Design and Development of Multiplexed MRM assays for Evaluation of Blood Protein Biomarkers

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29th October 2015
SRP Context

Chemistry – NMR and MS
John Hughes and Howard Morris
Biochemistry and 2D gels
Proteomics and mass spec
Mechanisms and markers
Frustration
Biomarkers: local to global to .... ...
... review
Mass-spectrometry based analysis of the human proteome
HUPO 2014

We are delighted to announce that the 13th Human Proteome Organization World Congress will be held in Madrid, Spain from 5 to 8, October 2014.

We gather annually to discuss about the most recent advances in proteomics technology and its present and future applications. On the occasion of the Madrid Congress, we speak to further promote the development of the young field to contribute to the well-being of future generations.
Tissue-based map of the human proteome

Matthias Uhlen,1 Lisa Fagerberg, Björn M. Hallström, Cecilia Lindskog, Per-Olaf Sjöblom, Adil Muratoglu, Ana Silverstone, Caroline Kampf, Evelina Stjördal, Anna Asplund, Ing-Marie Olsson, Karolina Edlund, Emma Lindberg, Sunil Navari, Cristina Al-Asheli Sittigato, Joakim Ohlsson, Désirée Øverby, Jenny Orimoa Sjöblom, Stephane Hebron, Thor Ahl, Per-Henrik Edgren, Helge Ruttvig, Hanna Tegel, Jan Mulder, Johan Rockberg, Peter Nilsson, Johan M. Schweda, Markus Hamsten, Kalle van Wijnen, Mattias Forsberg, Lukas Pernson, Fredrik Johannson, Martin Zoulin, Gunnar van Huppe, Jens Nilsen, Frederik Pranck

INTRODUCTION: Resolving the molecular details of protein variation in the different tissues and organs of the human body would greatly increase our knowledge of human biology and disease. Here, we present a map of the human tissue proteome based on quantitative mass spectrometry on a tissue and organ level combined with protein profiling using microarray-based immunohistochemistry to achieve spatial localization of proteins down to the single-cell level. We provide a global analysis of the secreted and membrane proteins, as well as an analysis of the expression profiles for all proteins targeted by pharmaceutical drugs and proteins implicated in cancer.

MATERIALS AND METHODS: We used an integrative omics approach to study the spatial human proteome. Samples representing all major tissues and organs (n = 44) in the human body have been analyzed based on 38,028 antibodies corresponding to 16,905 proteins, with a high content of secreted proteins. The antibodies have been used to produce more than 32 million tissue-based mass spectrometry images, each annotated by pathologists for all sampled tissues. To facilitate integration with other biological resources, all data are available for download and cross-referencing.

RESULTS: We report a genome-wide analysis of the tissue specificity of proteins and protein expression covering more than 90% of the putative protein-coding genes, complemented with analyses of various subproteomes, such as predicted secreted proteins (n = 107) and membrane-bound proteins (n = 550). The analysis shows that almost half of the genes are expressed in all analyzed tissues, which suggests that the gene products are needed in all cells to maintain "housekeeping" functions such as cell growth, energy generation, and basic metabolism. Furthermore, there is enrichment in metabolism among these genes, as 60% of all metabolic enzymes are expressed in all analyzed tissues. The largest number of tissue-enriched genes is found in the testis, followed by the brain and the liver.

CONCLUSIONS: A freely available interactive resource is presented as part of the Human Protein Atlas portal (www.proteinatlas.org), offering the possibility to explore the tissue-specific proteomes in tissues and organs and to analyze tissue profiles for specific protein classes. Comprehensive lists of proteins expressed at elevated levels in the different tissues have been compiled to provide a spatial context with localization of the proteins in the subcompartments of each tissue and organ down to the single-cell level.

The human tissue-enriched proteins. All tissue-enriched proteins are shown for 13 representative tissues or groups of tissues, stratified according to their predicted subcellular localization. Enriched proteins are mainly intracellular in testis, mainly membrane bound in brain and kidney, and mainly secreted in pancreas and liver.
THE HUMAN PROTEIN ATLAS

A Tissue-Based Map of the Human Proteome

Here, we summarize our current knowledge regarding the human proteome mainly achieved through antibody-based methods combined with transcriptomics analysis across all major tissues and organs of the human body. A large number of lists can be accessed with direct links to gene-specific images of the corresponding proteins in the different tissues and organs.

Read more
Proteomics Now

Proteomics: a pragmatic perspective

Parag Mallick1,2 & Bernhard Kuster3,4

NATURE BIOTECHNOLOGY VOLUME 28 NUMBER 7 JULY 2010
### Proteomics: a pragmatic perspective

Parag Mallick\(^1,2\) & Bernhard Kuster\(^3,4\)

*NATURE BIOTECHNOLOGY* VOLUME 28 NUMBER 7 JULY 2010
High **quality samples** are critical

Selecting which sample and technology to use to address a **specific question or hypothesis** is increasingly challenging.
Discovery and development of a blood-based protein signature markers to guide patient treatment decisions
Protein Biomarker Pipeline

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

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

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

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

Clinical Test

Sample Numbers

Statistical Methods
Biomarker Discovery Projects

- Tissue & cells: pancreatic cancer
- Cell models & serum markers: breast cancer
- Tissue & fluids – synovium, synovial fluid/serum: rheumatoid arthritis
- Tissues & fluids (serum/urine): drug metabolism and preclinical toxicity
- Fluids – serum/urine: prostate cancer

- Use of proteomics for the discovery of early markers of drug toxicity
- Stratification and Monitoring of Juvenile Idiopathic Arthritis Patients by Synovial Proteome Analysis
- Use of SELDI-TOF to discover and identify potential biomarkers in InnoMed PredTox: A multi-site study
- Discovery and confirmation of a protein biomarker panel with potential to predict response to biological therapy in psoriatic arthritis
- Urinary markers for prostate cancer
- Breast cancer proteomics: clinical perspectives
SEEMINGLY STRAIGHTFORWARD but SUCCESS for CONVERTING protein biomarkers to DIAGNOSTIC TESTS of CLINICAL VALUE is VERY LIMITED
Biomarker Futility

‘Omics have delivered

No new biomarkers to the clinic

A DROP IN THE OCEAN

Few of the numerous biomarkers so far discovered have made it to the clinic.

Estimated number of papers documenting thousands of claimed biomarkers

150,000

Estimated number of biomarkers routinely used in the clinic

100

Poor specimens
Fragmented approach
Why?
Key Considerations
Questions

Who does:
- Implementation?
- How to promote awareness and use?
  - Who:
  - Uses?
  - Pays?
- Regulatory path?
- Healthcare impact?

What samples?
- What markers?
- How many?
- How to combine?

Which platform?
- Discovery
- Validation
- Use
- Analytical performance?
  - Cost?
- Throughput?

Clinical Evaluation?
- Statistical Methods?

Clinical use & benefit
Questions

- Who does: Implementation?
- How to promote awareness and use?
- Who: Uses? Pays?
- Regulatory path? Healthcare impact?
- What samples?
- What markers?
- How many?
- How to combine?
- Which platform?
- Discovery Validation Use
- Analytical performance? Cost? Throughput?
- Clinical Evaluation?
- Statistical Methods?
- How much does it cost to get a molecular biomarker to market?

Clinical use & benefit
Patients

Clinical use & benefit

Responsibility

Statistics

Analytical

Rigour
So it’s not STRAIGHTFORWARD
Oncotype DX Breast Cancer Assay

- 21 genes within a tumor to determine a Recurrence Score.
- Provide physicians and their patients with critical information.
- To help guide treatment decision making in women with ductal carcinoma in situ (DCIS) or invasive carcinoma.
- Can predict the potential benefit of chemotherapy and likelihood of distant breast cancer recurrence in..
- Node negative or node positive, ER-positive, HER2-negative invasive breast cancer.
- Became available in 2004, it has been used by over 10,000 physicians to help guide treatment for over 200,000 patients in 60+ countries.
The Oncotype DX® Breast Cancer Test

Revealing the underlying tumour biology to help decide:

Chemo? No Chemo?

Introducing the Oncotype DX® Test
Oncotype DX® Breast Cancer Assay

Available to NHS clinicians in 2015

The only genomic test validated for its ability to predict the likelihood of chemotherapy benefit as well as risk of recurrence, the Oncotype DX® test will be available via an access scheme for National Health Service (NHS) in England starting April 1, 2015.

The access scheme follows the National Institute for Health and Care Excellence's (NICE) exclusive recommendation to use the test for assisting in chemotherapy treatment decisions for patients with early-stage, hormone receptor positive, HER2 negative, invasive breast cancer.

Read the press release
Oncotype DX Pipeline

- Perou et al., 2000
  - 62 samples
  - ~8100 genes

- Veer et al., 2002
  - 98 samples
  - Over 5000 genes

- Sørlie et al., 2001
  - 84 samples
  - Over 1700 genes

- Golub et al., 1999
  - 38 samples
  - ~6800 genes

- Other database and literature searches

282 Samples

250 Gene Signature

250 Candidates

Validation of 250 gene signature

437 Samples

Oncotype DX 21 Gene Panel

21 Gene Panel

Recurrence score

Validation of recurrence score

NSABP B-14 Trial

>4,100 Samples
Conclusion

followed a
STEPWISE APPROACH
to develop
QUANTITATIVE SERUM-BASED TEST
of
12 PROTEIN BIOMARKERS
consistently associated with
RA DISEASE ACTIVITY
Crescendo: Vectra DA

MYRIAD RBM

What is Vectra® DA?
- The only validated multibiomarker blood test to measure rheumatoid arthritis (RA) disease activity.
- Vectra DA integrates 12 key protein biomarkers into a single and objective score between 1 and 100 to classify RA disease activity.
- Validated for use in adults diagnosed with RA, and studied in more than 1700 RA patients from multiple cohorts.
- Vectra DA is covered by Medicare.
- Vectra DA is performed by Myriad RBM's sister company, Crescendo Bioscience, in their CLIA certified laboratory.
- Over 100,000 patients have been tested with Vectra DA.

Potential Roles for Vectra DA in Supporting Clinical Drug Development for RA
- Identify RA patient populations with active disease
- Expand pool of patients eligible for clinical trials by capturing patients excluded by conventional measures (CRP/ESR)
- Measure changes in disease activity in response to treatment
- Provide quantitative assessment of disease activity in longitudinal studies
- Minimize placebo effect through use of objective measure rather than subjective clinical assessments
- Provide response curves based on objective measure for more discrete dose/response analysis

How is Vectra DA different from other lab tests?
Some lab tests may measure inflammation indirectly (such as erythrocyte sedimentation rate [ESR]) or measure only one biomarker of inflammation (such as C-reactive protein [CRP]) whereas Vectra DA measures 12 key proteins that represent multiple RA biological pathways. Additionally, the utility of ESR and CRP is limited because they are normal in a large percentage of patients with RA. In contrast, Vectra DA can frequently detect elevated disease activity even when CRP is low.

A SINGLE OBJECTIVE SCORE TO CLASSIFY RA DISEASE ACTIVITY:

<table>
<thead>
<tr>
<th>Level of Disease Activity</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectra DA Score</td>
<td>1 to 29</td>
<td>30 to 44</td>
<td>45 to 100</td>
</tr>
</tbody>
</table>

- VCAM-1: Adhesion Molecules
- EGF: Growth Factors
- IL-6: Cytokine-Related Proteins
- MMP-1: Matrix Metalloproteinases
- MMP-3: Skeletal-Related Proteins
- YKL-40: Leptin
- Resistin
- SAA: Acute Phase Proteins

February 2014
issues

FDA regulations on LDT’s

Establishing Clinical Value

Re-imbursement/Revenues (monetary value)
How?
How can we discover and develop protein biomarkers of clinical utility and value?
“It's simple”
Need

Solution

Scale it

Dean Griffiths, Cambridge Consultants 2014
Imagine
Imagine this scene .....
Imagine this 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, a full blood count, thyroid function test (if clinically indicated) and a screening for goitre and hyperthyroidism
- PSA (Prostate Specific Antigen) recommended for men over the age of 40

*Laboratory testing at The Well Hammond by request

Lifestyle Analysis

- Stress questionnaire and analysis
- Lifestyle questionnaire and correlation 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 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 and enhance a healthy lifestyle.
Imagine this screen …..

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Laboratory tests

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- PSA (Prostate Specific Antigen) recommended for those over the age of 40
  (*Laboratory testing at The Well’s Medical Department)

Lifestyle Analysis

- Stress questionnaire
- Lipid profiles
- Body composition analysis
- Review of current diet and exercise regime and development of a personal lifestyle plan

Doctor’s consultations

- 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 those over the age of 40
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Blood – FBC, Hb & Fe, cholesterol, glucose, liver & kidney function

Urine

Heart

Hearing

Vision


Cost $550.00
All clear doc?

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

PSA 14.2ng/ml

“What now?”
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.
Digital rectal examination (DRE)

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.
TRUS Biopsy
Gleason Scoring of Biopsy
So, the result.....
PSA 14.2ng/ml
DRE – abnormal
Gleason 3 + 4
Prostate Cancer

What now?
Decisions....

The patient's treatment decision is a momentous one.

Must gather reliable information to participate in the diagnostic process and select the therapy most reasonable under the circumstances.

Prostate Cancer Coalition

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

As he confronts his condition and he must should take into account his personal goals the available therapies and their peculiar morbidities.
He will get differing medical opinions from his primary care physician urologist radiologist + second and third opinions from other specialists family members & friends other patients
From Personal to Population

Diagnosed with prostate cancer
Ireland >3,000 men
UK >25,000 men
Europe >400,000
USA > 250,000

Every Year
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

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)
To RP or not to RP?

That is the question…

Radical Prostatectomy
Radiation (with hormones)
EBRT/BT

To RP or not to RP?
That is the question…
‘Significant’ Morbidities

Urinary Dysfunction
Bowel Dysfunction
Erectile Dysfunction
Loss of Fertility
Side Effects of Hormone Therapy
Side Effects of Chemotherapy
Morbidities (36months)


NNSRP=non-nerve sparing radical prostatectomy, NSRP=nerve sparing radical prostatectomy, EBRT=external beam radiation therapy, BT=brachytherapy

Yellow = normal function
Blue = mild dysfunction
Red indicates = more severe dysfunction
Active Surveillance
The Problem

>60% of those diagnosed early with the disease could (but currently don’t) have active surveillance

estimated that in US alone over-treatment of PCa costs $3 billion each year

recent data indicates increased use of active surveillance would be just as effective – i.e. have no impact on clinical outcome from the disease
So, to summarise

1 in 6 men are diagnosed with prostate cancer
Most men die with not of prostate cancer
Overtreatment is far too common

Patient's treatment decision is a momentous one

PSA, DRE and biopsy do not adequately support this life-changing decision
Can we discover and develop new protein biomarkers to help recently diagnosed Prostate Cancer patients make their ‘treatment’ decision?
PIPELINE
Protein Biomarkers

DISCOVERY
- Sample accrual
- Protein Discovery
- Protein Identification and Characterisation
- Other analytes (anything measurable)
- Antibody-based Western blotting
- ELISA
- Mass Spectrometry-based Multiple Reaction Monitoring (MRM)
- Robust high-throughput assays
- Additional clinical samples
- Multicentre cohorts

Statistical Methods

CONFIRMATION
- Assay development
- Validation/Qualification
- Clinical Trials
- Approval & Adoption

VALIDATION PANEL
- Sample Numbers
- Approval & Adoption
- Regulatory Authorities
- Clinician Adoption
- Impact measurement

Start at the end
Define the Clinical Question/Need

Better treatment decision
Clinical Assay ‘Requirements’

- Non invasive
- Avoid tissue heterogeneity
- Suitable for repeat sampling
- Actionable information
- Available for use

- Serum
- Urine/prostatic fluid
- Seminal fluid
Need

Solution

Scale it
Biomarker Discovery

PCRC Serum Sample Bioresource

Biomarker discovery

I: 2D-DIGE

II: Label-free LC-MS/MS

7:OC

<7:OC

7:NOC

Biomarker Candidate list
**Discovery: Label free LC-MS/MS**

- **Serum samples**
  - GS5 (n = 10)
  - GS7 OC (n = 10)
  - GS7 NOC (n = 10)

**Affinity Depletion using MARS 14 column**

**Create reference pool sample from each pool depleted sample**

**Trypsin digestion**

**Depleted serum samples**

- **Protein concentration normalization**

**In-solution digestion**

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

**Trans-Proteomic Pipeline**

- **Progenesis, database search and result filtering**
- **Peptide/protein expression profile**
  - In-house MS/MS spectral library
  - Public MS/MS spectral library

- **Protein assay and 1D gel**
  - TPP and Skyline
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)
Assembly of protein candidates

- PCRC Serum Sample Bioresource
- Biomarker discovery
  - I: 2D-DIGE
  - II: Label-free LC-MS/MS
  - III: Literature review

64 Candidate Proteins

Biomarker Candidate list ➔ Biomarker Validation

MRM
MRM: Multiple Reaction Monitoring

1. Identify tryptic peptide unique (m/z) to protein of interest
   
   *(previous experimental data [ChipCube Q-Tof] or in silico)*

2. Complex mixture of peptides introduced into Triple Quad MS

<table>
<thead>
<tr>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Select Peptides</td>
<td>Fragment</td>
<td>Select Fragments</td>
</tr>
</tbody>
</table>

3. Measure relative or absolute quantification (consider normalisation)

**Advantages:**

- Potentially robust, sensitive and high-throughput
- No need for specific antibodies
- Multiplexing – flexible, minimal additional cost to assay
Ionise all peptides

(i)
Select peptide ion

(ii)
Fragment peptide ion

(iii)
Select and detect/monitor fragment

Q1
Q2
Q3

MRM
Iterative MRM Development

In-house Q-TOF Data

Protein List

Public MS/MS Data Repositories

Skyline

Optimised MRM Method

QQQ
Targeted proteomics

Analysis of a preselected group of proteins delivers more precise, quantitative, sensitive data to more biologists. Vivien Marx reports.

Although the number and identity of protein-coding genes in humans and many other organisms are known to a certain level of approximation, the numbers of proteins produced by each of these genes remains a mystery. Further complicating matters, given the many possible splice forms and post-translational modifications, the potential number of proteins is "staggering," says Arizona State University researcher Josh LaBaer, who is also president-elect of the US Human Proteome Organization. A protein is also dynamic. "It's phosphorylated at one minute; it's not phosphorylated the next minute," he says. This is fascinating science, but it makes proteins in a complex, dynamic sample hard to precisely measure.

Understanding disease-related changes, for example, calls for reliable, quantitative ways of assessing protein levels, and mass spectrometers are instruments able to nail that task. But the data from so-called discovery proteomics experiments in which mass spectrometry is used to identify a large number of proteins in a sample are not always useful to biologists. Enter targeted proteomics, in which the analysis focuses on a subset of proteins of interest in a sample—an approach that has been

"I personally can't wait until we stop hearing about someone describing how big of a list of proteins, peptides or phosphopeptides they detected," says one researcher critical of discovery proteomics who did not wish to be identified. Proteomics has been doing "my list is bigger than your list" for far too long: "It is more important to measure the one right protein than 10,000 wrong ones."

Scientists wanting to follow well-founded hunches about dozens or hundreds of proteins seeks a focused, reproducible, quantitative view of a small subset of the whole proteome in their lab vials. High-throughput biology experiments, which include DNA sequencing, genome analysis and gene expression analysis, are generating massive data sets pertaining to particular genes and pathways active in disease or in signaling processes of interest. The shifting of proteomics closer to data are not inherently the same," says Ruedi Aebersold, from the Institute of Molecular Systems Biology at the Swiss Federal Institute of Technology in Zurich. Neither person is necessarily wrong: the contradiction stems from their measurement of different subsets of the whole proteome, he says. "Because the space to sample is so huge, then the mass spectrometer pulls out, every time, a slightly different subset."
MRM

Targeted approach for measuring multiple proteins simultaneously

Features:
- Dynamic range of >4 orders of magnitude
- >50 proteins per assay (easily more)
- Can be quantitative
- Very robust: CV's of less than 10%
- NOT as sensitive as ELISA (in many 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.
Candidate Biomarkers

Workflow Map

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

64 Proteins

MS/MS data?

Yes

59 Proteins

MRM design

Up to 5 peptides/protein, 8 transitions/peptides
269 peptides, 275 precursor, 2049 transitions

Unscheduled MRM

Dotproduct >0.9, RT regression coef > 0.9
Good peak shape and high intensity

Yes

Calculated CV% for 50 peptides

10 replicates unscheduled runs

31 proteins, 50 peptides, 50 precursor, 149 transitions, 63 crude serum sample (G56, 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

MRM Transitions

Example:

APOA1_DLATVYVHDVLK

IP
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
- 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 MRM method
- Proteins: 48
- Peptides: 109
- Transitions: 545
- 5 transitions per peptide
- 1-5 peptides per protein

Initial SRM method
- Proteins: 52
- Peptides: 119
- Transitions: 609
- 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
- Drug treated or vehicle control
  (~51 hours instrument time)
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Fold change ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping</td>
<td></td>
</tr>
<tr>
<td>Proteomics (label-free LC-MS)</td>
<td></td>
</tr>
<tr>
<td>Transcriptomics (Affy array)</td>
<td></td>
</tr>
</tbody>
</table>

MRM measurement: 48 proteins
Can we move from MRM assays to Clinical Tests?
Prediction of Organ Confinement (Evaluation I)

OC (GS6 and 7) and NOC (GS7)

AUC=0.82

OC (GS7) and NOC (GS7)

AUC=0.78

PLS-DA with 200 times bootstrapping
A 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

PSA 14.2ng/ml

Blood Test for Organ Confinement

Clinical Test

Better Decision for Individual Patient
Confinement Score Test

Multiplexed Protein Blood Test

Some answers – still some questions

**Atturos Business Planning**
How to promote awareness and use?
Who: Uses? Pays?
Regulatory path? Healthcare impact?

**WP3**

**WP1**
1500

**WP2**
1 or 2

**Serum Proteins**
Up to 63 Algorithm

**Content**

**Use**

**Platform**

**Clinical benefit**

**Clinical Evaluation**
Bayesian Statistical Methods

**LC-MS**
Discovery Validation Use
Analytical performance
Cost? Throughput
MRM Assays

Identify and measure peptide which is unique to the protein of interest - measure it (mass/charge ratio) and fragments of it generated in the MS
Biomarker measurements (now)

Biomarker assembly

Biomarker Prioritization

Biomarker Validation

Triple Quad with UPLC

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

Over 14 consecutive batches of serum samples %CV for RT was < 0.63%

<table>
<thead>
<tr>
<th>Peptide</th>
<th>RT (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGPGAPGAPGPK</td>
<td>2.17</td>
</tr>
<tr>
<td>ALDSGITGAR</td>
<td>3.18</td>
</tr>
<tr>
<td>VTSIQDWVQK</td>
<td>5.09</td>
</tr>
<tr>
<td>NFPSPVDAAFR</td>
<td>7.15</td>
</tr>
<tr>
<td>ASSIIDELFQDR</td>
<td>11.91</td>
</tr>
<tr>
<td>EIPAWVPFDPAQITK</td>
<td>13.06</td>
</tr>
<tr>
<td>VAGLESGLDFPNLTVIR</td>
<td>17.32</td>
</tr>
<tr>
<td>VPTADLEDVLPLAEDITNILSK</td>
<td>19.61</td>
</tr>
</tbody>
</table>
Endogenous/Synthetic Spike

Bar charts of ratio for endogenous/synthetic peptides.

Endogenous/Synthetic ratio for 5 QC peptides across 42 analytical runs. CV = 11.3%
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.
Clinical Utility: What will it take?

‘End user’ driven question/ clinical need

Designed to be fit for purpose

Discovery experiment(s) match clinical question

Well planned validation strategy ....

ANALYTICAL PLATFORM

Incorporation of appropriate statistical methods

Then, science ends ... product development begins

€/£/$
Acknowledgements

Prostate Cancer Research Consortium

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

Movember Serum GAP Team
Stella Ademowo, Bill Watson
Jian Chen, Trevor Clancy, Moyez Dharsee, Ken Evans,
Lorelei Mucci, Kristen Tasken
Brian Flatley

Ben Collins
Yue Fan
Brian Morrissey
Rosanna Inzitari
Lisa Staunton
Claire Tonry
Belinda Hernandez
Andrew Parnell
Cathy Rooney
Giuliano Elia
Kieran Wynne
Christine Miller
Input: Information and Ideas

Need

Solution

Scale it
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%.
Making Better Decisions

Multiplexed Protein Blood Test to Improve Prostate Cancer Patient Decisions

Steve Pennington
Founder and CSO
Next Steps: Prostate Cancer

Multiplexed Protein Blood Test
to Improve Prostate Cancer Patient Decisions

Steve Pennington
Founder and CSO