Development of a serum protein assay for organ confined prostate cancer

15th June 2014

Steve Pennington
UCD Conway Institute, UCD, Dublin
Protein Biomarker Discovery and Development

**Discovery**
- Sample accrual
- Protein Discovery
- Protein Identification and Characterisation
- Other analytes (anything measurable)

**Confirmation**
- **Antibody based**
  - Western blotting
  - ELISA
- **Mass Spectrometry based**
  - Multiple Reaction Monitoring (MRM)
- Multi-analyte assays

**Validation/Qualification**
- ‘Robust’ high-throughput assays
- Additional clinical samples
  - Large Multicentre Cohorts
  - Large Scale Clinical Trials

**Approval & Adoption**
- Regulatory Authorities
- Clinician Adoption
- Impact measurement

Sample Numbers
Protein Biomarker Discovery and Development

 Discovery

 Confirmation
 Assay development

 Validation/
 Qualification

 Approval &
 Adoption

 Discovery

 Confirmation

 Assay development

 Validation/
 Qualification

 Approval &
 Adoption

 Statistical Methods
Biomarker Futility

Biomarkers

Too many research teams, operating in isolation, have made fragmented claims about disease-associated biomarkers, argues George Poste. They are now calling for a coordinated ‘big science’ approach to deliver effective biomarkers to the clinic.

The lack of standardization in the collection and storage of specimens that have already been fixed (and sometimes even stored) can hinder subsequent research.

Olins have delivered no new biomarkers to the clinic.

Estimated number of papers documenting thousands of claimed biomarkers

150,000

Estimated number of biomarkers routinely used in the clinic

100
Clinical Utility

2006
Clinical Utility: 8 years on

Will the **protein** biomarkers we discover be useful?
How will we proceed to them gaining 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 Antibody) recommended for those over the age of 40
  *(Laboratory testing at The Well is carried out by Mediblu)

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 men's 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

Imagine the screen

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  *(Laboratory testing at The Well Medical Centre - Mallow)*

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* (Laboratory testing at The Well carried out in Medlab)

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Diagnosis & Treatment

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All clear doc?

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PSA 14.2ng/ml
**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.
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TRUS Biopsy
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

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.

Personalised - Population

Prostate Cancer: GET THE FACTS
Other than skin cancer, prostate cancer is the most common cancer in American men.

1 in 6 men will be diagnosed with prostate cancer during his lifetime.

Prostate cancer can be a serious disease, but most men diagnosed with prostate cancer do not die from it. In fact, more than 2.5 million men in the United States who have been diagnosed with prostate cancer at some point are still alive today.

Gleason's Grade 5-7

Gleason's Grades 5-6 and Gleason's Grade 7 are similar in prognosis. Somewhere around 50% of men with these total Grades will be alive after 12 years. However, consider that a Gleason's Grade 2+3=5 will have a better prognosis than a Gleason's Grade of 3+4=7. Even a 3+4=7 will have a better prognosis than a 4+3=7 (both have a total Grade of 7, but Pattern 4, which is more aggressive than 3, is more prevalent in 4+3=7).

All 7’s aren’t equal

3+4 ≠ 4+3
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

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.
Decisions, Decisions, Decisions

Radical Prostatectomy (RP)

Radiation (with hormones)

No treatment (Active Surveillance)
Diagnosis and Treatment

Diagnostic Test

PSA

DRE

Biopsy

Diagnosis

Normal

BPH

Confined Prostate cancer

Non Confined Prostate cancer

Treatment

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
Biomarker Panel Development

PCRC Serum Sample Bioresource

Biomarker discovery

2D-DIGE  Label-free LC-MS/MS

Biomarker Candidate list
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

Serum samples

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

Mars 14 column

Trypsin digestion

Create reference pool sample from each pool depleted sample

Affinity Depletion using MARS 14 column

Depleted serum samples

Protein concentration normalization

Protein assay and 1D gel

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

In-solution digestion

Progenesis, database search and result filtering

Peptide/protein expression profile

Public MS/MS spectral library

In-house MS/MS spectral library

Trans-Proteomic Pipeline
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)
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  Literature review

64 Candidate Proteins

Biomarker Candidate list

MRM

Biomarker Validation
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
Multiplexed quantification

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

Rosalind E. Jenkins¹, Neil R. Kitteringham¹, Christie L. Hunter², Sally Webb², Tony J. Hunt², Robert Elsby³, Rod B. Watson⁴, Dominic Williams⁵, Stephen R. Pennington⁶ and B. Kevin Park⁷

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

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<th>95% Cls Upper</th>
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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.
Another protein panel assembly

Protein/gene list

Gene expression
dataset
200 highest ranked transcripts

Mapped to 168
Uniprot accessions

51 proteins with
library MS/MS
spectra available

35 Proteins
after inspection
and filtering

12 overlap
Px + Gx

Label-free LC
proteomics dataset
53 highest ranked
IPI accessions

Mapped to 33
Uniprot accessions

33 proteins with
library MS/MS
spectra available

31 Proteins
after inspection
and filtering

1 overlap
Px + Lit

Literature
27 candidate
biomarkers

Mapped to 27
Uniprot accessions

17 proteins with
library MS/MS
spectra available

16 Proteins
after inspection
and filtering

‘Housekeeping’
17 proteins with no
change in Px and
Gx datasets

Mapped to 17
Uniprot accessions

7 proteins with
library MS/MS
spectra available

5 Proteins
after inspection
and filtering

57 proteins

Manual inspection
and filter (proteotypic,
pep. size, methionine,
ragged ends, etc.)
**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)

**Technical variance measurement**

- 10 injections pooled sample
(\~17 hours instrument time)
- Mean CV = 5.7%

**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

**Measurement in 30 individual samples**

- Drug treated or vehicle control
(\~51 hours instrument time)
Development of a Pharmaceutical Panel Using a
Hepatotoxicity Biomarker Approach to Targeted
Proteomics

Ben C. Collins,†‡ Christine A. Miller,† Alexandre Sposmi,§ Phillip Hewish§
Martin Wells†, William M. Gallaghers, and Stephen R. Penningtont§§

[Graph showing fold change ratio with various treatment groups and housekeeping genes]
### Workflow Map

- **Biomarker from 2D-DIGE**
- **Biomarker from Label-free LC-MS/MS**
- **Literature review**

**64 Proteins**
- **MS/MS data?**
  - Yes: 59 Proteins
  - No: Unscheduled MRM

**59 Proteins**
- **MRM design**
- **Up to 5 peptides/protein, 8 transitions/peptides**
  - 269 peptides, 275 precursor, 2049 transitions

**Calculated CV% for 50 peptides**
- 10 replicates unscheduled runs

**31 proteins, 50 peptides, 50 precursor, 149 transitions, 63 crude serum sample (GS6, G7,G7ECE)**
- **Short the gradient to 38 mins, Unscheduled MRM**

**Calculated CV% for 53 peptides for crude and depleted serum samples**
- **Scheduled MRM on 6 replicates on crude and depleted samples**

**32 proteins, 53 peptides, 53 precursor, 158 transitions**
- **Select up to 2 peptides/protein, 3 transitions/peptide**

**33 proteins, 87 peptides, 87 precursor, 653 transitions**

### 31 Candidates

- **1-5 peptides/protein**
- **8 transitions/peptide**

**Example:** APOA1, DPLATVYDVLK

### MRM Transitions

**Key Points**
- 31 candidate biomarkers
- Schedule MRM on crude and depleted samples
Candidate Biomarker MRMs
Prediction of Organ Confinement (initial data)

OC (GS6 and 7) and NOC (GS7)

AUC = 0.82

PLS-DA with 200 times bootstrapping
Use global data to assemble panel

Biomarker discovery

2D-DIGE

Label-free LC-MS/MS

Literature review

Biomarker Validation

MRM

Skyline

64 Candidate Proteins

1st Generation

Biomarker Candidate list

136 Candidate Proteins

2nd Generation
Biomarker measurement (now)

**Biomarker assembly**

**Biomarker Prioritization**

**Biomarker Validation**

**X Candidate Proteins**

**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)*

Blood Test for Organ Confinement

PSA 14.2ng/ml

Best Decision for Individual Patient

Clinical assay
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

ToPCaP
Transdisciplinary Prostate Cancer Partnership

Movember GAP

National Prostate Cancer Research Group

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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Christine Miller
“The philosophies of one age have become the absurdities of the next…..”

William Osler
MRM for Lung Cancer

- 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

Crude Serum Sample
Blank
Current Biomarker Pipeline

Programme

Discovery    Assay Development    Validation    Approval/Adoption

Prostate Cancer
63/64

Psoriatic Arthritis
47/102

Pre-Clinical Tox (Liver)
48/48

Cytochrome P450s
14 P450’s

Cardiovascular
24/24

Breast Cancer

Numbers: MRMs developed/Candidates

= Intellectual Property Filings

500 patient samples
Abundant protein removal

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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