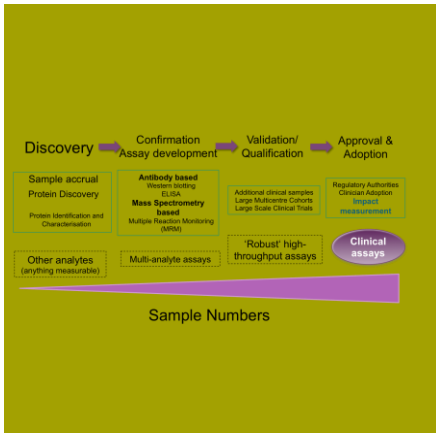
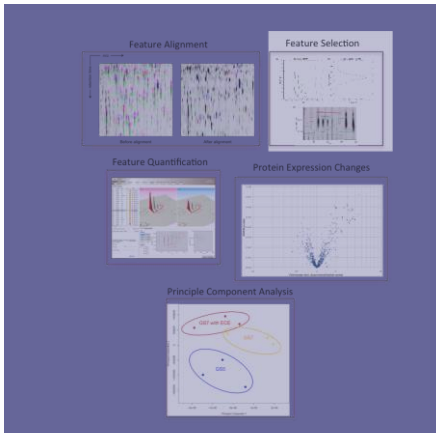
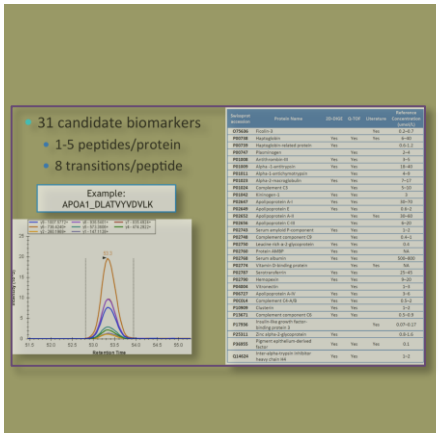
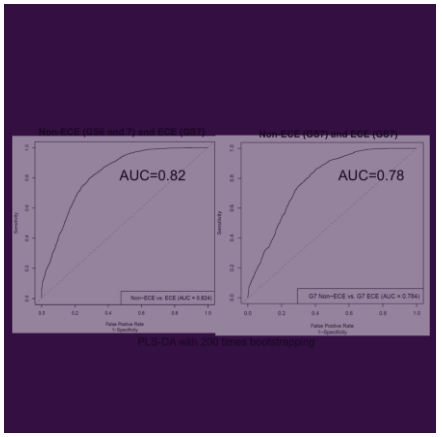
Development of a serum protein assay for organ confined prostate cancer







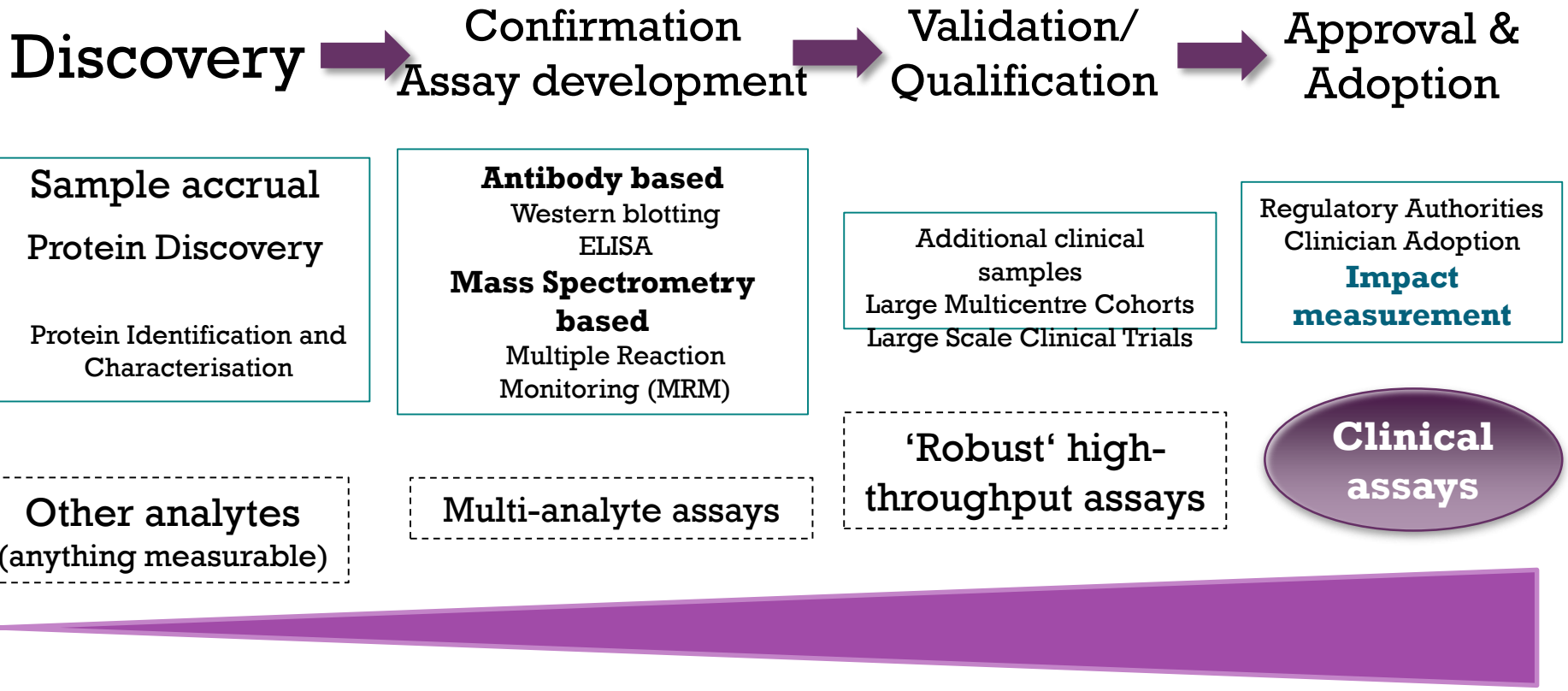


15th[th] June 2014

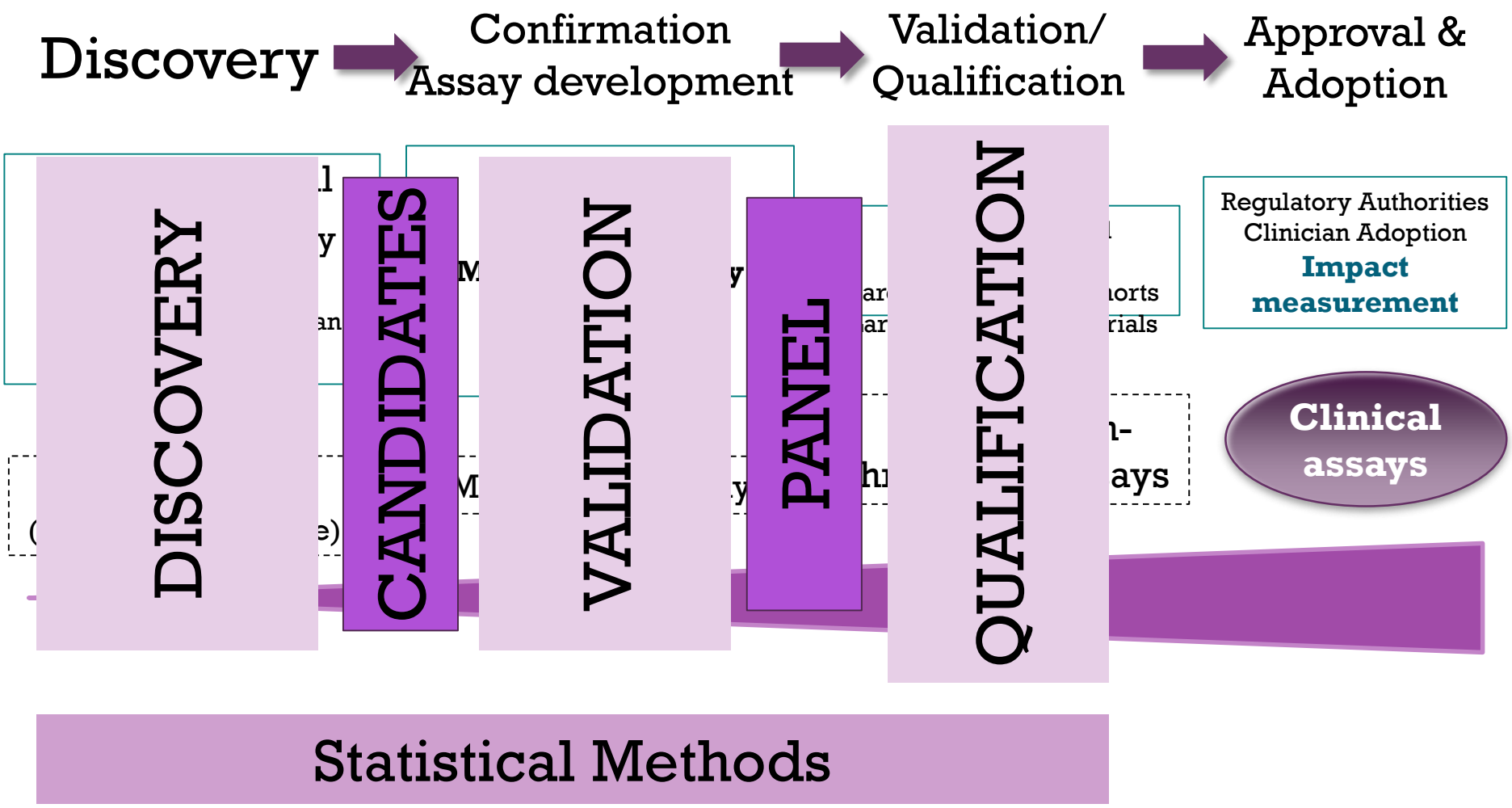
Steve Pennington

UCD Conway Institute, UCD, Dublin

Protein Biomarker Discovery and Development



Protein Biomarker Discovery and Development



Biomarker Futility

COMMENT

WELLCOME IMAGES

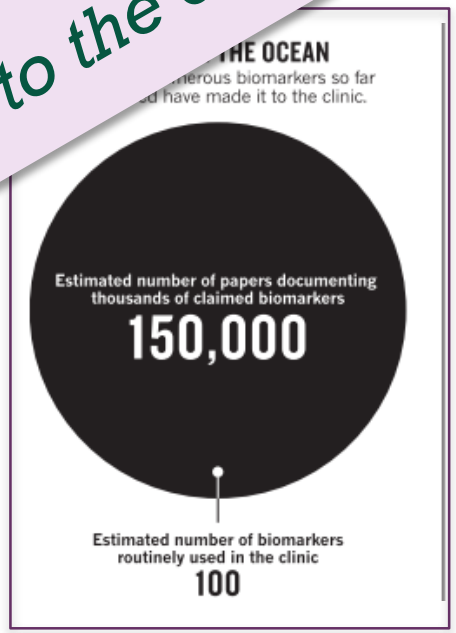
Too many resea… specimens the… from local insti… be fixed tis-sues … tories, the useful-… limited by inadequate … or a lack of donor consent.

The lack of standardization in the coll… …tured) can hinder subsequent research.

Br… the biomarkers

… of fragmented research on disease-associated biomarkers … by a coordinated 'big science' approach, argues **George Poste**.

…ATURE | VOL 469 | 13 JANUARY 2011

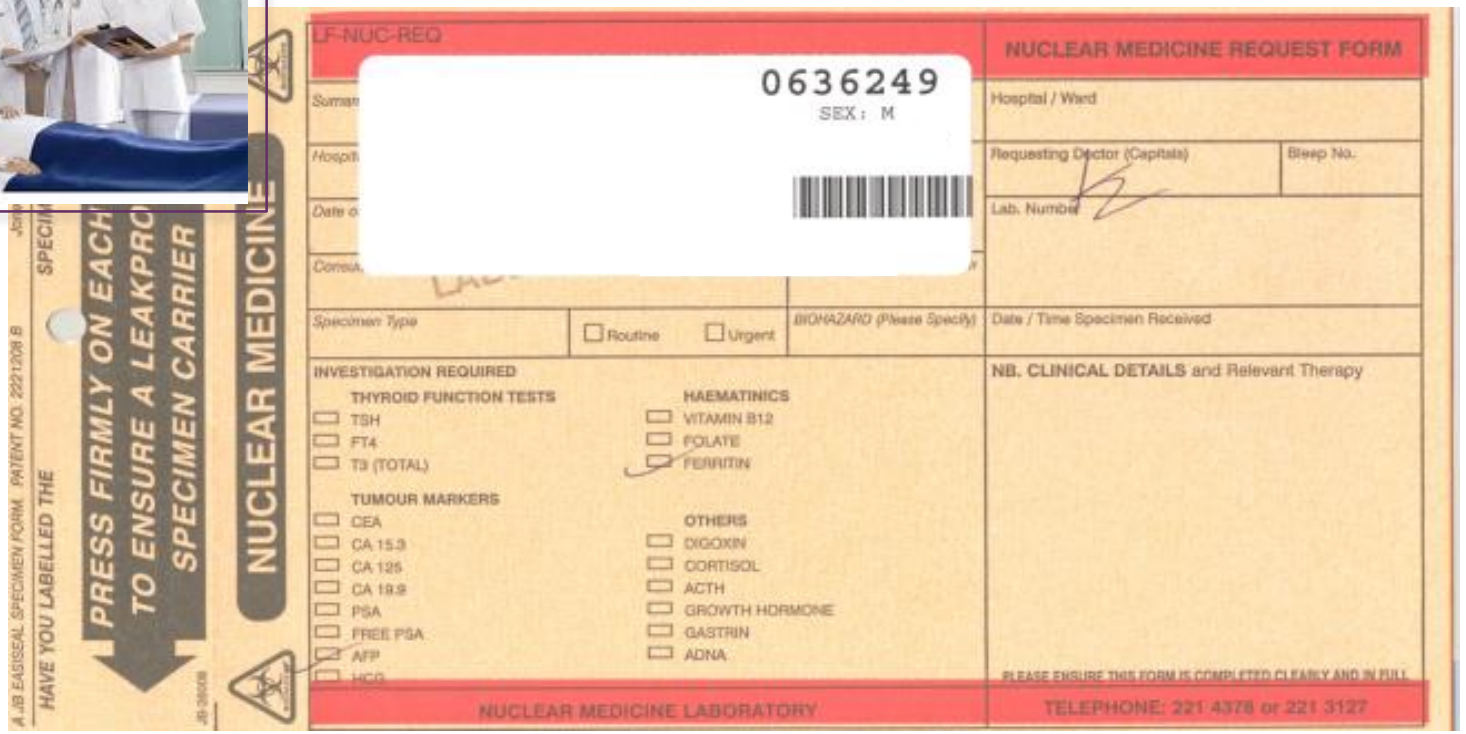


'Omics have delivered no new biomarkers to the clinic

Specimens
Fragmented approach

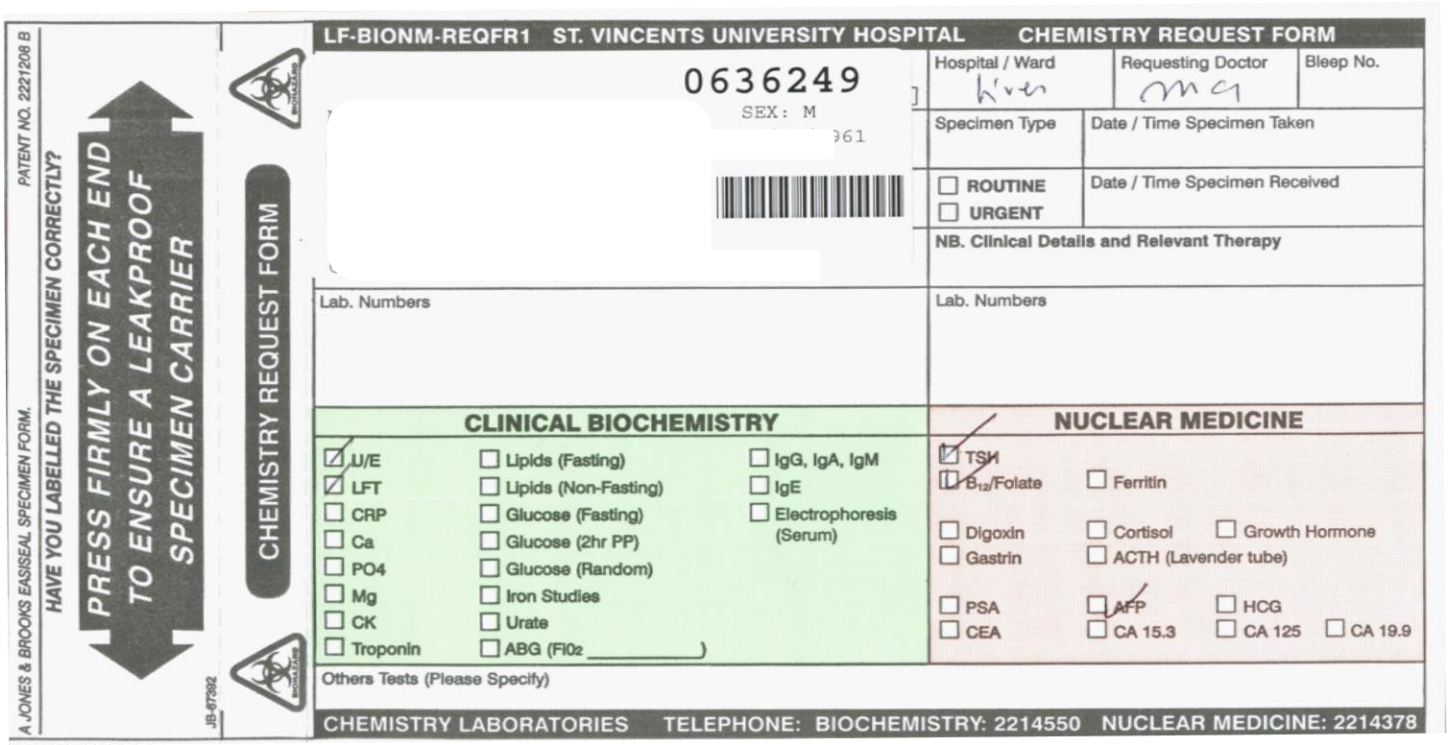
Clinical Utility

2006



Clinical Utility: 8 years on

2014



Will the **protein** biomarkers we discover be useful?

How will we proceed to them gaining utility?

From Biomarkers to Diagnostics

COMMENTARY

www.ScienceTranslationalMedicine.org 31 July 2013 Vol 5 Issue 196 196cm6 1

TUMOR-BIOMARKER DIAGNOSTICS

Breaking a Vicious Cycle

Daniel F. Hayes,[1*] Jeff Allen,[2] Carolyn Compton,[3] Gary Gustavsen,[4] Debra G. B. Leonard,[5] Robert McCormack,[6] Lee Newcomer,[7] Kristin Pothier,[4] David Ransohoff,[8] Richard L. Schilsky,[9] Ellen Sigal,[2] Sheila E. Taube,[10] Sean R. Tunis[11]

Despite prodigious advances in tumor biology research, few tumor-biomarker tests have been adopted as standard clinical practice. This lack of reliable tests stems from a vicious cycle of undervaluation, resulting from inconsistent regulatory standards and reimbursement, as well as insufficient investment in research and development, scrutiny of biomarker publications by journals, and evidence of analytical validity and clinical utility.

Tests must have analytical validity, clinical value and financial value.

From Biomarkers to Diagnostics

Biomarkers should be fit for purpose and their purpose known

1. Reform regulatory review
2. Increase re-imbursement of tumour tests with clinical utility
3. Increase investment in research (cf. therapeutics)
4. Increase rigour for assessment - publication
5. Adhere to high-level evidence based recommendations for use

Tests must have analytical validity
as well as clinical and financial value.

Can we identify and develop protein biomarkers of **clinical value** in prostate cancer ?

Tests to guide treatment decisions

Imagine this scene …..

Imagine the screen

Health Screening for Men

Comprehensive health screening for men. It takes about three hours to complete and incorporates an exhaustive list of health screening features with an emphasis on modern men's health issues and lifestyle.

Physiological Assessment

- Blood pressure, heart rate, weight, height, body mass index measurement
- Urinalysis to check liver and kidney function and for infection
- FOB test for those over the age of 50
- Heart Assessment (Resting ECG)
- Lung Function tests (Spirometry)
- Hearing test (Audiometry)
- Eye assessment to check visual acuity, near and far vision, macular and retinal problems and other potential problems regarding the retina and fundus

Laboratory tests

- An extensive blood screen to include an assessment of cholesterol and glucose levels, liver and kidney function, measurement of haemoglobin and iron levels, full blood count, thyroid function test (if clinically indicated) and screen for gout and haemochromatosis
- PSA (Prostate Specific Antigen) recommended for those over the age of 40
  **(Laboratory testing at The Well is carried out by Medlab)*

Lifestyle Analysis

- Stress questionnaire and analysis
- Lifestyle questionnaire, body composition analysis
- Review of current diet and exercise regime and development of a personal lifestyle plan

Doctor consultation

- Full physical examination and assessment of the body systems
- Awareness regarding testicular cancer and colorectal examination
- Results of all tests (including the blood results) are explained and any health issues that may have been identified as part of the medical will be discussed
- Advice around stress management and lifestyle modification
- Digital Prostate Exam for over those over the age of 40
- An open opportunity for the visitor to discuss any underlying concerns they may have

Reporting

All results are explained on the day of the medical. A written report and full interpretation of results is sent out to your designated address within 7 working days of completion of the mens health screening including a personalised lifestyle plan to maintain motivation to enhance a healthy lifestyle.

Blood – FBC, Hb & Fe, cholesterol, glucose, liver & kidney function
Urine
Heart
Hearing
Vision

http://www.thewell.ie/executive_medicals_men.asp

Imagine the screen

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  (Laboratory testing at The W[illegible] by [illegible])

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Doc[illegible]sultatio[illegible]

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Cost €510.00

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**(Laboratory testing at The W[illegible] carried out by Mediab[illegible]*

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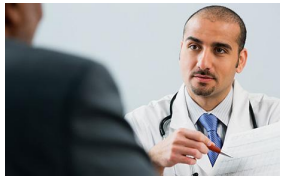
Cost $720.00

http://www.thewell.ie/executive_medicals_men.asp

All clear doc?



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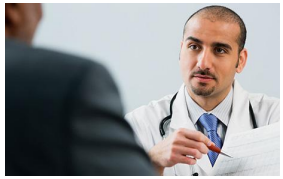
✓✓✓✓✓✓✓✓

PSA

All clear doc?



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√√√√√√√√

PSA 14.2ng/ml

DRE

Digital rectal examination (DRE)

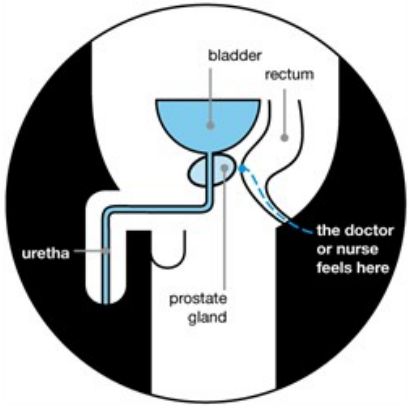
The DRE is a common way of helping to diagnose a prostate problem. Your doctor or nurse feels the prostate gland through the wall of the back passage (rectum).

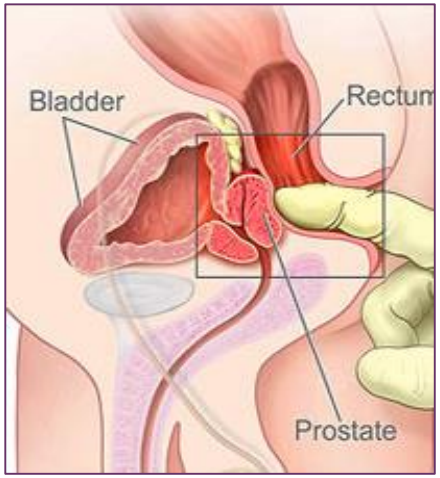
The DRE may be carried out by your GP and will be repeated by the hospital specialist if your GP thinks you should see one. If you are having a PSA test as well, the DRE should be done after the PSA test if possible. This is because having a DRE straight before a PSA test might raise your PSA level.

You will lie on your side, on an examination table, with your knees brought up towards your chest. If you find it easier, you can stand and lean over the back of a chair or across the examination table instead.

The doctor or nurse will slide their finger gently into your back passage. They will wear gloves and put some gel onto their finger to make it more comfortable. Some men understandably find it embarrassing but it is over quickly and shouldn't be painful.

They will feel the back surface of the prostate gland for any hard or irregular areas and to estimate its size.





If your prostate gland is larger than expected, this could be a sign of an enlarged prostate. A prostate gland with hard bumpy areas may suggest prostate cancer.

If your DRE result shows anything unusual, you will be referred to a hospital specialist. The DRE is not a completely accurate test. A man with prostate cancer may have a DRE that feels normal.

DRE

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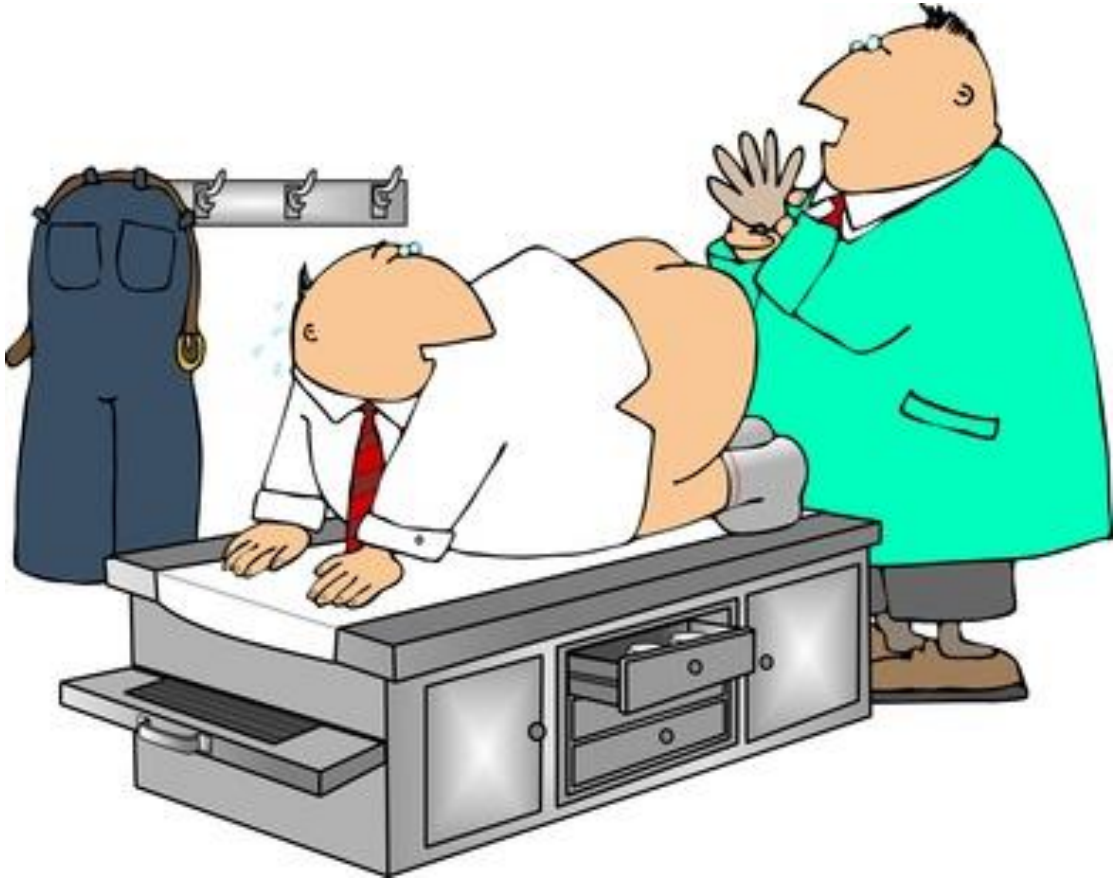
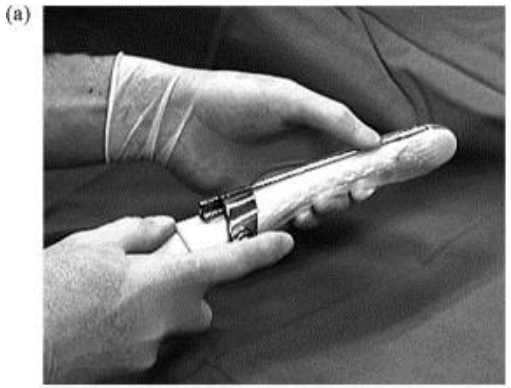
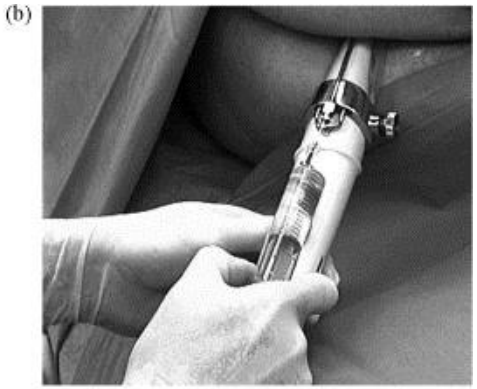
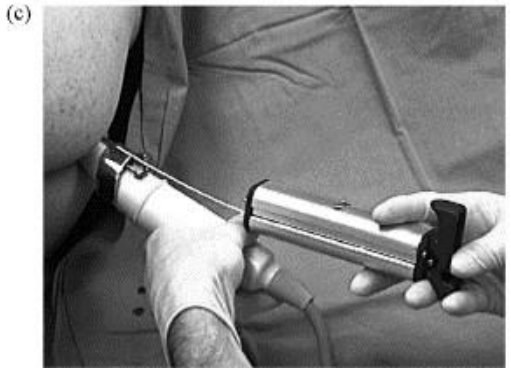
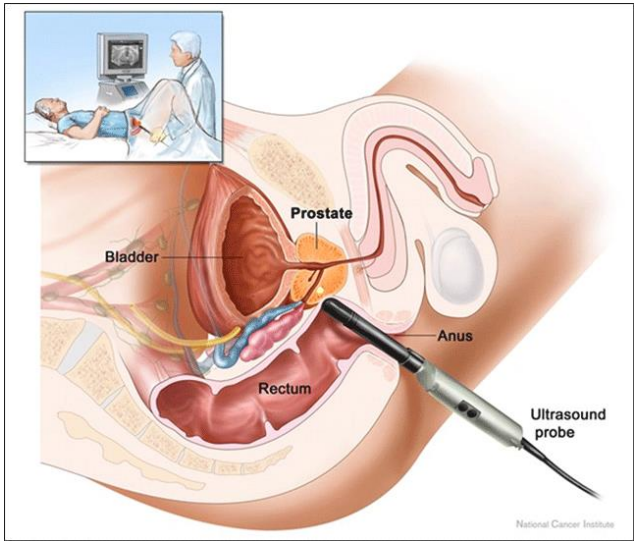


TRUS Biopsy

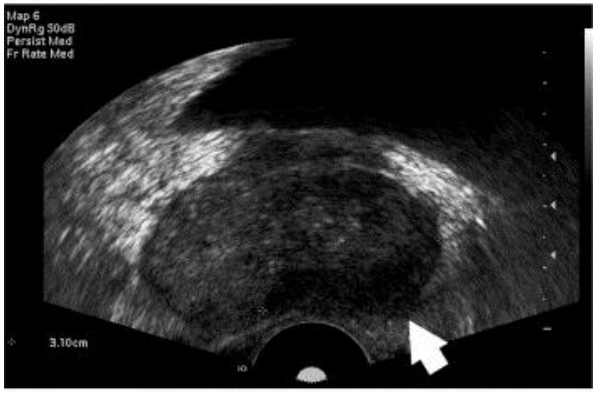








(a)



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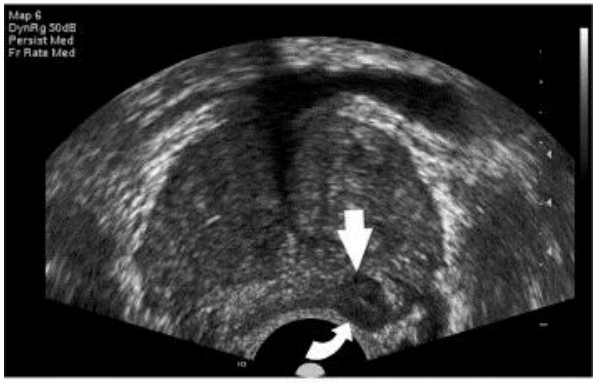
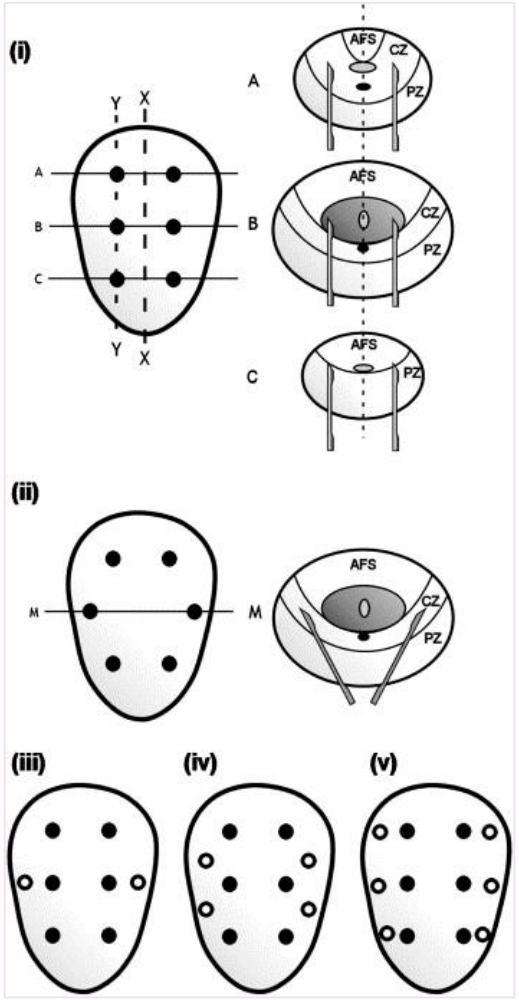
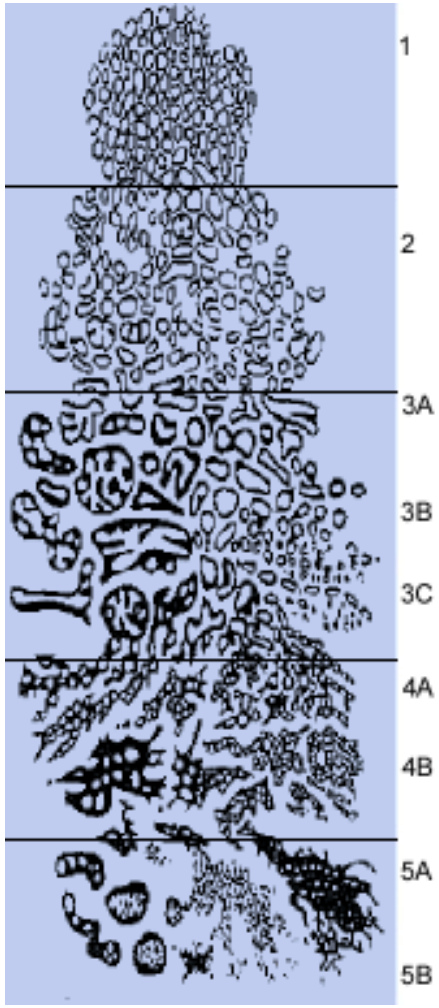
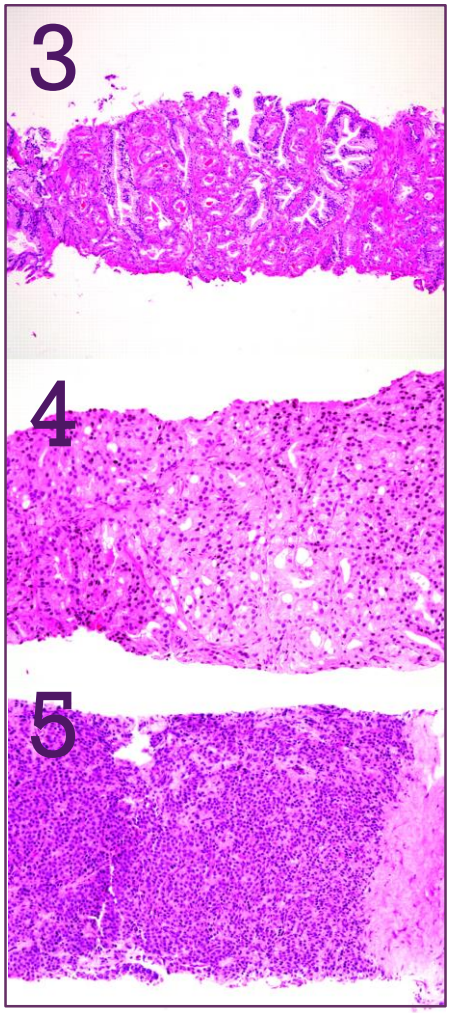


(c)

Gleason Scoring of Biopsy







So, the result…..

Gleason 3 + 4

DRE – abnormal

PSA 14.2ng/ml

"What now?"

Decisions….

- The patient's treatment decision is a **momentous** one.
- He must gather all the reliable information he can so he can participate in the diagnostic process, then ultimately select the therapy most reasonable under the circumstances.
- As the patient confronts his condition - and he must do so - he should take into account his personal goals regarding the **available therapies** and their peculiar morbidities.
- In his **decision** process he may get differing medical opinions

Prostate Cancer Coalition

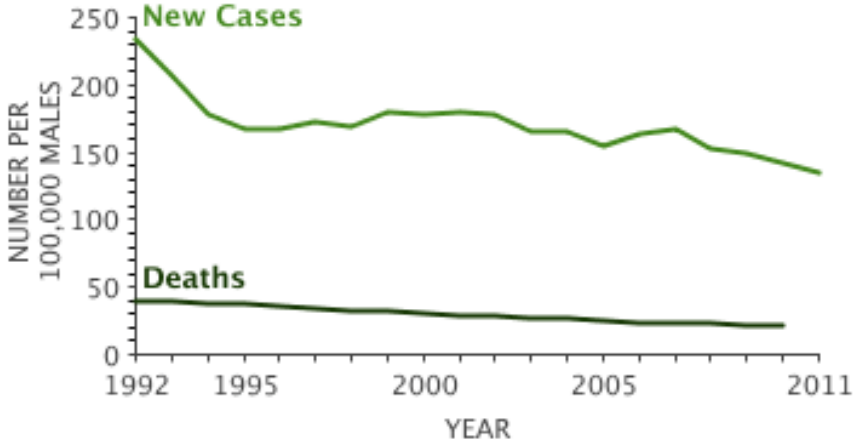
http://www.pccnc.org/patient_resources/understanding_diagnosis/

NCI Statistics

Prostate Cancer: GET THE FACTS

Other than skin cancer, prostate cancer is the most common cancer in American men.

Estimated New Cases in 2014	233,000
% of All New Cancer Cases	14.0%
Estimated Deaths in 2014	**29,480**
% of All Cancer Deaths	**5.0%**



Percent Surviving 5 Years

98.9%

2004–2010

Number of New Cases and Deaths per 100,000: The number of new cases of prostate cancer was 147.8 per 100,000 men per year. The number of deaths was 23.0 per 100,000 men per year. These rates are age-adjusted and based on 2007–2011 cases and 2006–2010 deaths.

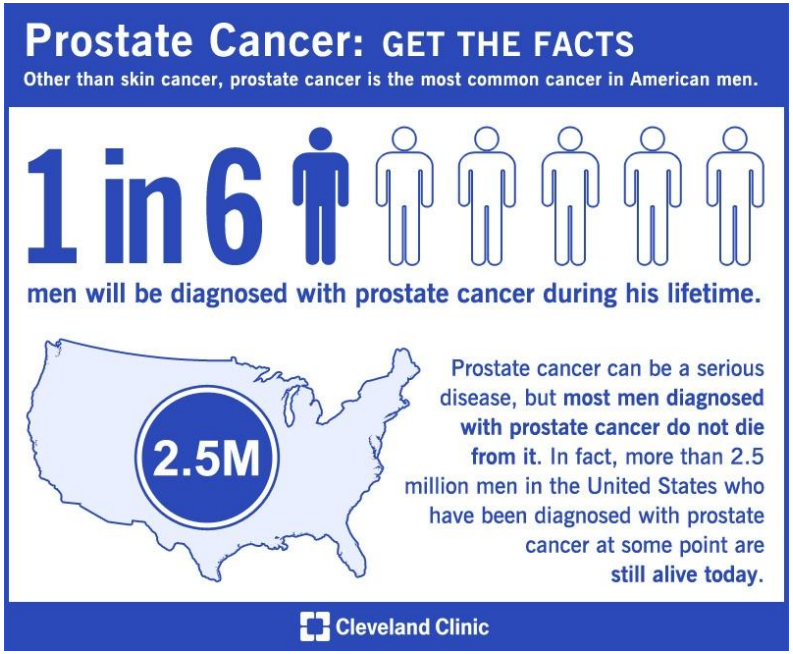
Lifetime Risk of Developing Cancer: Approximately 15.3 percent of men will be diagnosed with prostate cancer at some point during their lifetime, based on 2008–2010 data.

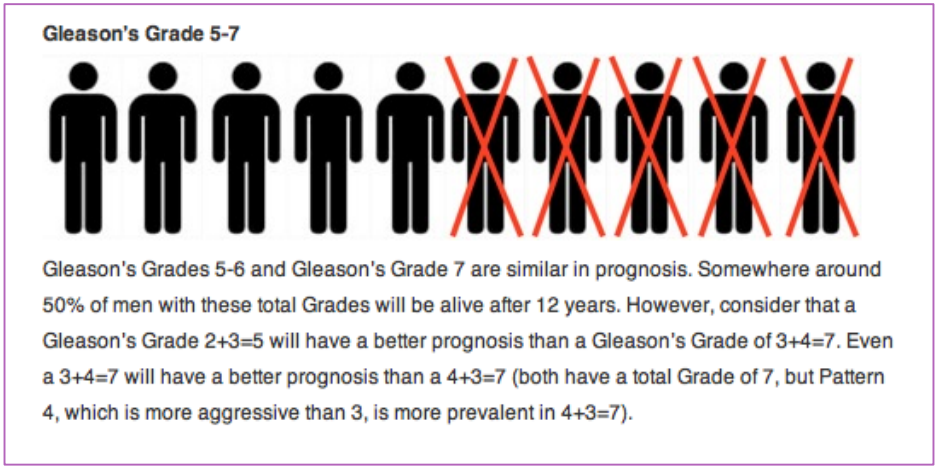
Prevalence of this cancer: In 2011, there were an estimated 2,707,821 men living with prostate cancer in the United States.

4, which is more aggressive than 3, is more prevalent

http://seer.cancer.gov/statfacts/html/prost.html

Personalised - Population





All 7's aren't equal

$3+4 \neq 4+3$

NCI Statistics

Over-diagnosis and over-treatment is a major problem

Most men die **with** rather than **of** prostate cancer

But, there is currently no effective treatment for metastatic prostate cancer

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Decisions, Decisions, Decisions

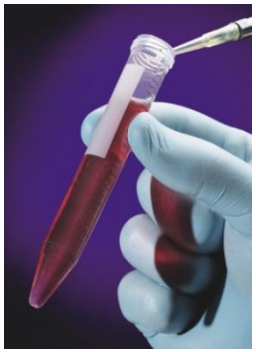
Radical Prostatectomy (RP)

Radiation (with hormones)

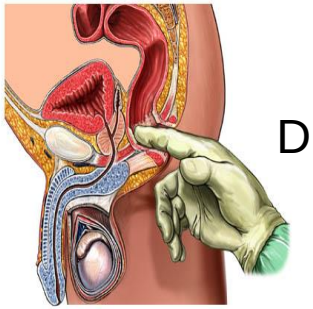
No treatment (Active Surveillance)

Diagnosis and Treatment

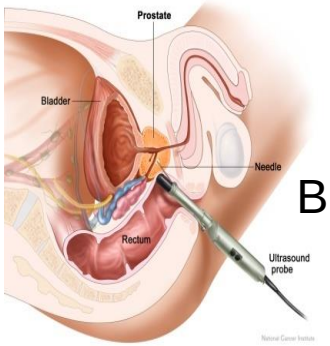
Diagnostic Test	Diagnosis	Treatment



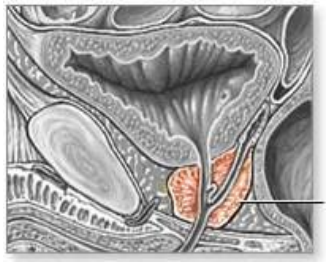
PSA



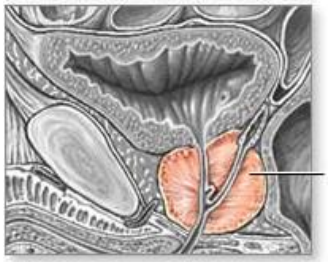
DRE



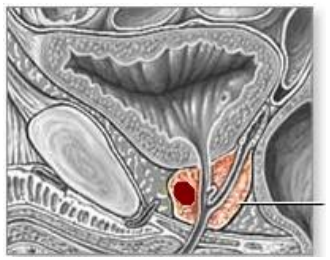
Biopsy



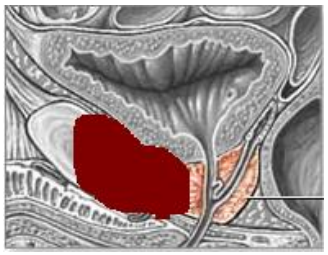
Normal



BPH



Confined Prostate cancer



Non Confined Prostate cancer

Active Surveillance

Surgery

Radiation

RP

no RP

Can we identify and develop protein biomarkers of **clinical value** in prostate cancer ?

To guide treatment decisions

Accessible, Repeatable, Reliable

PCa Multidisciplinary Teams

UCD Conway Teams

Prostate Cancer Research Consortium

National Prostate Cancer Research Group

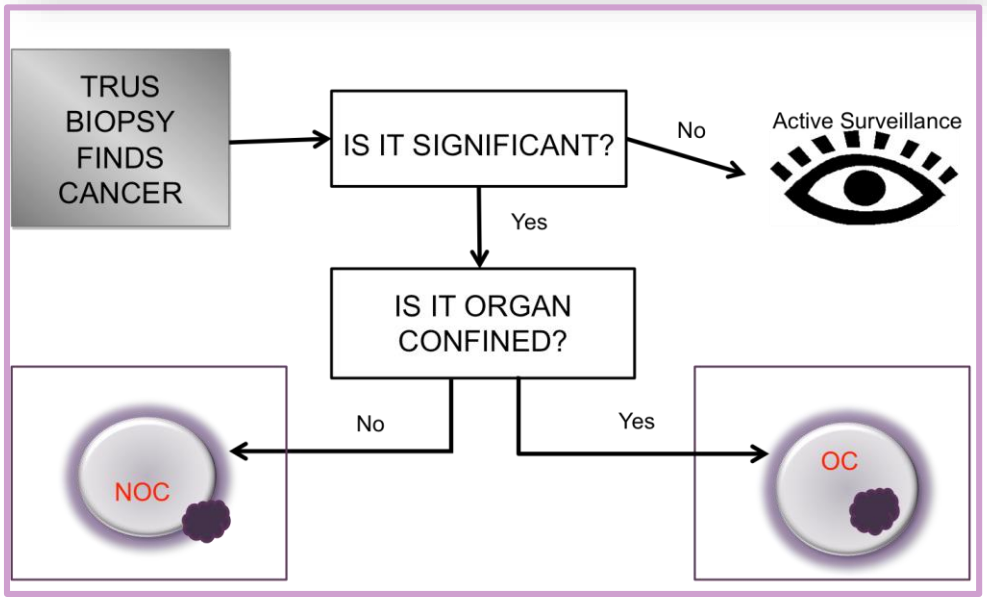
Define the Clinical Question First

nature REVIEWS UROLOGY

Biomarker research in prostate cancer —towards utility, not futility

Nature Reviews Urology **8**, 131-138 (March 2011) | doi:10.1038/nrurol.2011.11

Sheng Fei Oon, Stephen R. Pennington, John M. Fitzpatrick & R. William G. Watson



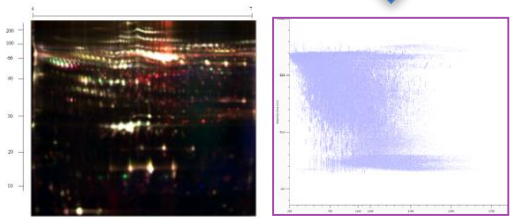
No RP

RP

Biomarker Panel Development

PCRC Serum Sample Bioresource

Biomarker discovery



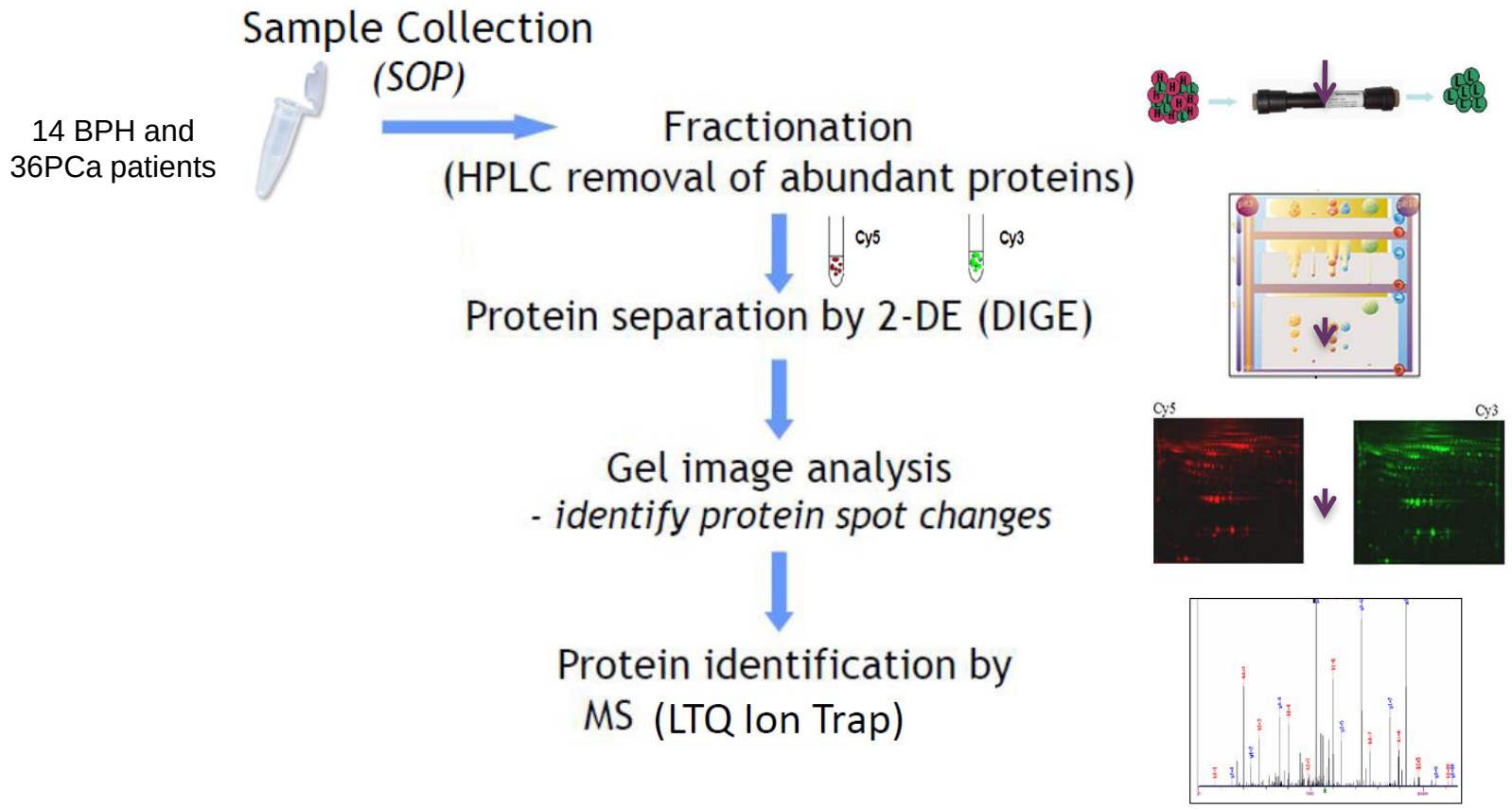
2D-DIGE

Label-free LC-MS/MS

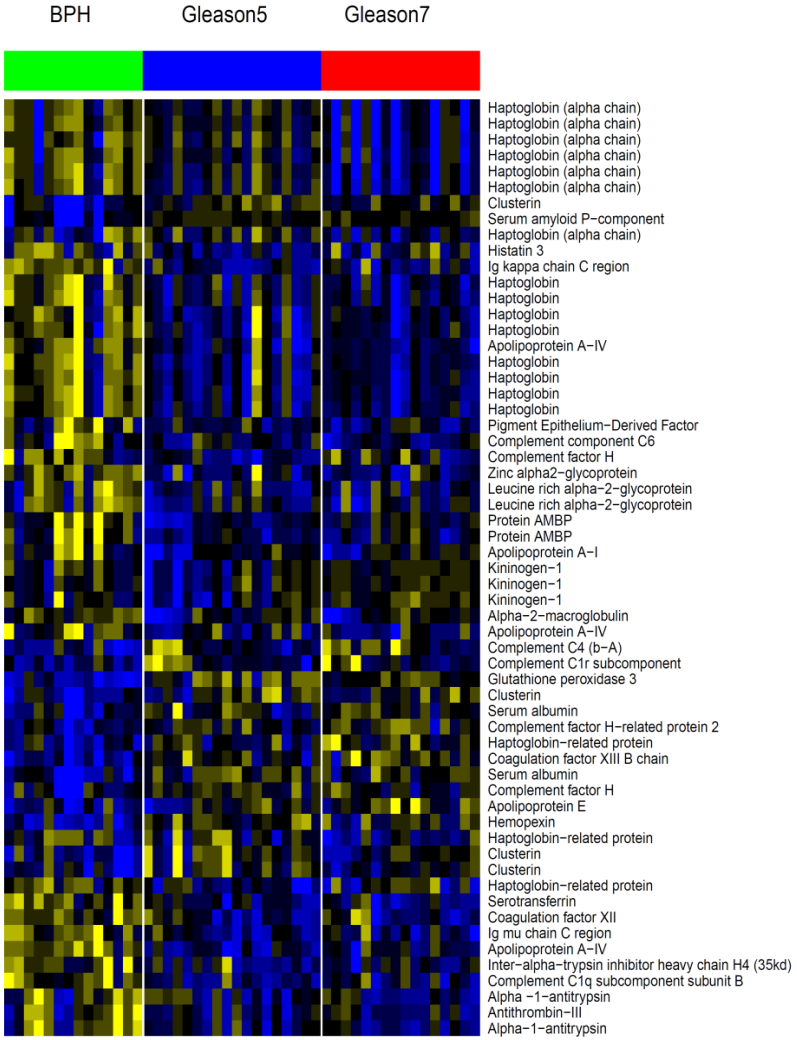
Biomarker Candidate list

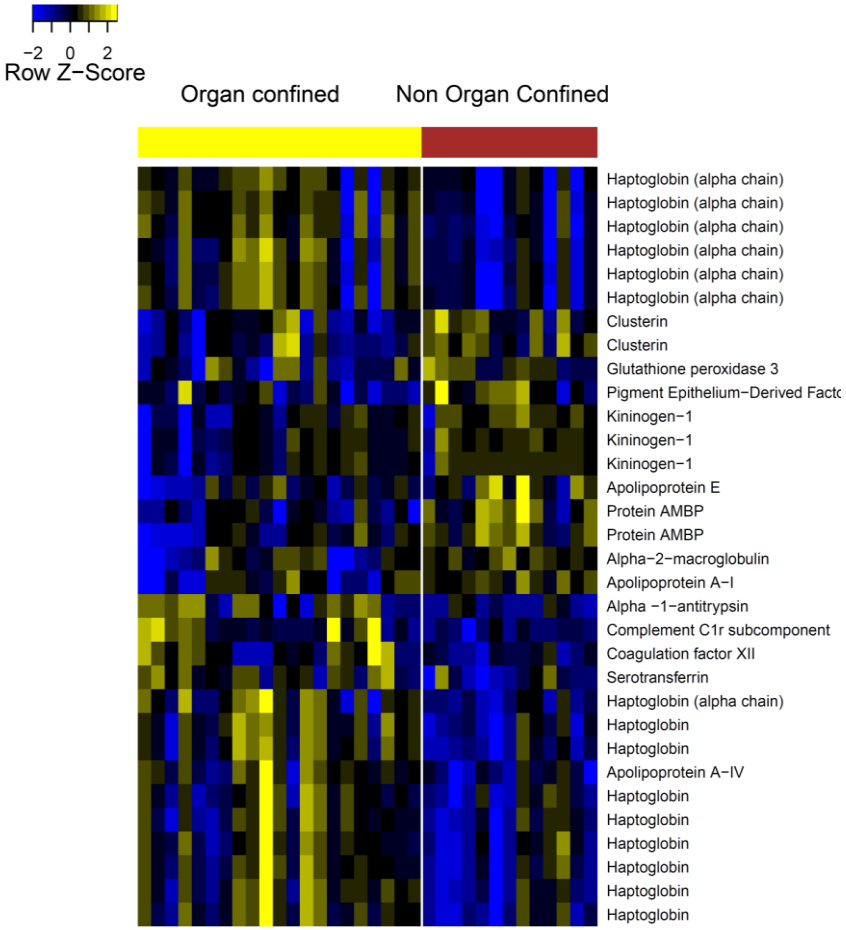
Discovery: 2D-DIGE

- 50 age matched serum samples from PCRC
  - 14 BPH, 36 PCa patients (Organ Confined and Non Organ Confined)

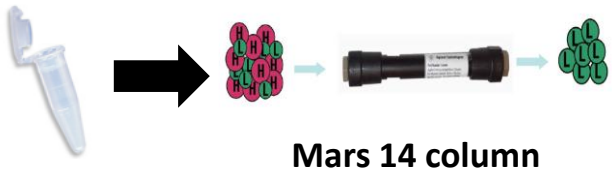


2D-DIGE candidates



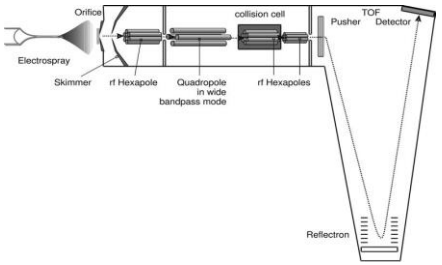


Discovery: Label free LC-MS/MS

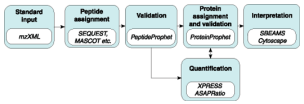


Mars 14 column

Trypsin digestion



Progenesis LC-MS



Trans-Proteomic Pipeline

GS5 (n = 10)
GS7 OC (n = 10)
GS7 NOC (n = 10)

Serum samples

Affinity Depletion using MARS 14 column

Depleted serum samples

Create reference pool sample from each pool depleted sample

In-solution digestion

Label-free LC-MS/MS on Q-TOF

Progenesis, database search and result filtering

Peptide/protein expression profile

Protein assay and 1D gel

Protein concentration normalization

TPP and Skyline

Public MS/MS spectral library

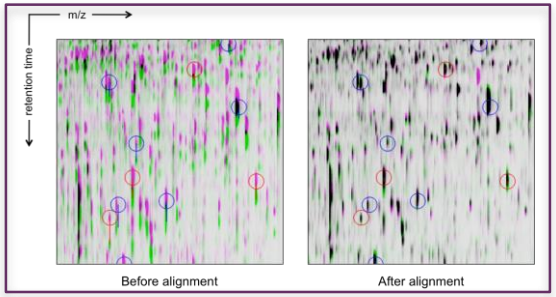
+

In-house MS/MS spectral library

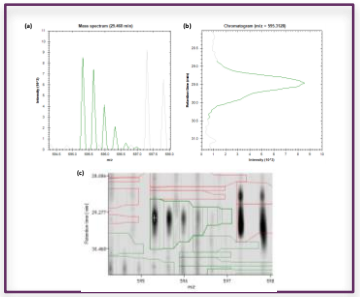
Label free LC-MS/MS data

- >90,000 features
- Ion counting for quantification
  - Alignment using Progenesis
- Mascot search for protein id
  - Mascot Score > 34 (FDR = 3.08%)
  - Remove non-unique mapping peptides
- MS/MS library construction
  - Trans-Proteomic Pipeline (TPP)
- Peptide to protein roll up
- Analysis of differential protein expression
- 59 Proteins differentially expressed (p-value<0.05)

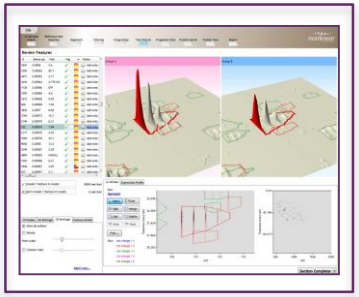
Feature Alignment



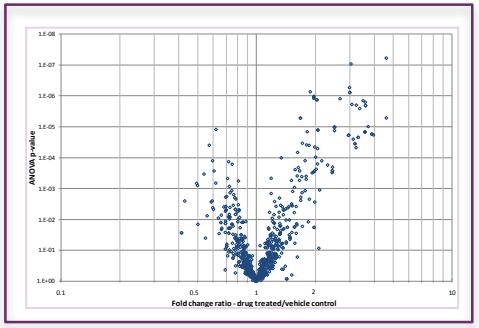
Feature Selection



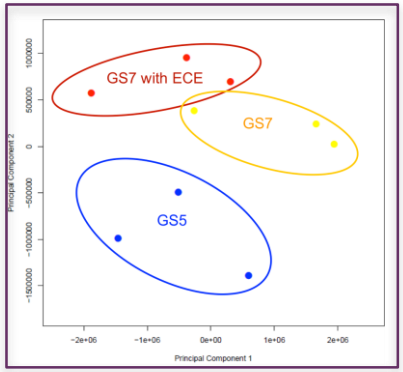
Feature Quantification



Protein Expression Changes



Principle Component Analysis

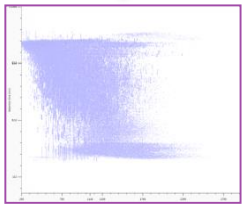


PCRC OC Biomarker Candidates

PCRC Serum Sample Bioresource

Biomarker discovery

2D-DIGE



Label-free LC-MS/MS

Literature review

64 Candidate Proteins

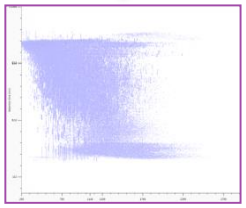
Biomarker Candidate list

PCRC OC Biomarker Candidates

PCRC Serum Sample Bioresource

Biomarker discovery

2D-DIGE



Label-free LC-MS/MS

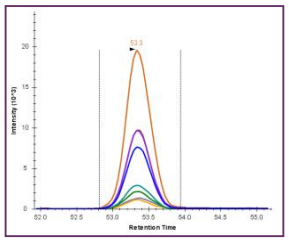
PubMed

Literature review

64 Candidate Proteins

Biomarker Candidate list

MRM

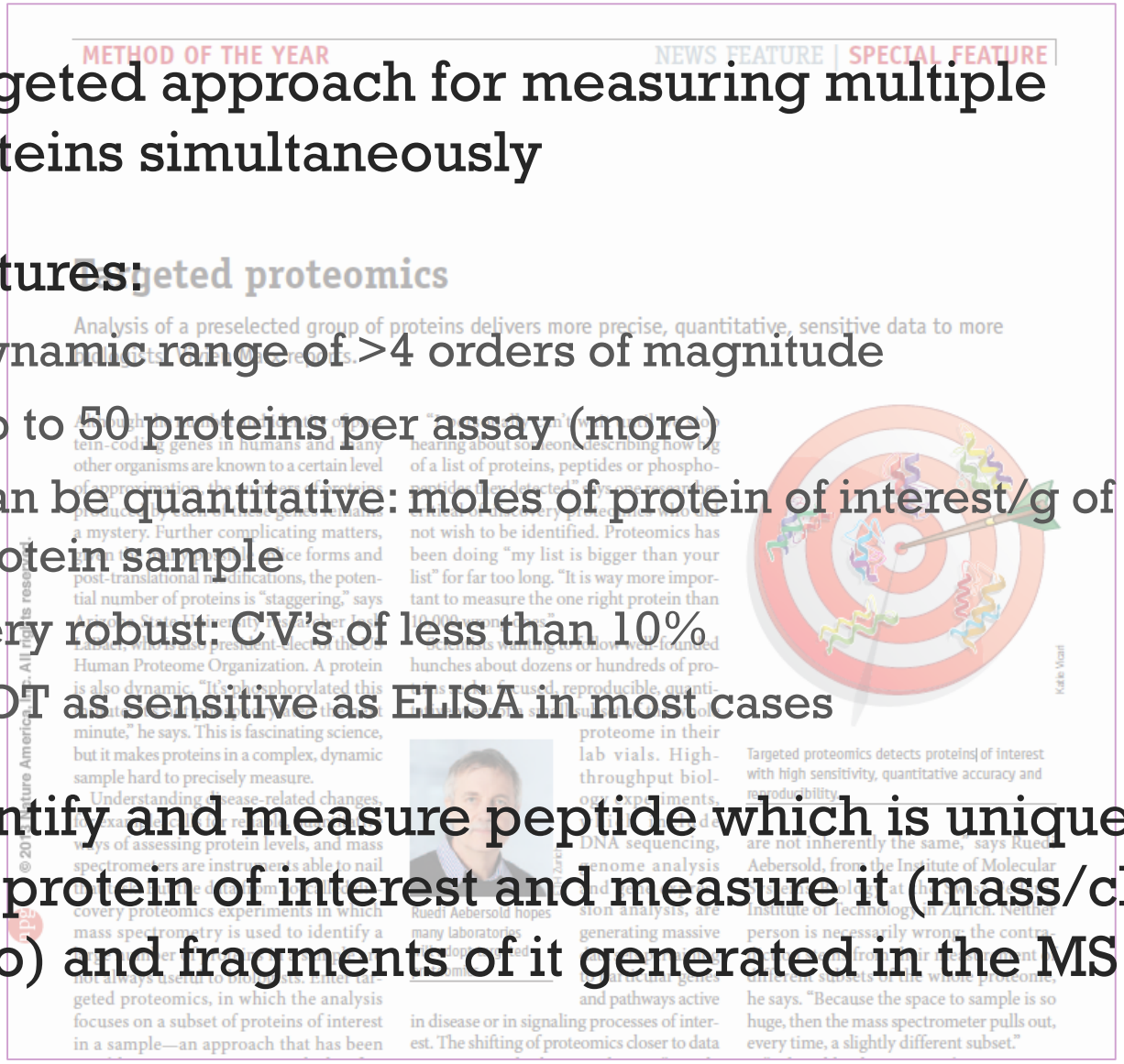


Biomarker Validation

UCD DUBLIN

MRM

- Targeted approach for measuring multiple proteins simultaneously
- Features:
  - Dynamic range of >4 orders of magnitude
  - Up to 50 proteins per assay (more)
  - Can be quantitative: moles of protein of interest/g of protein sample
  - Very robust: CV's of less than 10%
  - NOT as sensitive as ELISA in most cases
- Identify and measure peptide which is unique to the protein of interest and measure it (mass/charge ratio) and fragments of it generated in the MS


METHOD OF THE YEAR

NEWS FEATURE | SPECIAL FEATURE

geted proteomics

Analysis of a preselected group of proteins delivers more precise, quantitative, sensitive data to more

Targeted proteomics detects proteins of interest with high sensitivity, quantitative accuracy and

Multiplexed quantification

1934 DOI 10.1002/pmic.200500432 Proteomics 2006, 6, 1934–1947

RESEARCH ARTICLE

Relative and absolute quantitative expression profiling of cytochromes P450 using isotope-coded affinity tags

Rosalind E. Jenkins[1], Neil R. Kitteringham[1], Christie L. Hunter[2], Sally Webb[2], Tony J. Hunt[2], Robert Elsby[3], Rod B. Watson[4], Dominic Williams[1], Stephen R. Pennington[5] and B. Kevin Park[1]

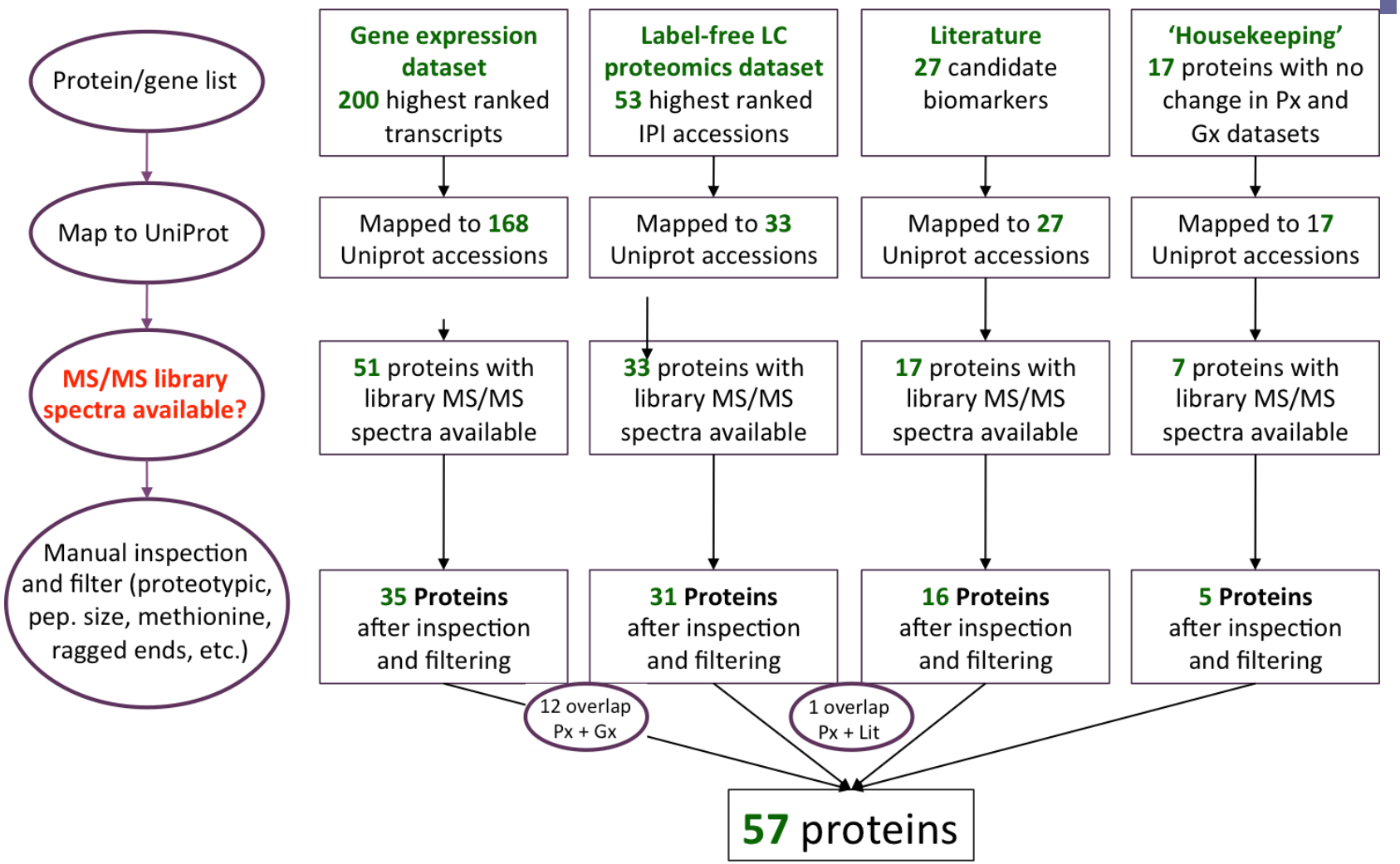
[1] Department of Pharmacology, University of Liverpool, Liverpool, UK
[2] Applied Biosystems, Framingham, MA, USA
[3] AstraZeneca, Loughborough, UK
[4] Applied Biosystems, Warrington, UK
[5] Conway Institute, University College Dublin, Dublin, Ireland

Swiss-Prot acc. no.	Cytochrome P450	Peptide	Level in control (fmol/μg total protein)	95% uncertainty	Level in PB induced (fmol/μg total protein)	95% uncertainty	Average ratio PB:control	95% CIs Upper	95% CIs Lower	p value (t-test)
P00184	1a1	CIGETIGR	5.38	0.35	5.21	1.14	0.96	1.18	0.79	0.670
P00186	1a2	CIGEIPAK	1.38	0.24	1.25	0.18	0.91	0.95	0.87	0.013
Q64429	1b1	CIGEELSK	14.11	1.01	14.99	0.51	1.06	1.13	1.00	0.031
P15392	2a4	YCFGEGLAR	11.53	1.22	13.02	0.26	1.13	1.28	1.00	0.043
P56593	2a12	FCLGDSLAK	15.07	1.62	14.99	1.32	1.00	1.07	0.93	0.905
P12790	2b9/10/13/20	ICLGESIAR	11.41	1.95	68.97	5.24	6.07	7.24	5.09	<0.0001
Q64458	2c29/37/50	ICAGEGLAR	55.84	4.03	171.18	23.03	3.06	3.53	2.66	0.001
P56655	2c38/39	VCAGEGLAR	7.58	1.09	7.49	0.96	0.99	1.06	0.92	0.844
P56657	2c40	ICVGESLAR	16.15	1.93	15.65	1.97	0.98	1.03	0.93	0.625
P11714	2d9/11	SCLGEALAR	12.42	0.70	7.56	1.39	0.61	0.70	0.52	0.002
P24456	2d10/22/26	SCLGEPLAR	21.68	1.05	19.61	0.42	0.90	0.96	0.86	0.007
Q05421	2e1	VCVGEGLAR	35.13	1.58	30.38	2.78	0.86	0.91	0.82	0.006
P33267	2f2	LCLGEPLAR	21.74	3.34	16.27	1.96	0.75	0.78	0.72	0.0003
O54749	2j5	ACLGEQLAK	9.05	0.94	8.82	0.62	0.99	1.02	0.93	0.009
Q64459	3a11/13/16	NCLGMR	5.48	0.63	19.56	1.16	3.58	3.96	3.23	<0.0001
O88833	4a10/11/12/14	NCIGK	2.71	0.96	4.32	0.93	1.61	1.98	1.31	0.003

a) Levels listed are in fmol/μg total protein. Ratios in bold were subjected to the first tier statistical analysis (comparisons with the mean ratios of all peptides), whereas the rest were subjected to the second tier analysis (comparisons with the mean ratios of all peptides minus the three induced). This is described in greater detail in Section 2.

16 Cytochrome P450's

Another protein panel assembly



MRM development pipeline

Initial SRM method

Proteins - **57**
Peptides - **174**
Transitions - **1681**
- 8-10 transitions per peptide
- 1-5 peptides per protein

Survey run – determine detectability of peptides

15 injections of pooled sample
(~ 13 hours instrument time)

Refined method

Proteins - **52**
Peptides - **119**
Transitions - **609**
- 5 transitions per peptide
- 1-5 peptides per protein

Collision energy optimisation
16 injections of pooled sample
(~14 hours of instrument time)

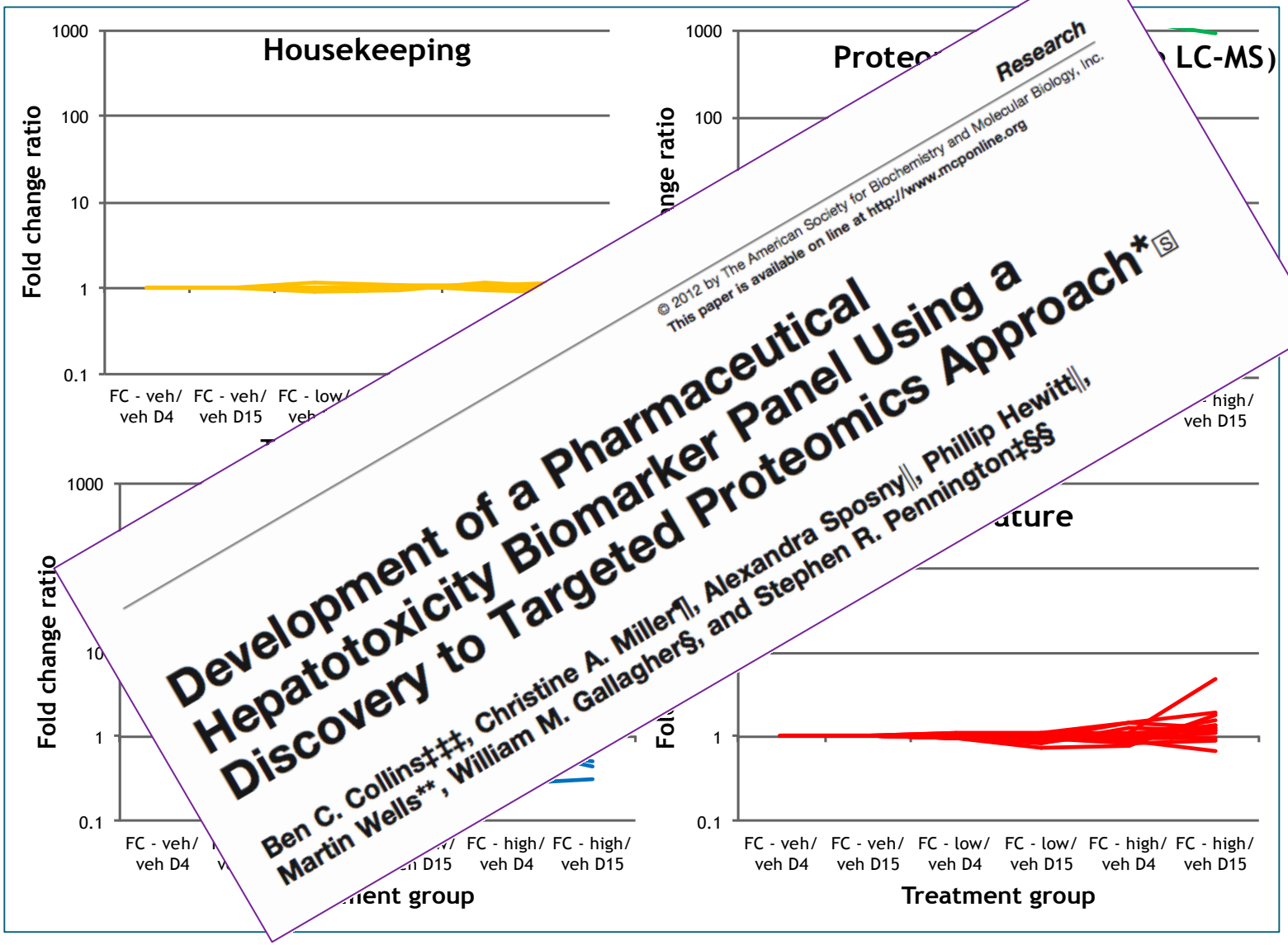
Final SRM method

Proteins - **48**
Peptides - **109**
Transitions - **545**
- 5 transitions per peptide
- 1-5 peptides per protein

Technical variance measurement
10 injections pooled sample
(~17 hours instrument time)
→**Mean CV = 5.7 %**

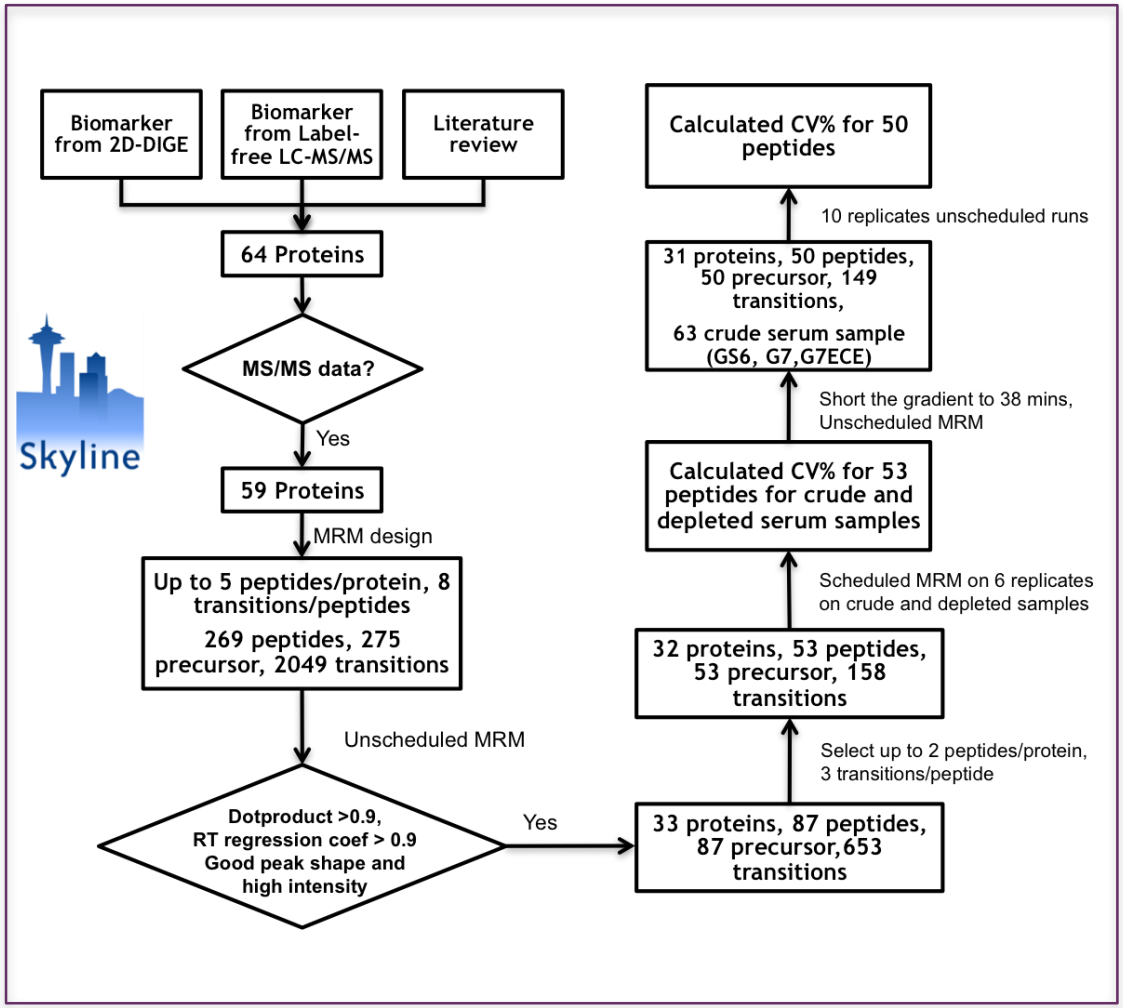
Measurement in 30 individual samples
(~51 hours instrument time)
- drug treated or vehicle control)

MRM measurement:48 proteins



PCa OC Candidate Biomarkers

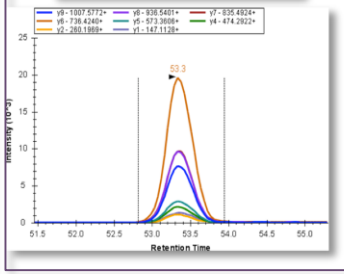
Workflow Map



31 Candidates

- 31 candidate biomarkers
  - 1-5 peptides/protein
  - 8 transitions/peptide

Example: APOA1_DLATVYVDVLK

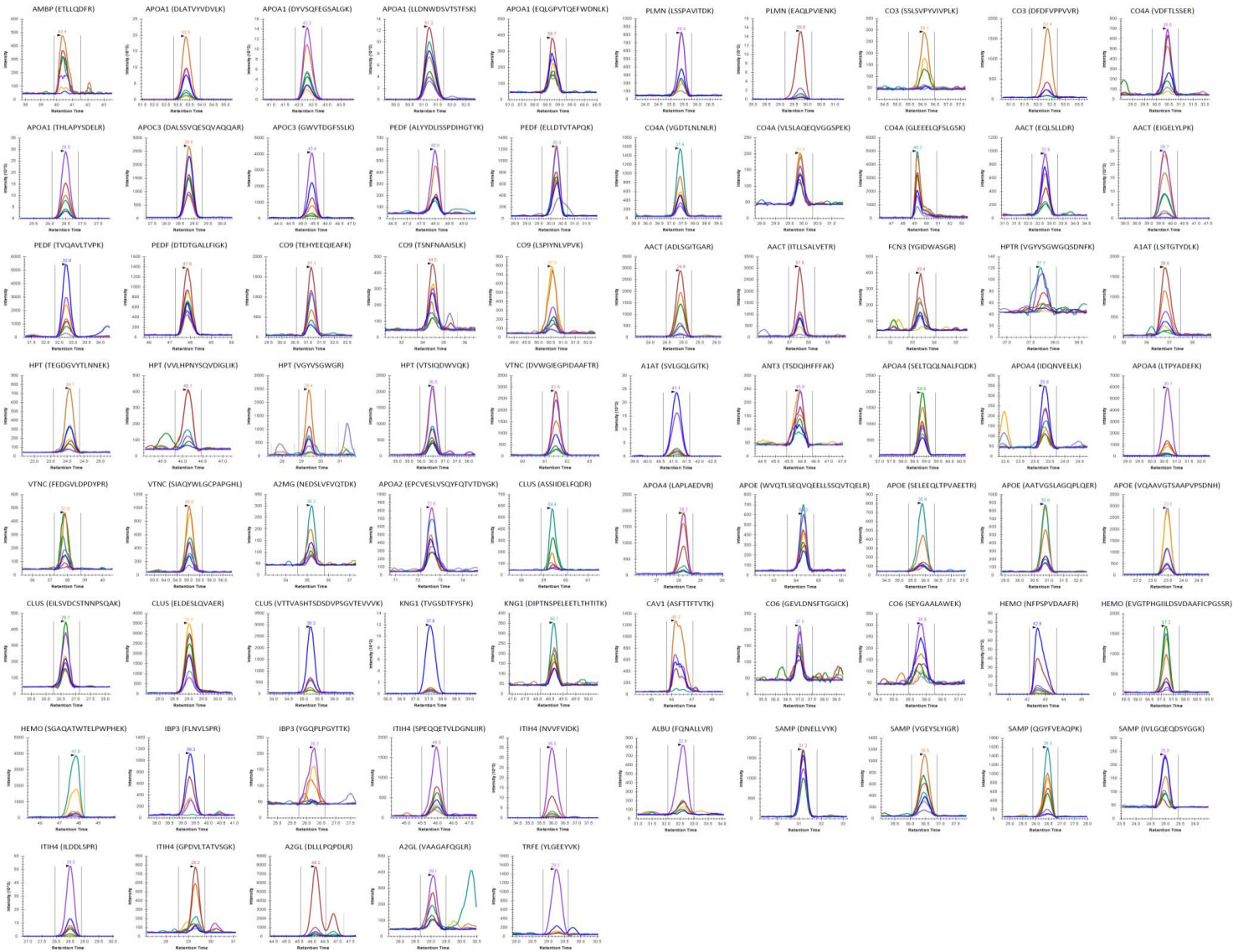


Swissprot accession	Protein Name	2D-DIGE	Q-TOF	Literature	Reference Concentration (umol/L)
O75636	Ficolin-3			Yes	0.2–0.7
P00738	Haptoglobin	Yes	Yes	Yes	6–40
P00739	Haptoglobin-related protein	Yes			0.6-1.2
P00747	Plasminogen		Yes		2–4
P01008	Antithrombin-III	Yes	Yes		3–5
P01009	Alpha -1-antitrypsin	Yes	Yes		18–40
P01011	Alpha-1-antichymotrypsin		Yes		4–9
P01023	Alpha-2-macroglobulin	Yes	Yes		7–17
P01024	Complement C3		Yes		5–10
P01042	Kininogen-1	Yes	Yes		3
P02647	Apolipoprotein A-I	Yes	Yes		30–70
P02649	Apolipoprotein E	Yes	Yes		0.6–2
P02652	Apolipoprotein A-II		Yes	Yes	30–60
P02656	Apolipoprotein C-III		Yes		6–20
P02743	Serum amyloid P-component	Yes	Yes		1–2
P02748	Complement component C9		Yes		0.4–1
P02750	Leucine rich α-2-glycoprotein	Yes	Yes		0.4
P02760	Protein AMBP	Yes	Yes		NA
P02768	Serum albumin	Yes	Yes		500–800
P02774	Vitamin D-binding protein		Yes	Yes	NA
P02787	Serotransferrin	Yes	Yes		25–45
P02790	Hemopexin	Yes	Yes		9–20
P04004	Vitronectin		Yes		1–3
P06727	Apolipoprotein A-IV	Yes	Yes		3–6
P0C0L4	Complement C4-A/B	Yes	Yes		0.5–2
P10909	Clusterin	Yes	Yes		1–2
P13671	Complement component C6	Yes	Yes		0.5–0.9
P17936	Insulin-like growth factor-binding protein 3			Yes	0.07–0.17
P25311	Zinc alpha-2-glycoprotein	Yes			0.8-1.6
P36955	Pigment epithelium-derived factor	Yes	Yes	Yes	0.1
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	Yes	Yes		1–2

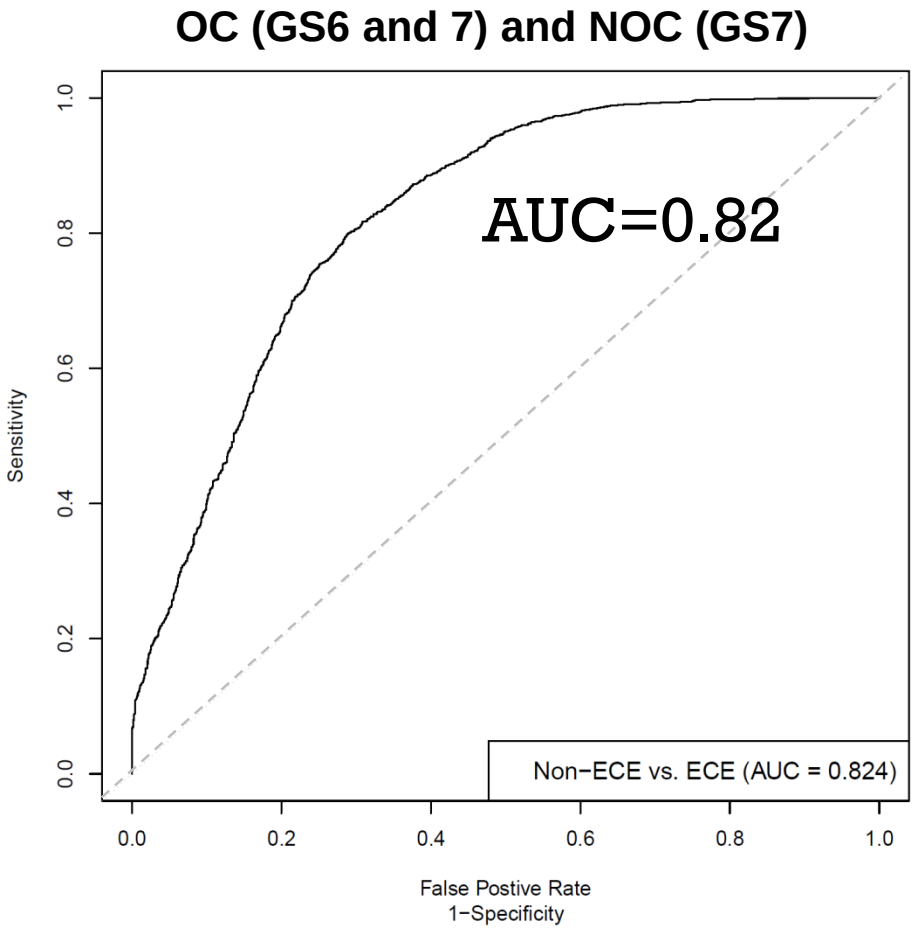
MRM Transitions

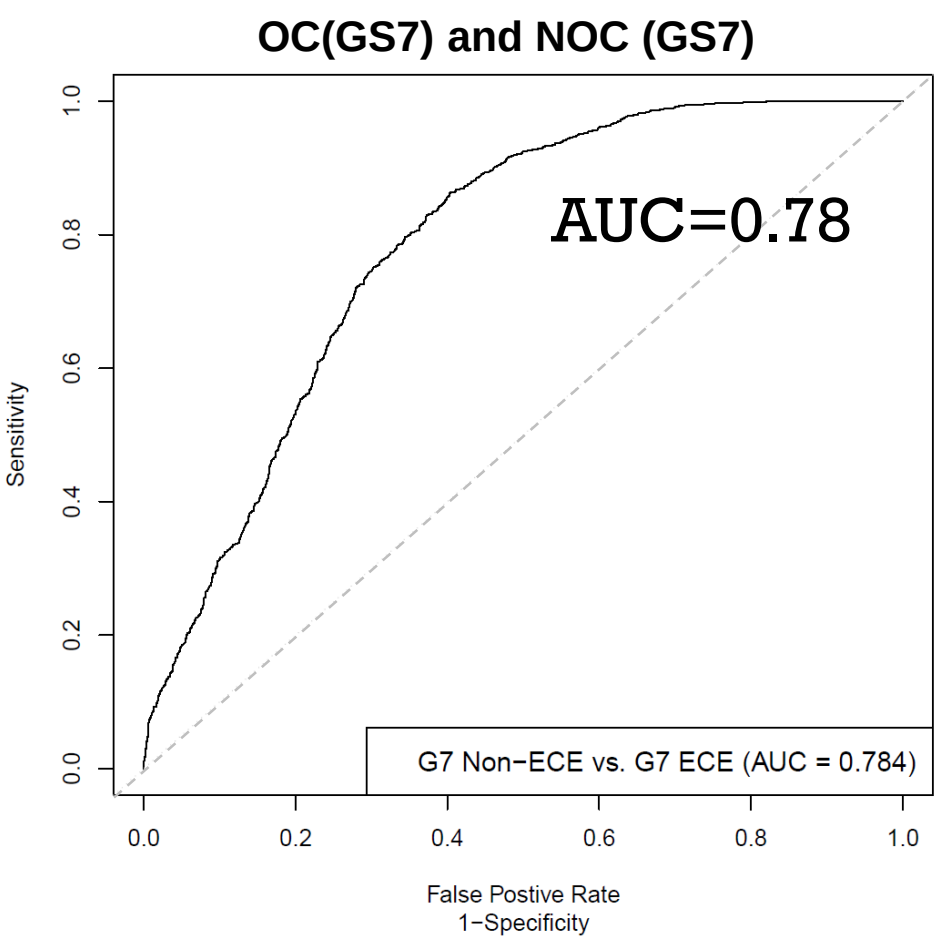
IP

Candidate Biomarker MRMs



Prediction of Organ Confinement (initial data)



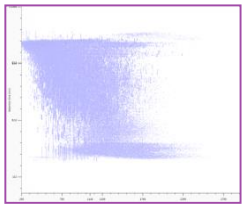


PLS-DA with 200 times bootstrapping

Use global data to assemble panel

Biomarker discovery

2D-DIGE



Label-free LC-MS/MS

PubMed

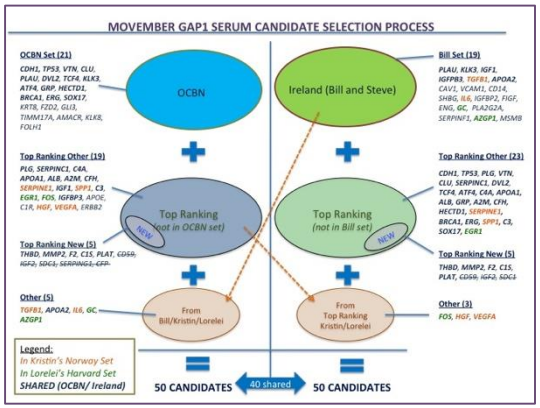
Literature review

1st Generation

64 Candidate Proteins

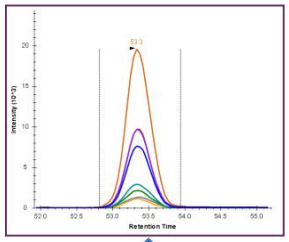
2nd Generation

Movember GAP



Biomarker Candidate list

136 Candidate Proteins



MRM

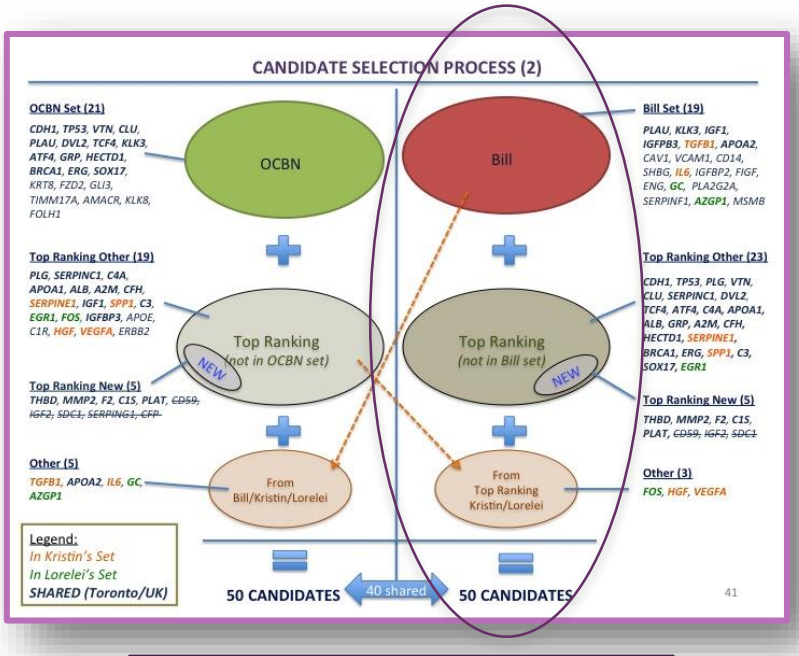
Skyline

Biomarker Validation

Biomarker measurement (now)

Biomarker assembly

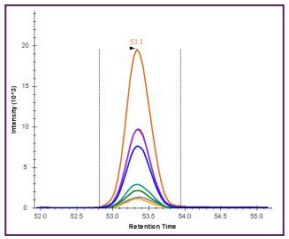
Biomarker Prioritization



X Candidate Proteins

Biomarker Validation

MRM

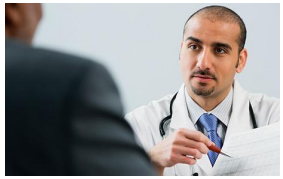


Agilent 6490 Triple Quad with UPLC: Agilent Partner Lab

Samples

Assembly of Reference Pool (method development and QC)
Test (150) Samples: False Indolent; True Indolent

Conclusion?



Health Screening for Men

Comprehensive health screening for men. It takes about three hours to complete and incorporates an exhaustive list of health screening features with an emphasis on modern men's health issues and lifestyle.

Physiological Assessment

- Blood pressure, heart rate, weight, height, body mass index measurement
- Urinalysis to check liver and kidney function and for infection
- FOB test for those over the age of 50
- Heart Assessment (Resting ECG)
- Lung Function tests (Spirometry)
- Hearing test (Audiometry)
- Eye assessment to check visual acuity, near and far vision, macular and retinal problems and other potential problems regarding the retina and fundus

Laboratory tests

- An extensive blood screen to include an assessment of cholesterol and glucose levels, liver and kidney function, measurement of haemoglobin and iron levels, full blood count, thyroid function test (if clinically indicated) and screen for gout and haemochromatosis
- PSA (Prostate Specific Antigen) recommended for those over the age of 40

**(Laboratory testing at The Well is carried out by Medlab)*

√√√√√√√

PSA 14.2ng/ml

Blood Test for Organ Confinement

Clinical assay

Best Decision for Individual Patient

Clinical Utility: What will it take?

- 'End user' driven question/ clinical need
- Design of discovery experiment(s) to match clinical question
- Well planned validation strategy ….. sample numbers and type
- Incorporation of appropriate statistical methods
  - For selection of candidates from discovery
  - For selection of signatures from candidate panels
- Then, science ends … product development begins

PCa Multidisciplinary Teams

UCD Conway Teams

Prostate Cancer Research Consortium

National Prostate Cancer Research Group

ToPCaP
Transdisciplinary Prostate Cancer Partnership

Movember GAP

GLOBAL ACTION PLAN

MOVEMBER

Acknowledgements

Prostate Cancer Research Consortium

Teams: Nurses, clinicians, pathologists, training clinician scientists, non-clinical scientists, research assistants

The PATIENTS

Movember Serum GAP Team

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Agilent Technologies

HRB Health Research Board

irish cancer society

Molecular Medicine Ireland

sfi Science Foundation Ireland

GLOBAL ACTION PLAN
Accelerating research outcomes through global collaboration

William Osler

"The philosophies of one age have become the absurdities of the next….."

MRM for Lung Cancer

RESEARCH ARTICLE *Sci Transl Med* **5**, 207ra142 (2013);

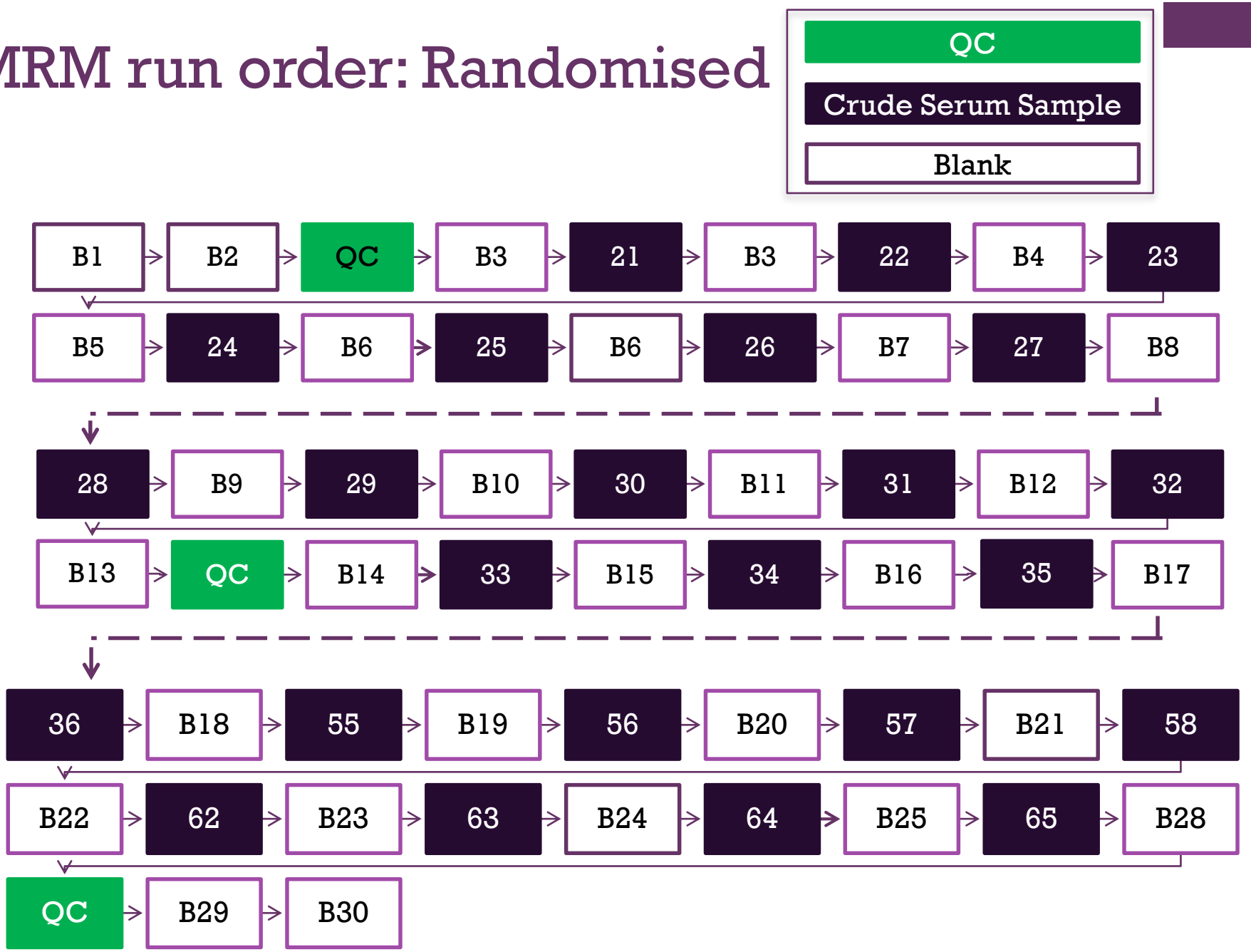
LUNG DISEASE

A Blood-Based Proteomic Classifier for the Molecular Characterization of Pulmonary Nodules

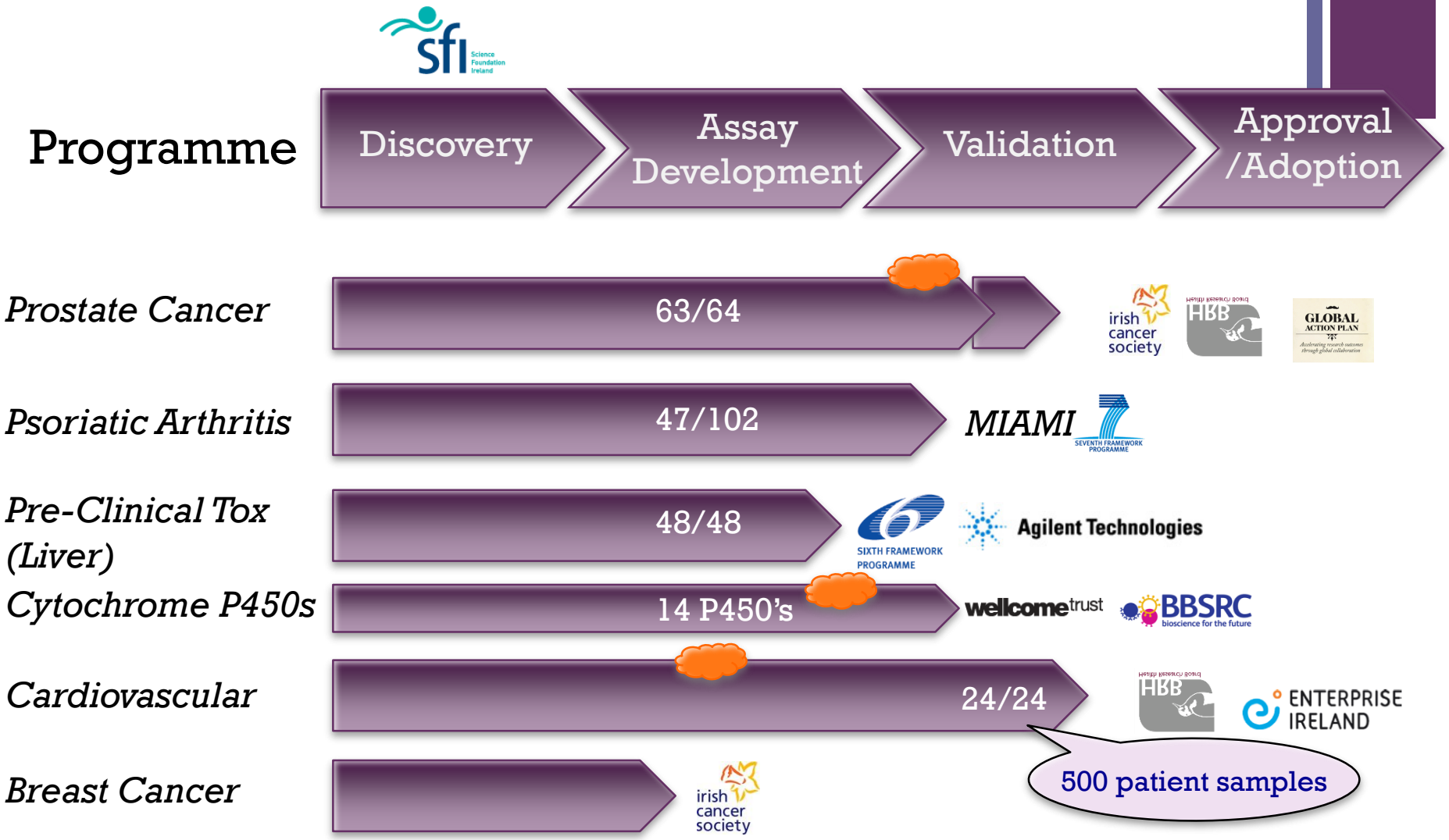
Xiao-jun Li,[1]* Clive Hayward,[1] Pui-Yee Fong,[1] Michel Dominguez,[1†] Stephen W. Hunsucker,[1] Lik Wee Lee,[1] Matthew McLean,[1‡] Scott Law,[1] Heather Butler,[1§] Michael Schirm,[2] Olivier Gingras,[2] Julie Lamontagne,[2] Rene Allard,[2] Daniel Chelsky,[2] Nathan D. Price,[3] Stephen Lam,[4] Pierre P. Massion,[5,6] Harvey Pass,[7] William N. Rom,[8] Anil Vachani,[9] Kenneth C. Fang,[1] Leroy Hood,[3] Paul Kearney[1]*

- Used a systems biology strategy to identify 371 protein candidates
- Developed a multiple reaction monitoring (MRM) assay for each.
- MRM assays applied in a three-site **discovery** study **(n = 143)**
- Used plasma samples from patients with benign and stage IA lung cancer
- Produced a 13-protein classifier.
- Classifier validated on an independent set of plasma samples **(n = 104)** exhibiting a negative predictive value (NPV) of over 90%.

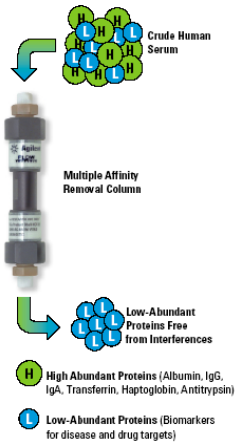
MRM run order: Randomised

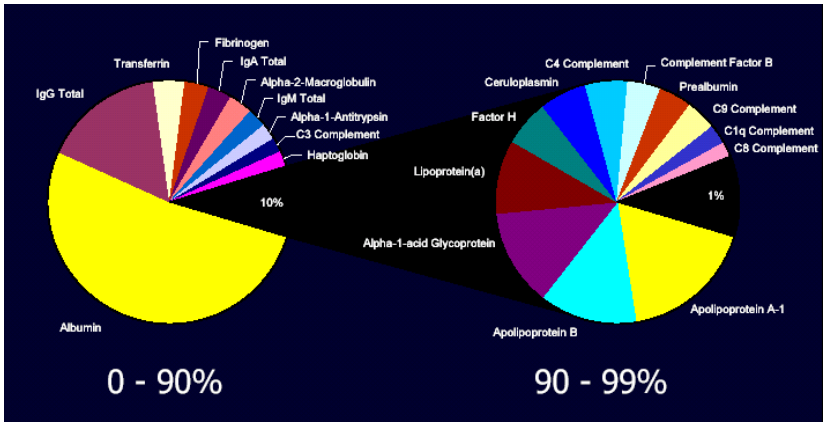


Current Biomarker Pipeline

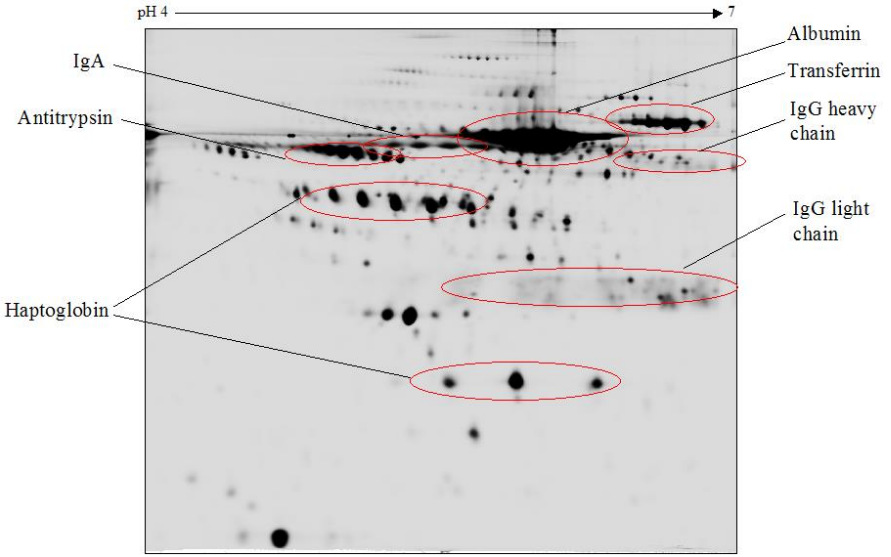


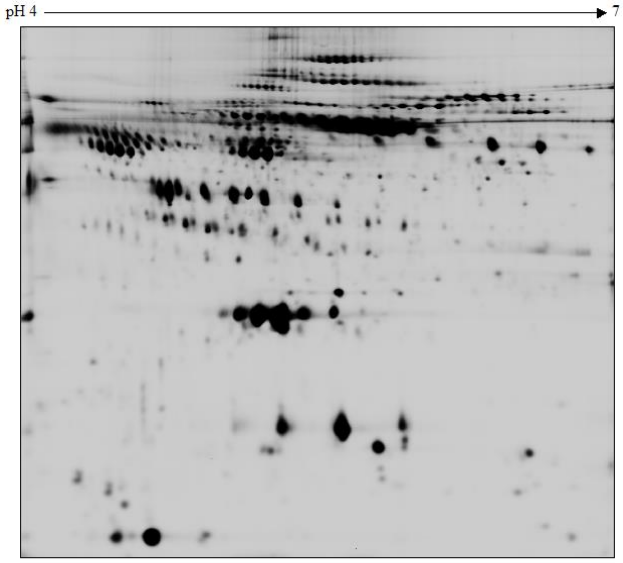
Abundant protein removal





Anderson and Anderson, Mol. Cell. Proteomics 2002 1: 845





Serum Proteins: Dynamic Range

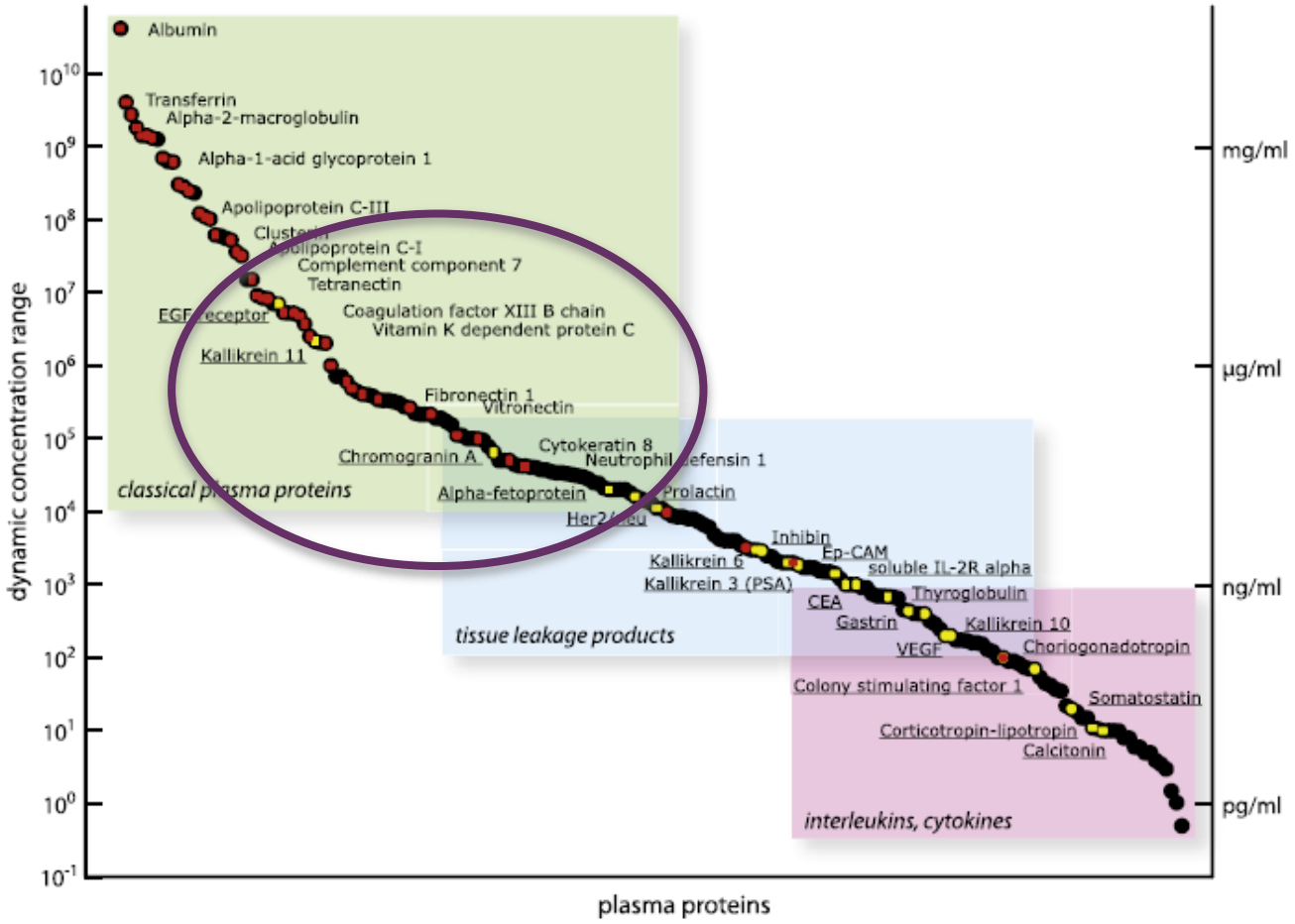
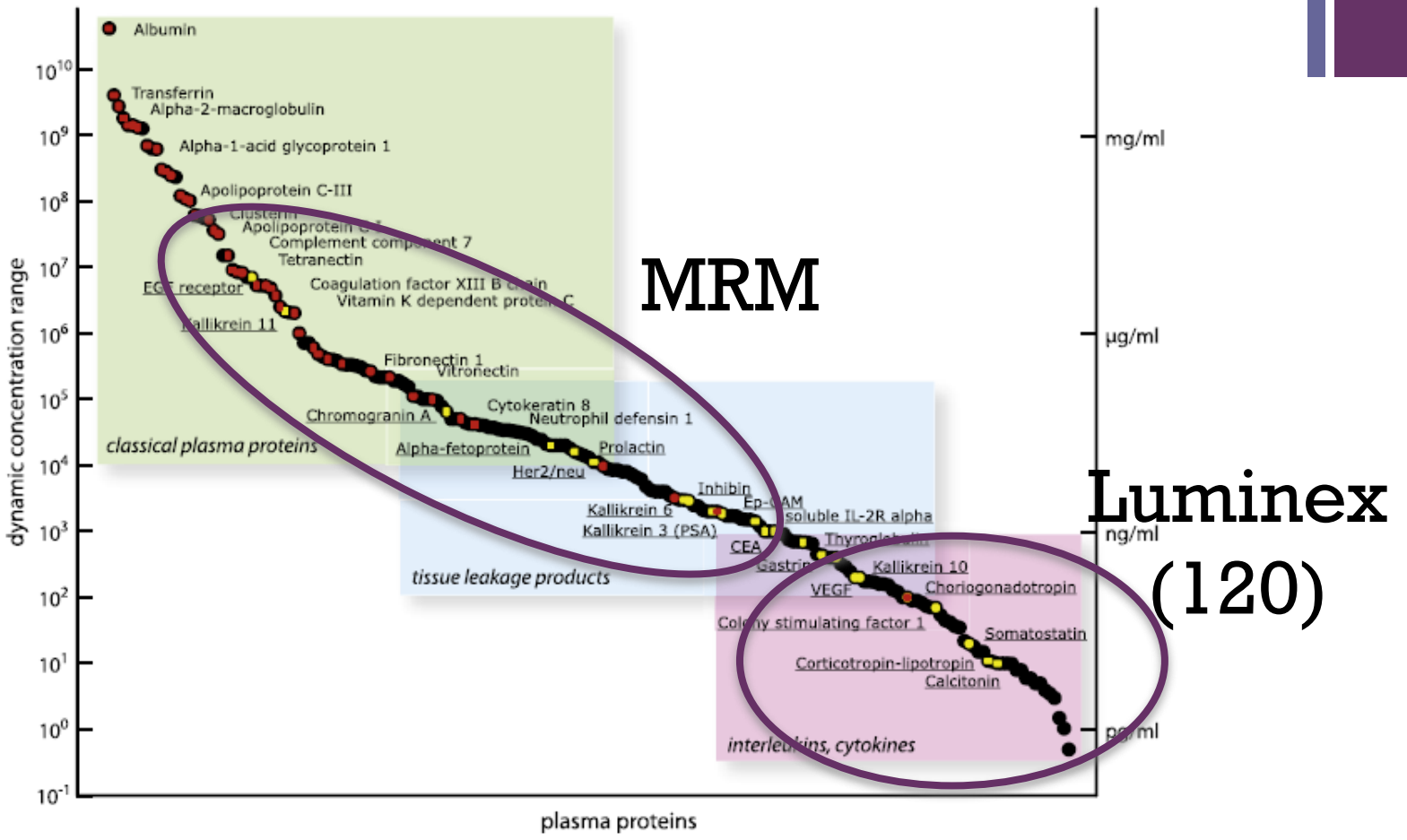


Figure 1 – Depicted are the plasma protein concentration as described by Anderson and Anderson (2002). The proteins can be grouped in three main categories (classical plasma proteins, tissue leakage products, interleukins/cytokines). Red dots indicate proteins that were identified by the HUPO plasma proteome initiative (States et al., 2006) and yellow dots represent currently utilized biomarkers (Polanski and Anderson, 2006).

Serum Proteins: Dynamic Range



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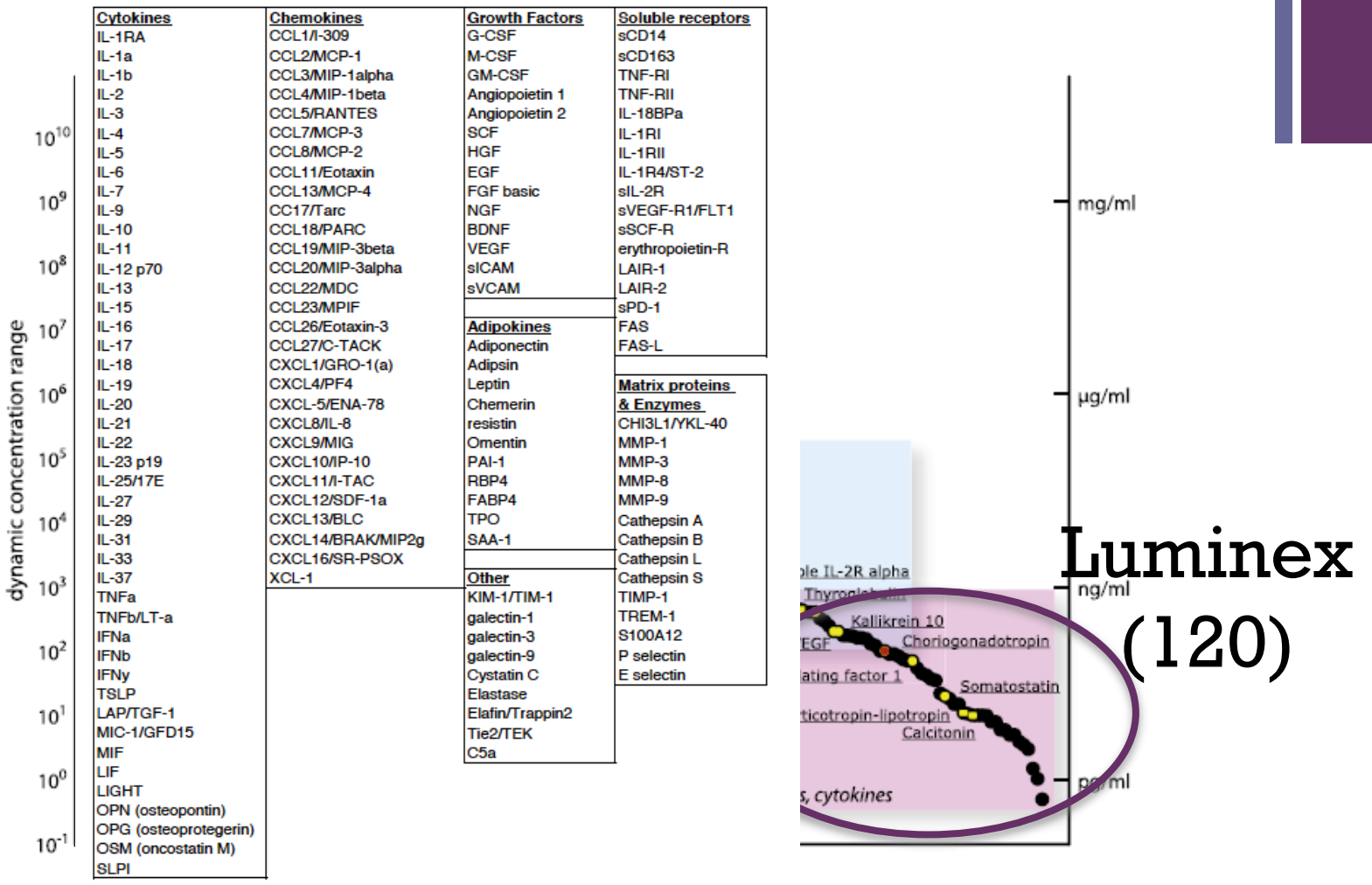

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