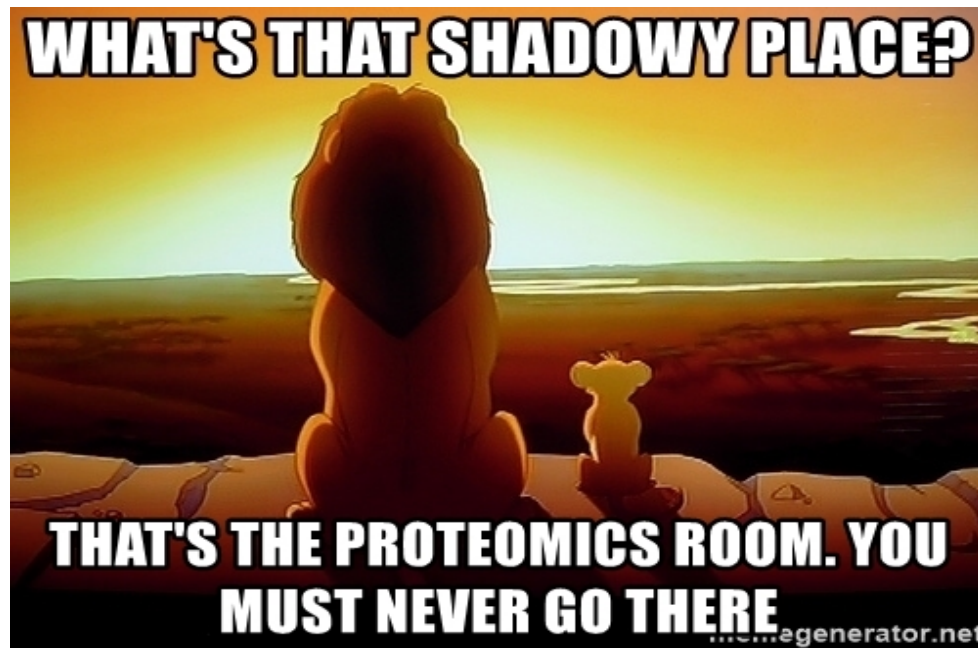
Hypothesis: The **3D-iTRAQ-LC-MS** Methodology (Garbis et al, 2008, Al-Dhagri et al, 2014) protocol is selective, sensitive and specific enough to reveal novel and clinically relevant **biomarkers** that can stage Pca progression.

PoC

Materials and Methods
Which Patient Cohorts/Samples?

Prof. Pandha SUN Study

Group	PSA Value	Number of pts
Pca Null	<1 ng/ml	20
Putative Benign Disease (BPH, prostatitis, PIN, inflammation, atrophy)	4.7–12 ng/ml	15
T1-T2 stage Pca	3.9–4.8 ng/ml	20
T3-T4 stage Pca	6.7–17.65 ng/ml	20

COHORT 1
Discovery Experiment (MS)

Prof. Clarke ProMPT Study

Group	PSA Value	Number of pts
Pca Null	Same	20
Putative Benign Disease (BPH, prostatitis, PIN, inflammation, atrophy)	Same	20
T1-T2 stage Pca	0.7–31 ng/ml	20
T3-T4 stage Pca	0.5–1400 ng/ml	20

COHORT 2
Validation Experiment (ELISA)

LC-MS

Determines mass-to-charge ratio, converts into molecular mass

😖 **Depletion Strategies**

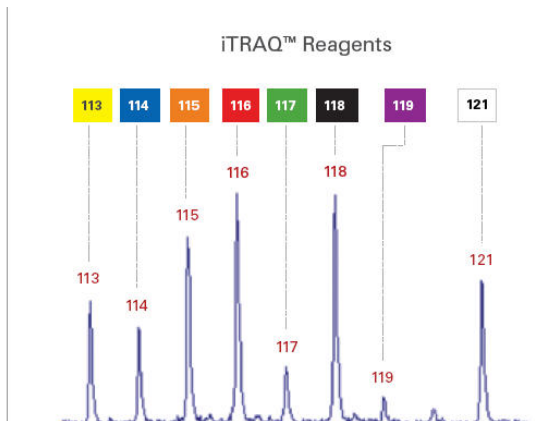Eliminate high abundance proteins (Albumin, Igs) which *mask* low abundance proteins
but
Inevitable loss -sweep away (eg exosomal, proteins bound to albumin)
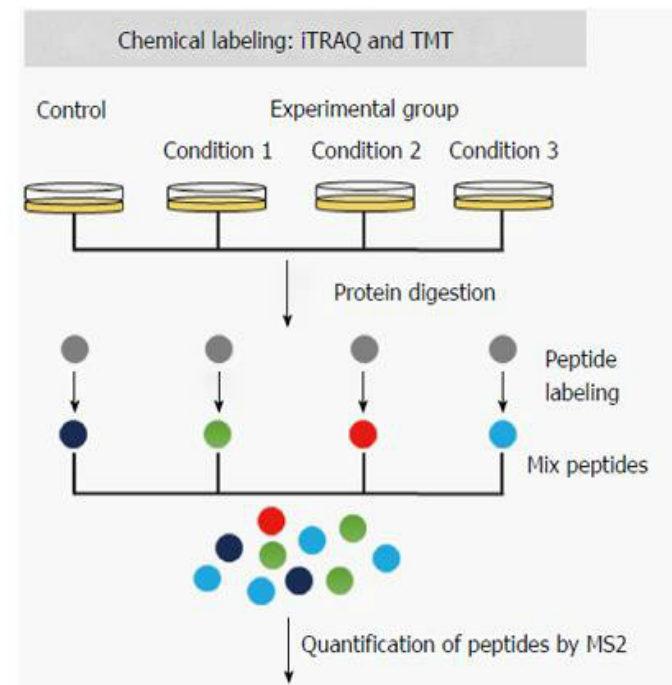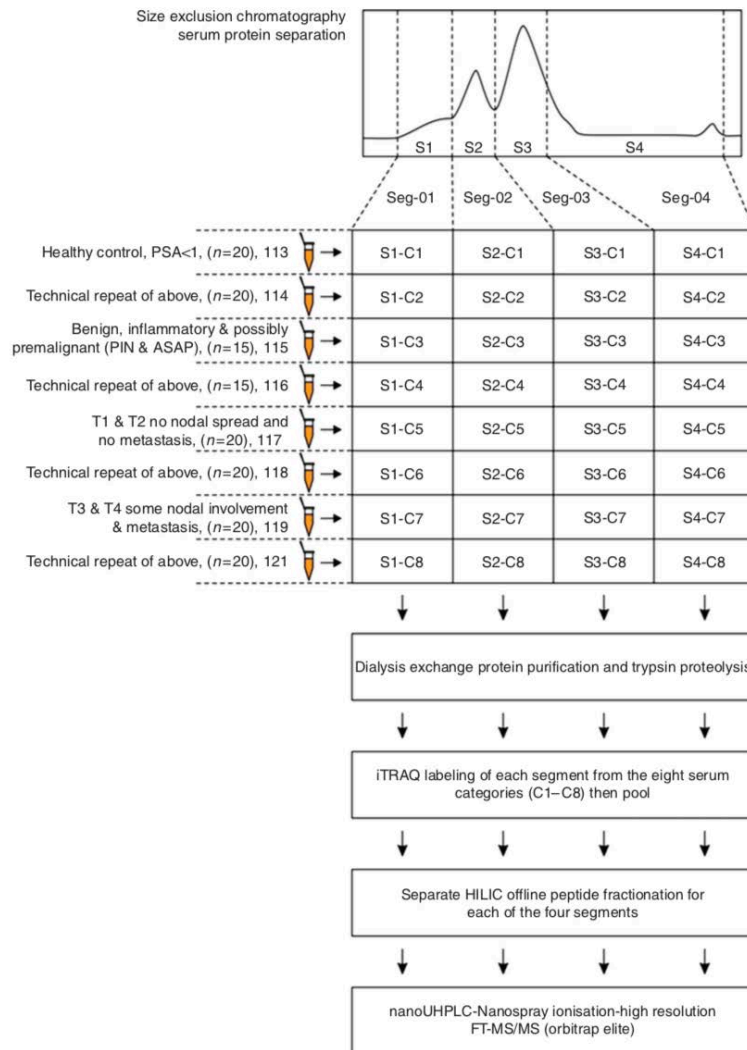
VS

😊 **Depletion-FREE Strategy**

Preservation through **iTRAQ Labeling and SuPrE-SEC**

How to best identify the proteome?

- Solubilize fresh neat serum (6M Gua/10% MeOH)

- SEC protein separation/purification

- Trypsin digestion→ Peptides

- **iTRAQ labeling**

- Pooling

- Offline peptide fractionation with HILIC and on-line RP LC-MS

- Statistical Analysis

- Literature and Network Analysis-Bioinformatics (STRING Database)

Chemical labeling: iTRAQ and TMT

Control | Experimental group

Condition 1   Condition 2   Condition 3

Protein digestion

Peptide labeling

Mix peptides

Quantification of peptides by MS2

Size exclusion chromatography
serum protein separation

| | S1 | S2 | S3 | S4 |

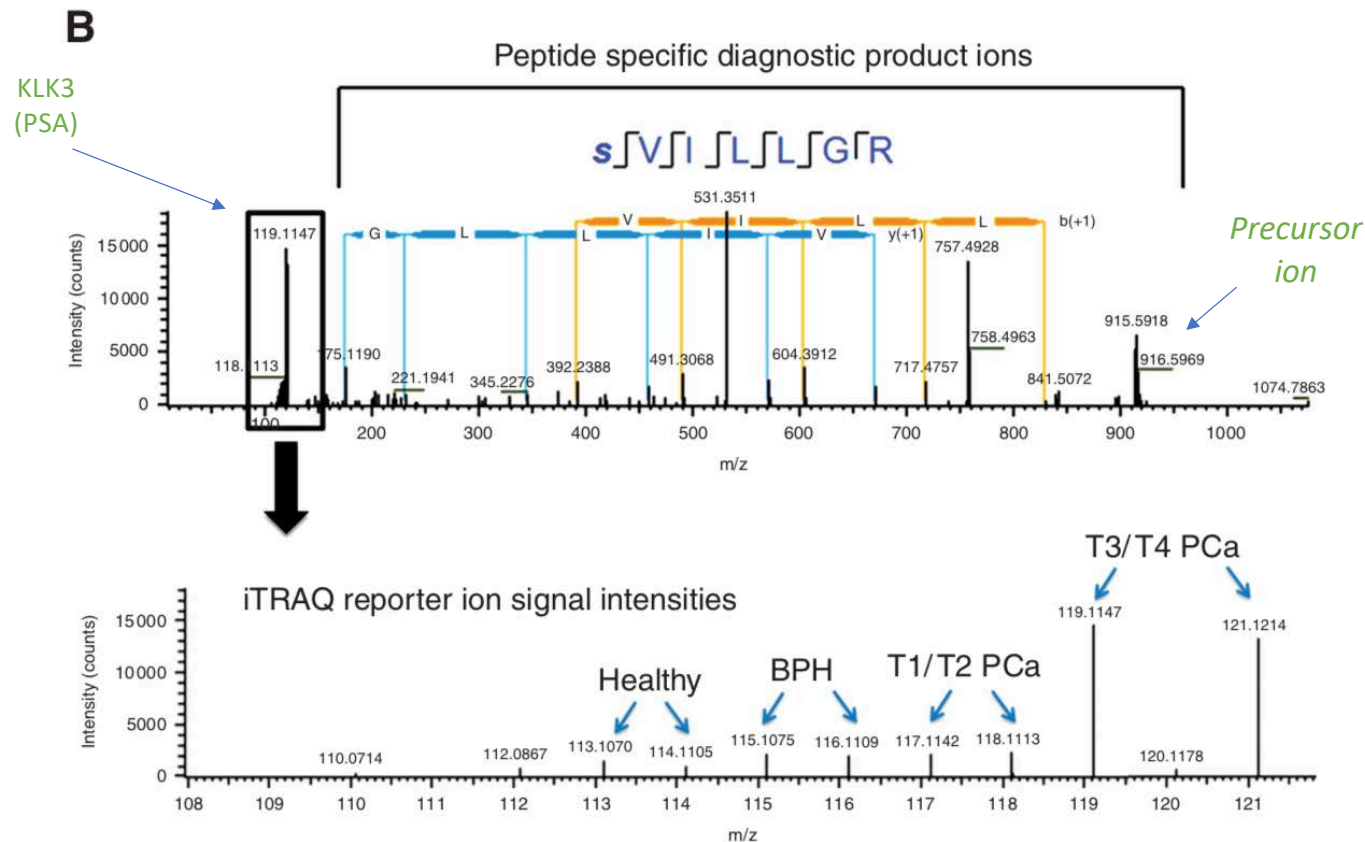|	Seg-01	Seg-02	Seg-03	Seg-04
Healthy control, PSA<1, (n=20), 113	S1-C1	S2-C1	S3-C1	S4-C1
Technical repeat of above, (n=20), 114	S1-C2	S2-C2	S3-C2	S4-C2
Benign, inflammatory & possibly premalignant (PIN & ASAP), (n=15), 115	S1-C3	S2-C3	S3-C3	S4-C3
Technical repeat of above, (n=15), 116	S1-C4	S2-C4	S3-C4	S4-C4
T1 & T2 no nodal spread and no metastasis, (n=20), 117	S1-C5	S2-C5	S3-C5	S4-C5
Technical repeat of above, (n=20), 118	S1-C6	S2-C6	S3-C6	S4-C6
T3 & T4 some nodal involvement & metastasis, (n=20), 119	S1-C7	S2-C7	S3-C7	S4-C7
Technical repeat of above, (n=20), 121	S1-C8	S2-C8	S3-C8	S4-C8

Dialysis exchange protein purification and trypsin proteolysis

iTRAQ labeling of each segment from the eight serum categories (C1–C8) then pool

Separate HILIC offline peptide fractionation for each of the four segments

nanoUHPLC-Nanospray ionisation-high resolution FT-MS/MS (orbitrap elite)

S1: High MW Proteome
S2: Ig Proteome
S3: Albumin
S4: Low MW Proteome

Mass Spectrometry (MS)

The Mass Spectrometry Experiment

mass analyzer

ionizer

Stream of gas phase molecules

Stream of molecular ions and fragments

Ions are deflected by magnetic field

detector

liquid delivery

● = molecule of interest

into MS

+ ve   - ve

With Voltage applied
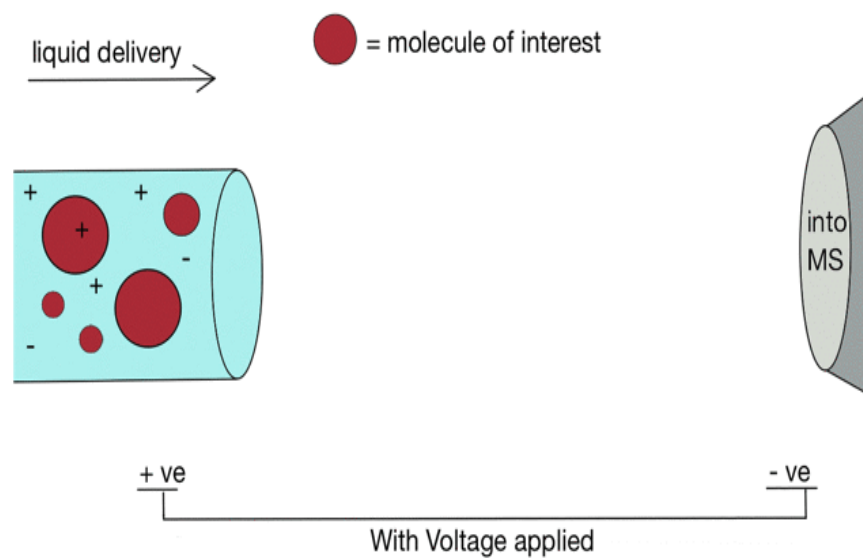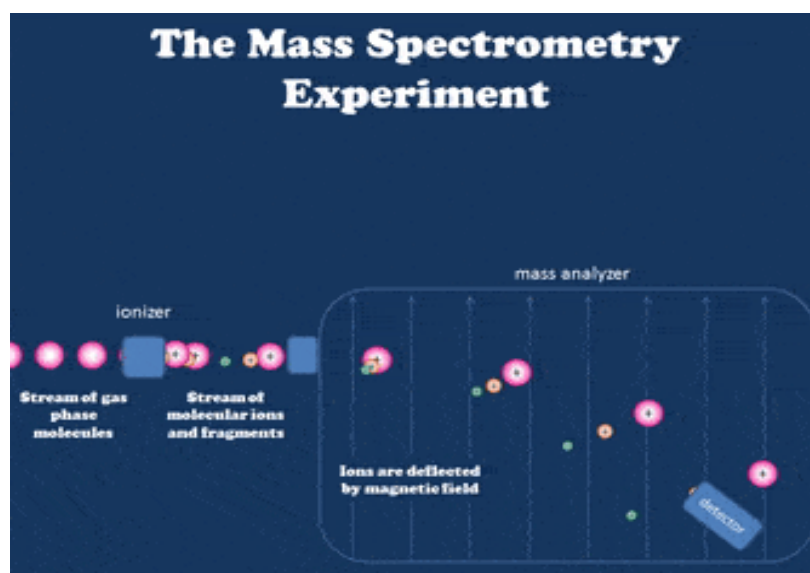
Qualitative Data
Quantitave Data

Results-What did the MS output look like?

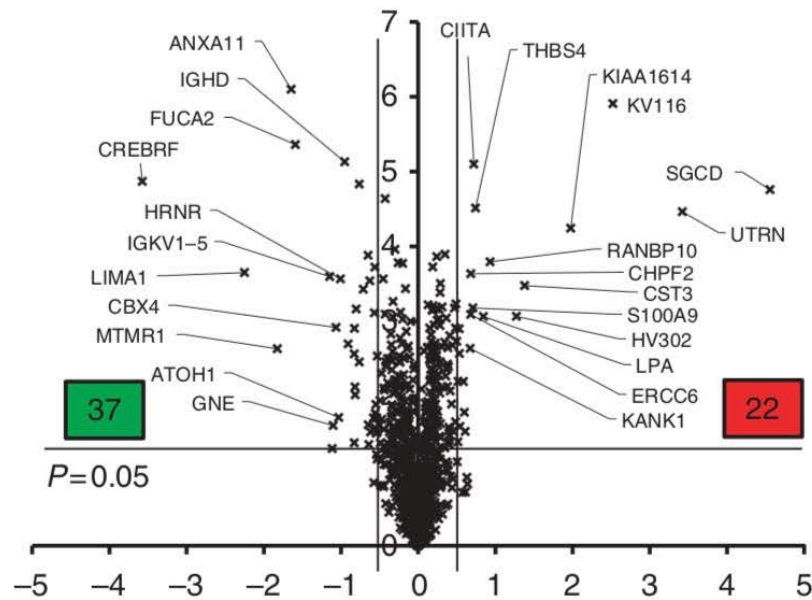# Results-Statistical Analysis
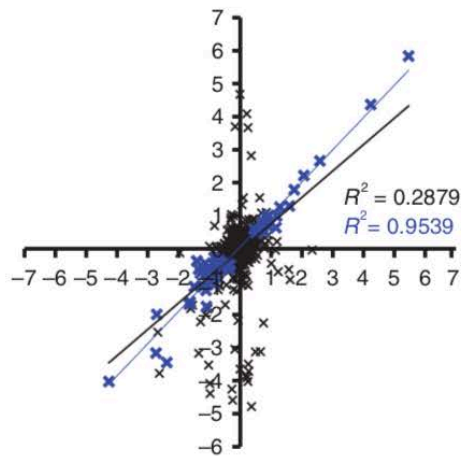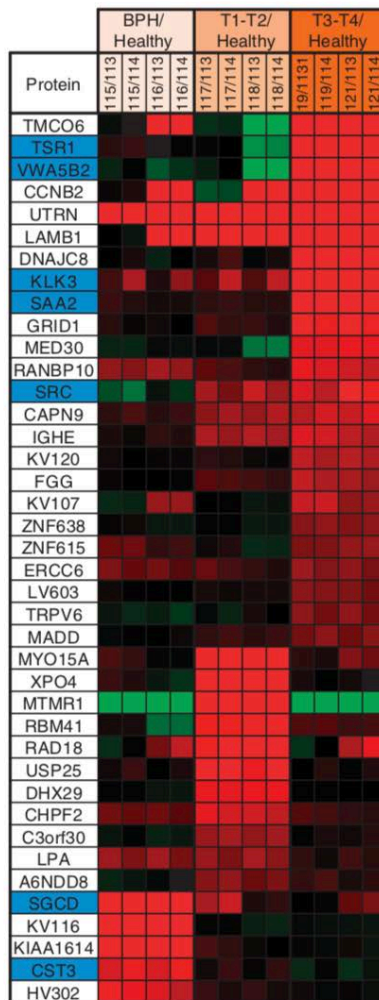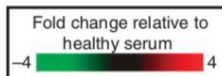

Regulation Score:

$$\frac{\bar{x}}{(s.d.+1)}$$

- **Heat Map** of **top 40** overabundant proteins(P<0.05) sorted by **regulation score**, across **BPH, T1–T2** and **T3–T4** samples relative to healthy serum.

- **7** proteins shortlisted to undergo **ELISA** validation

  TSR1
  VWA5B2
  KLK3
  SAA2
  SRC
  SGCD
  CST3

Results-Summary Workflow Map

1. Discovery Experiment

Discovery samples:
Prof Pandah SUN study,
REC reference 08/H1306/115.
Categorised as follows:
i) Prostate cancer null (n=20)
ii) Putative benign disease (n=15)
iii) T1-T2 stage prostate cancer (n=20)
iv) T3-T4 prostate cancer (n=20)

iTRAQ 3D LC Mass spectometery identified differentially regulated proteins. **1039 proteins**.

Ranking by regulation score and selection of top **40 proteins**.

Validation panel selection based on differential expression between disease groups and commercially availably kits. **7 proteins**.

2. Validation Experiment

Validation samples:
Prof Clarke ProMPT study,
REC reference MREC/01/4/061.
Categorised as follows:
i) Prostate cancer null (n=20)
ii) Putative benign disease (n=20)
iii) T1-T2 stage prostate cancer (n=20)
iv) T3-T4 prostate cancer (n=20)

Validation of protein panel by ELISA **2 proteins**.

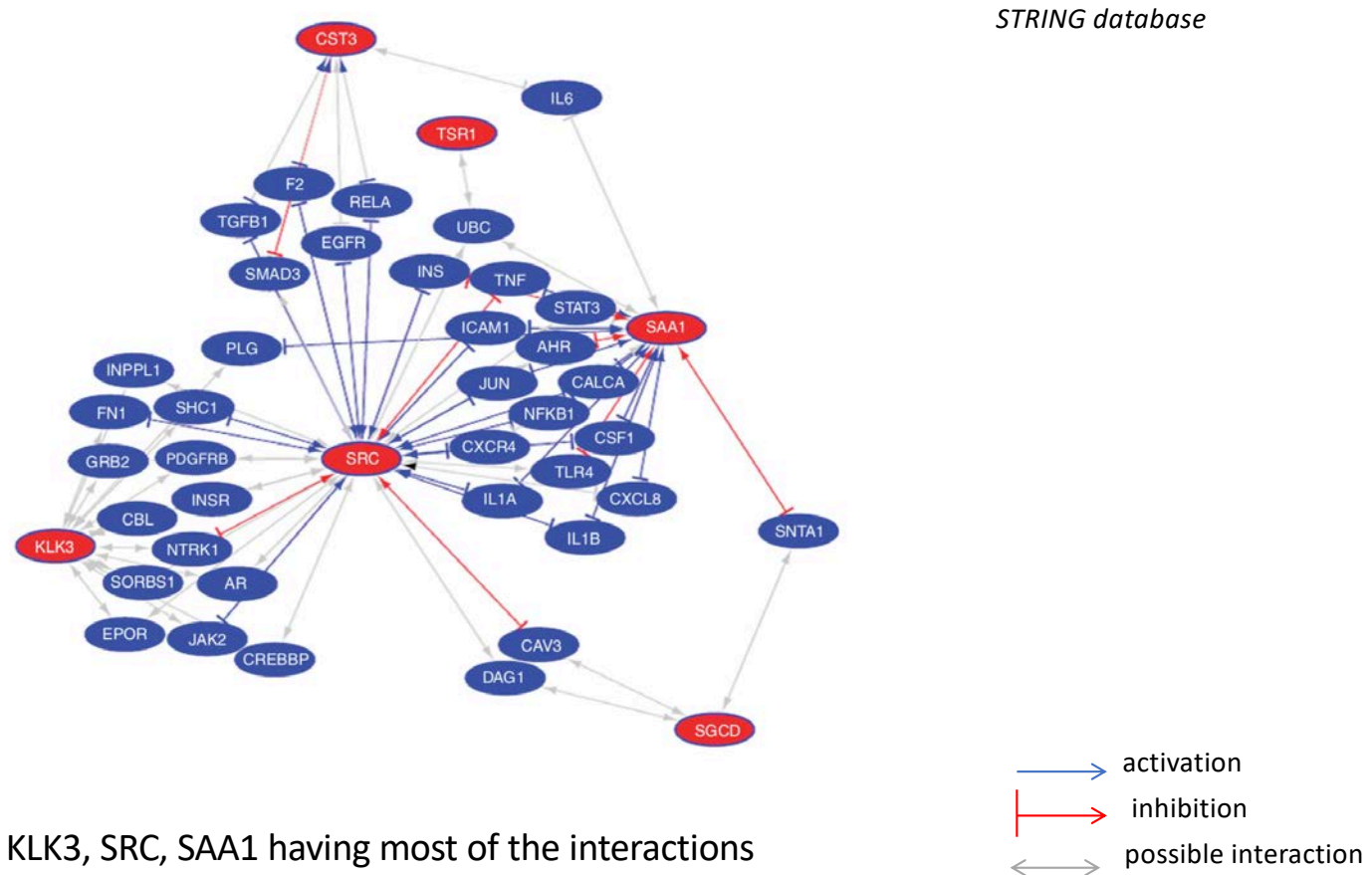# How much different is the expression profile at the 3 different stages?

Protein	log2 benign/control				log2 T1–T2/control				log2 T3–T4/control			
Delta-sarcoglycan	5.329	5.457	5.643	5.837	1.159	1.385	0.232	0.377	−0.021	0.104	0.699	0.846
Pre-rRNA-processing protein TSR1 homologue	0.334	0.452	0	0.078	−0.058	0.014	−1.11	−1.012	4.555	4.676	4.642	4.77
Kalikrein 3	0.498	1.174	0.261	0.988	0.633	1.32	0.596	1.295	3.204	3.892	3.161	3.818
von Willebrand factor A domain-containing protein 5B2	−0.312	−0.026	−0.682	−0.435	−0.253	−0.012	−1.836	−1.569	3.991	4.28	4.096	4.393
Serum amyloid A protein	0.471	0.288	0.181	0.209	0.379	0.449	0.325	0.327	2.046	1.992	2.309	1.965
Proto-oncogene tyrosine-protein kinase Src	−0.633	−0.879	−0.151	−0.437	1.118	0.827	1.366	1.1	1.506	1.263	2.044	1.809
Cystatin-C	1.476	1.749	1.461	1.785	0.172	0.456	0.097	0.394	−0.345	−0.06	−0.405	−0.15

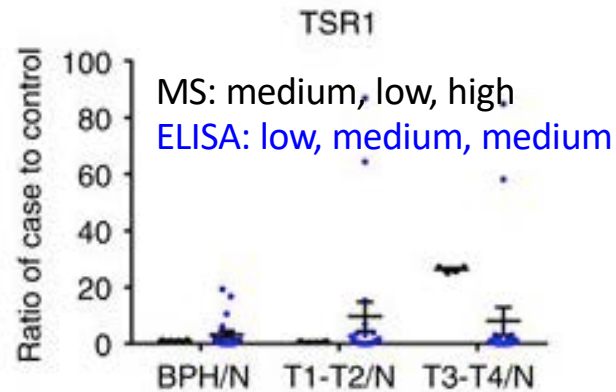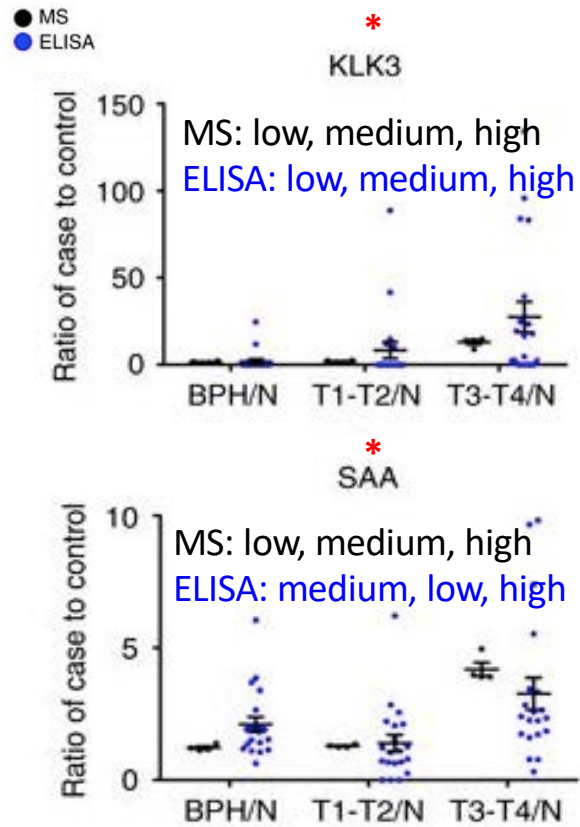KLK3: Benign average=0,73, T1-2 average=0,961, T3-4 average=3,519
SAA: Benign average=0,287, T1-2 average=0,37, T3-4 average=2,078
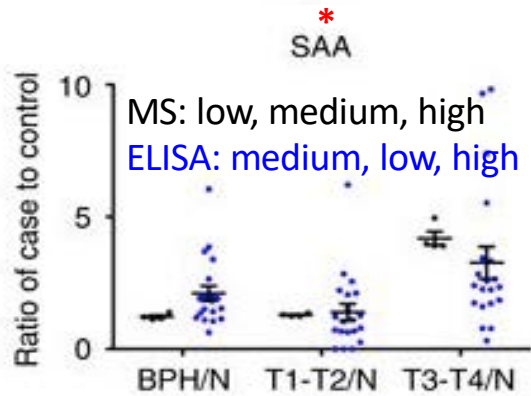TSR1: Benign average=0,216 T1-2 average=-0,51, T3-4 average=4,66

What is the interactome of the selected markers?

STRING database

KLK3, SRC, SAA1 having most of the interactions

Expression profile of the selected markers in ELISA

KLK3

MS: low, medium, high
ELISA: low, medium, high

TSR1

MS: medium, low, high
ELISA: low, medium, medium

SAA
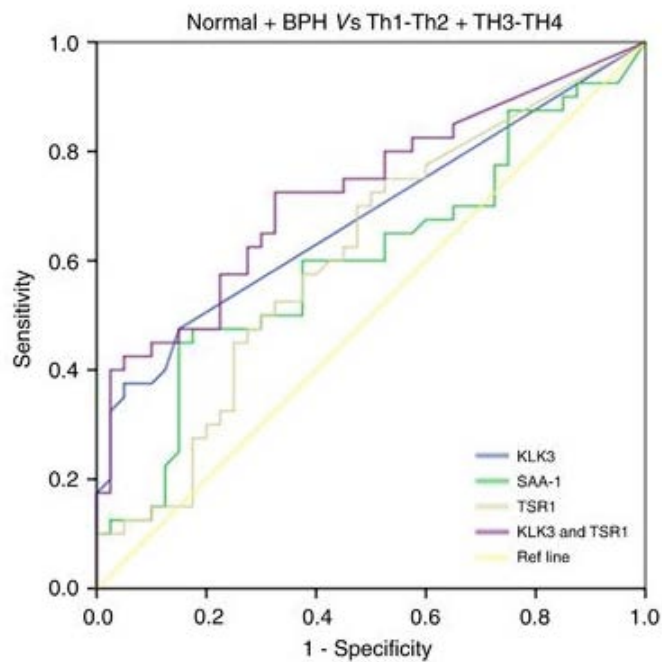
MS: low, medium, high
ELISA: medium, low, high

Why do you think ELISA for TSR1 has failed here?

2 proteins KLK3, SAA showed different expression profile
at the different Prostate Cancer stages

Sensitivity & specificity analysis

Protein	AUC	95% Confidence interval	Asymptotic sig
KLK3	0.679	0.561–0.798	$P = 0.006$
SAA-1	0.602	0.476–0.728	$P = 0.117$
TSR1	0.613	0.489–0.737	$P = 0.081$
KLK3 TSR1	0.727	0.591–0.821	$P < 0.0005$

KLK3+SAA-1 might be another promising combination

Literature review

Marker	Total publications	PCa publications	PCa publications enrichment *P*-value
SGCD	323	3	0.60
TSR1	112	2	0.29
VWA5B2	3	0	1.00
CST3	4431	60	5.85E−3
SRC	7805	618	5.28E−342
SAA1	3767	58	4.21E−4
KLK3	44 017	19 295	1.75E−25 391

| Marker	PCa biomarker publications	PCa biomarker publications enrichment *P*-value
SGCD	2	0.90
TSR1	0	1.00
VWA5B2	0	1.00
CST3	50	1.00
SRC	305	1.19E−1201
SAA1	48	1.00
KLK3	4908	1.92E−3105

Limitations (proof of concept paper)

- Small sample size
- Protein inference issue
- PCa heterogeneity -> low validation rate
- ELISA validation: **intact** interaction between an epitope and antigen is a must
- Literature review Vs systematic review

Conclusions

- PSA screening remains controversial and limited however still the gold standard as a predictive marker for follow-up
- Serum proteomics discovery pipeline is feasible in prostate cancer
- SAA and TSR1 can add to the predictability of KLK3 (KLK3: 0.679 TSR1 + KLK3: 0.727)
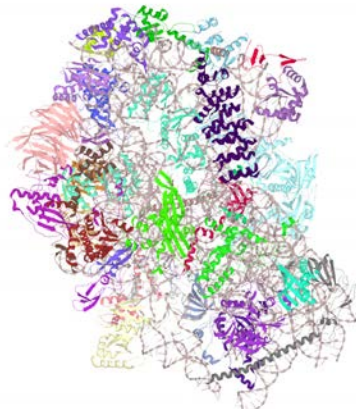
Pre-rRNA-processing protein TSR1 homolog
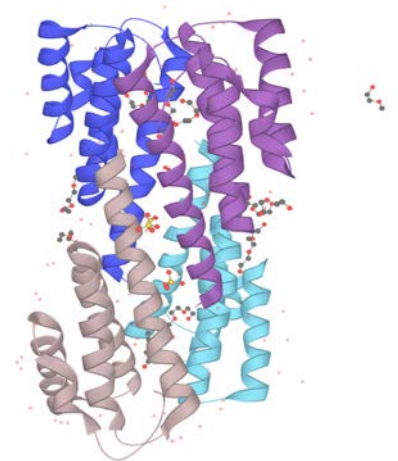
Serum amyloid A-1 protein

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(esp. Penny Georgakopoulou and Prof. Diomedes Logothetis)