

Metabolomics in cancer, with a focus on breast cancer

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Introduction

- Metabolomics: the study of metabolites as inference of cell processes and biological systems.
- In cancer cells, metabolic pathways are significantly altered, leading to detectable downstream changes in metabolites produced.
- The host immune response can also vary considerably.
- Combined, this offers a picture of both the tumour phenotype and the host's local and systemic response.

Introduction

- A range of potential applications
 - Novel metabolite discovery
 - Early diagnosis
 - Prognostics
 - Measurement of residual disease
 - Predictor or early detector of toxicity

- Currently still in experimental stage

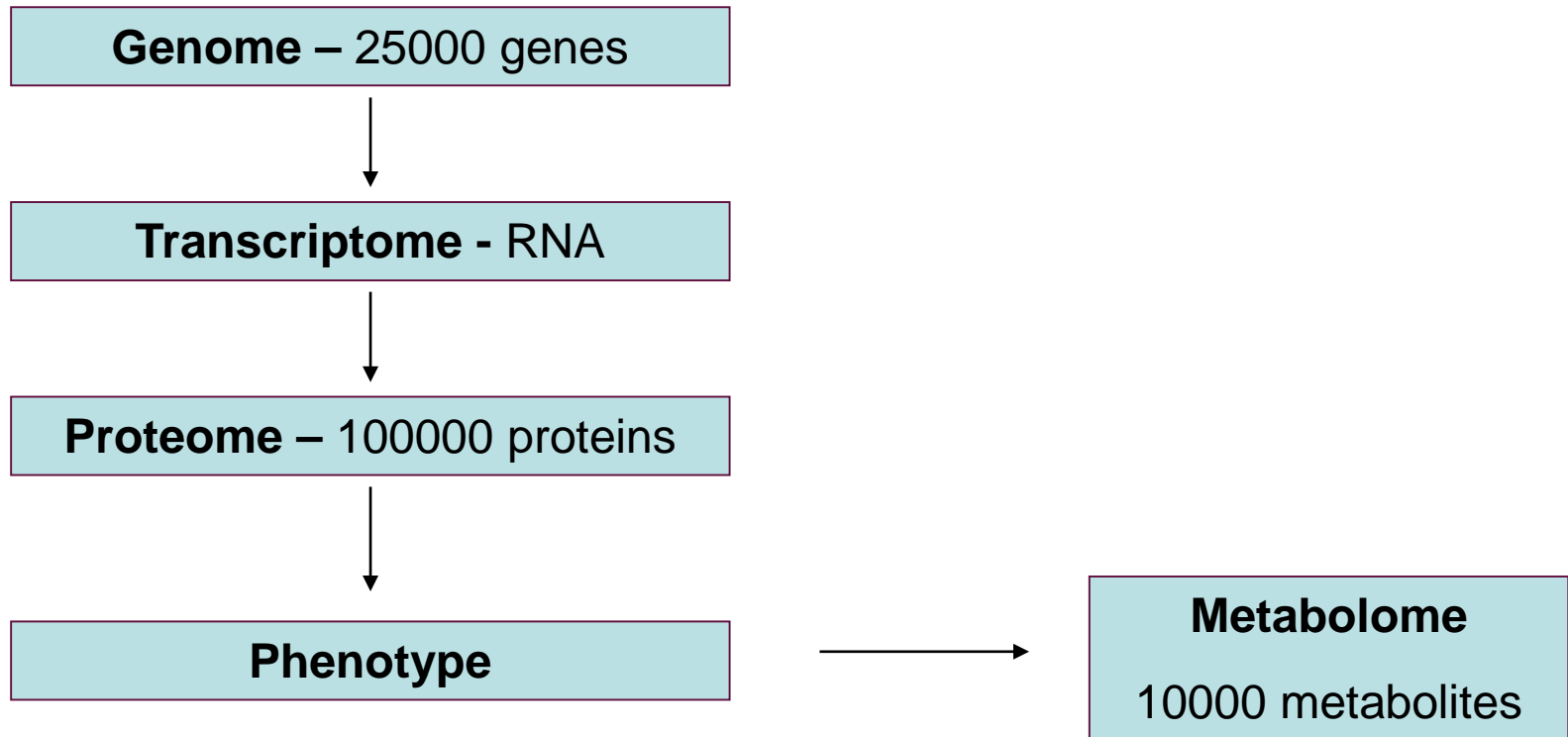
Outline

- Metabolomics as an ‘omics’ science
- Cancer metabolism
- Metabolomic techniques and Data Analysis
- Metabolomics of cancer– uncovering the fingerprint
- Clinical application - patterns and dynamic changes to predict prognosis

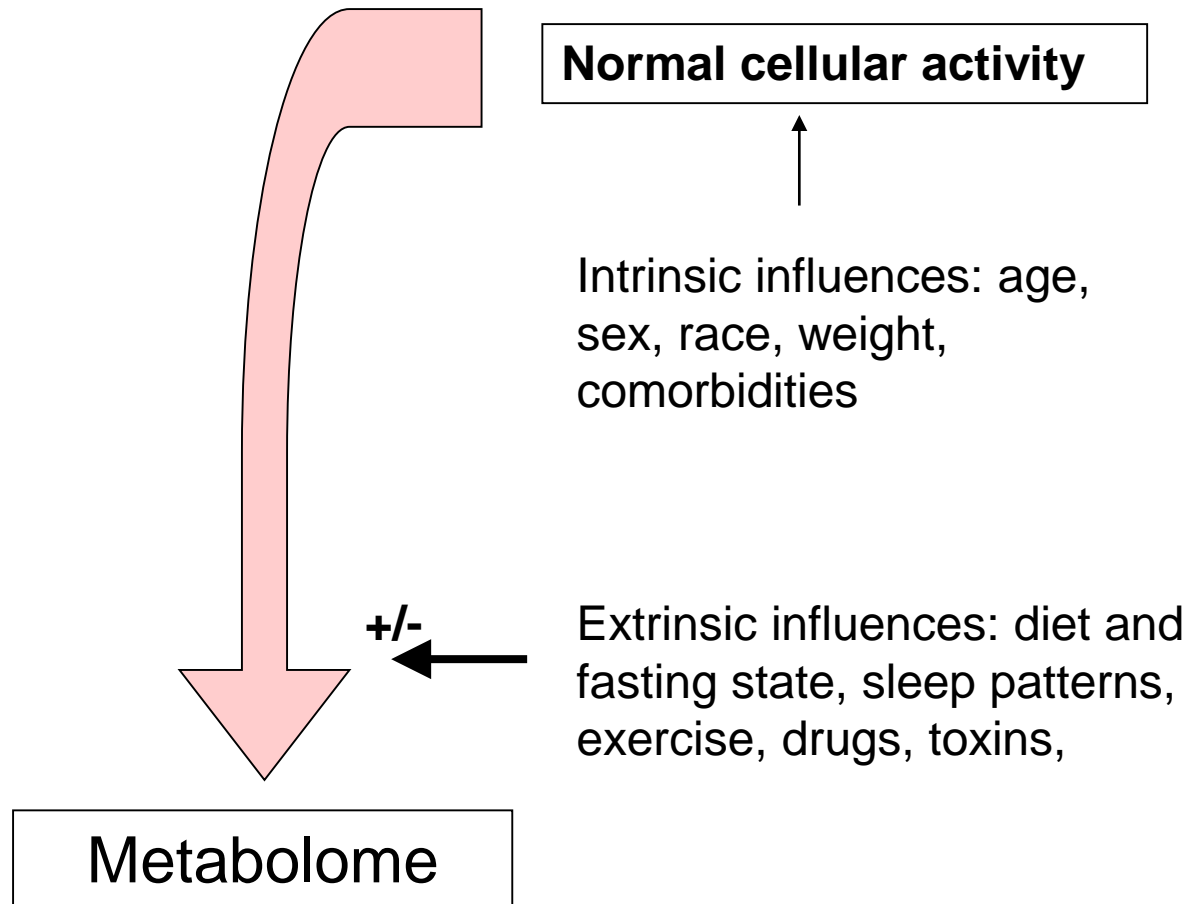
Metabolomics as an omics science

- Several ways to assess the identity of a cell
 - IHC of histopathologic sample – protein expression
 - Genes - sequencing
 - RNA – Gene expression profiling
 - Proteins
 - Output from cells - metabolites

Metabolomics as an omics science



Metabolomics as an omics science



Cancer metabolism

- Cancer cells have significantly altered metabolism
 - Upregulated glycolysis - Warburg effect
 - Increased phospholipids
 - Altered pyruvate and fatty acid synthesis
 - Altered signaling pathways

- Reflected in the pool of metabolites

Cancer metabolism – ‘Reverse Warburg’ effect

- Evidence that the aerobic glycolysis occurs in neighbouring stromal fibroblasts (rather than the cancer cells themselves).
- In this theory
 - cancer cells induce metabolic alteration in stroma, via oxidative stress, to become cancer associated fibroblasts(CAF).
 - Stromal cells undergo phenotypic change, become highly catabolic, and secrete pyruvate, lactate and other ‘fuels’.
 - These nutrients are taken up by the malignant cells.
- Thus the microenvironment is harnessed by the cancer as an energy source.
 - May explain cachexia of cancer

Agents that affect cancer cell metabolism

- Older
 - Methotrexate – anti folate, dihydrofolate reductase inhibitor
 - 5-Fluorouracil – thymidylate synthase (TS) inhibitor

- Newer
 - Capecitabine – 5-FU prodrug
 - Pemetrexed – TS, DHFR inhibitor
 - Bortezomib – proteasome inhibitor

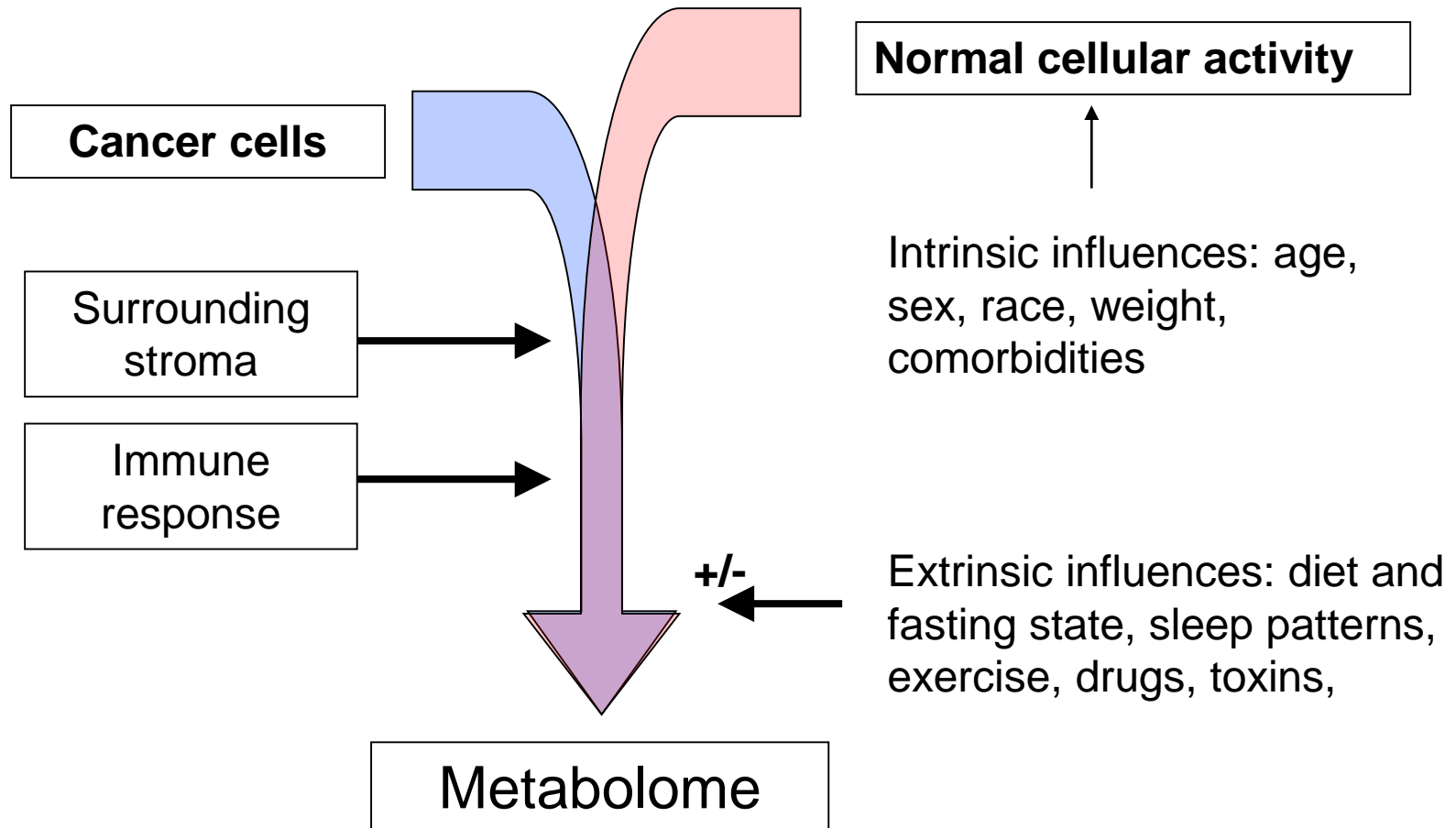
- Potential others – metformin?

Other metabolic changes

- Tumour cells *in vivo* do not act in isolation
 - Influenced by the host response
 - surrounding stroma
 - immune system
 - competing cancer subclonal populations
 - and the same external influences

- All represented in the metabolome

Metabolomics in cancer



Techniques

■ Biosamples

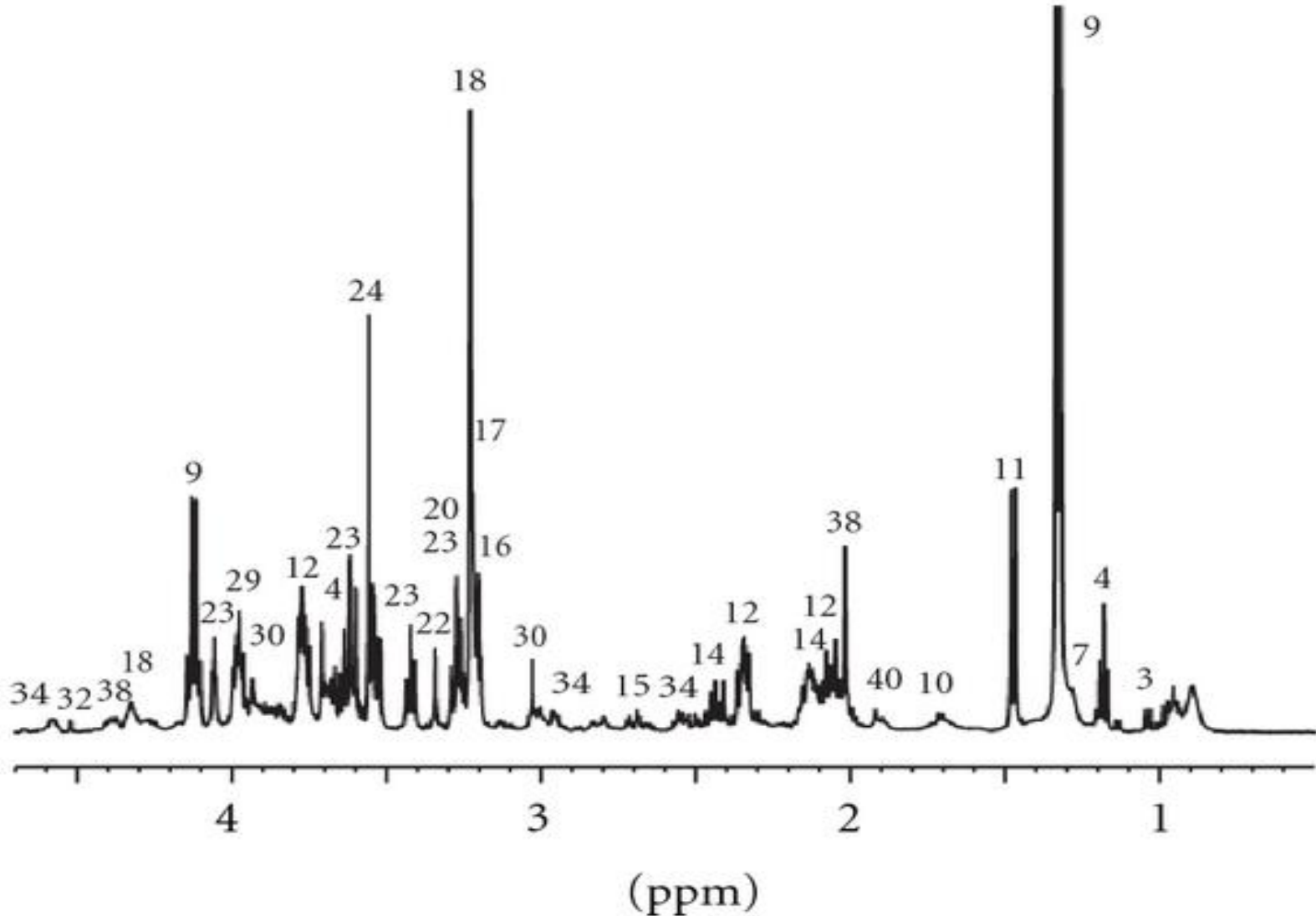
- tumour tissue
- serum, urine
- sweat, saliva, breath, GI secretions, ascites

Techniques

- Mass spectrometry (MS)
 - High sensitivity, identifies unknown metabolites
 - Destroys the tissue/sample

- Proton - Nuclear Magnetic Resonance spectroscopy (H-NMR)
 - Fast, less expensive, higher reproducibility, preserves the sample
 - Less sensitive, unable to identify unknown metabolites, limited availability

Example of 1-D H-NMR spectrum



Analysis

- Supervised
 - Searching for a prespecified panel of metabolites
 - Potential for bias
- Unsupervised
 - Exploratory analysis, open to new discovery
- Huge amounts of data
- Multitude of metabolites
- Superimposed on the sea of normal metabolites from normal processes

Analysis

- A panel or group of metabolites in isolation can make up a tumour metabolic profile.
- The pattern of metabolites represents the metabolomic signature or fingerprint for that sample.
- Recognising meaningful differences between complex spectra, and controlling for the many variables is a significant challenge.
- Complex chemometric techniques and statistical analysis required to detect patterns and allow comparison.

Why bother?

- Preclinical
 - cell lines: pathways and targets
- Clinical: Biomarkers
 - We need to get a better picture of the presence and extent of cancer, what it is doing, and what it might do
- Biopsies are limited: invasive *etc.*
- Search for easier sampling, which can offer a broader picture
 - Circ free DNA, CTCs, metabolomics

Why bother?

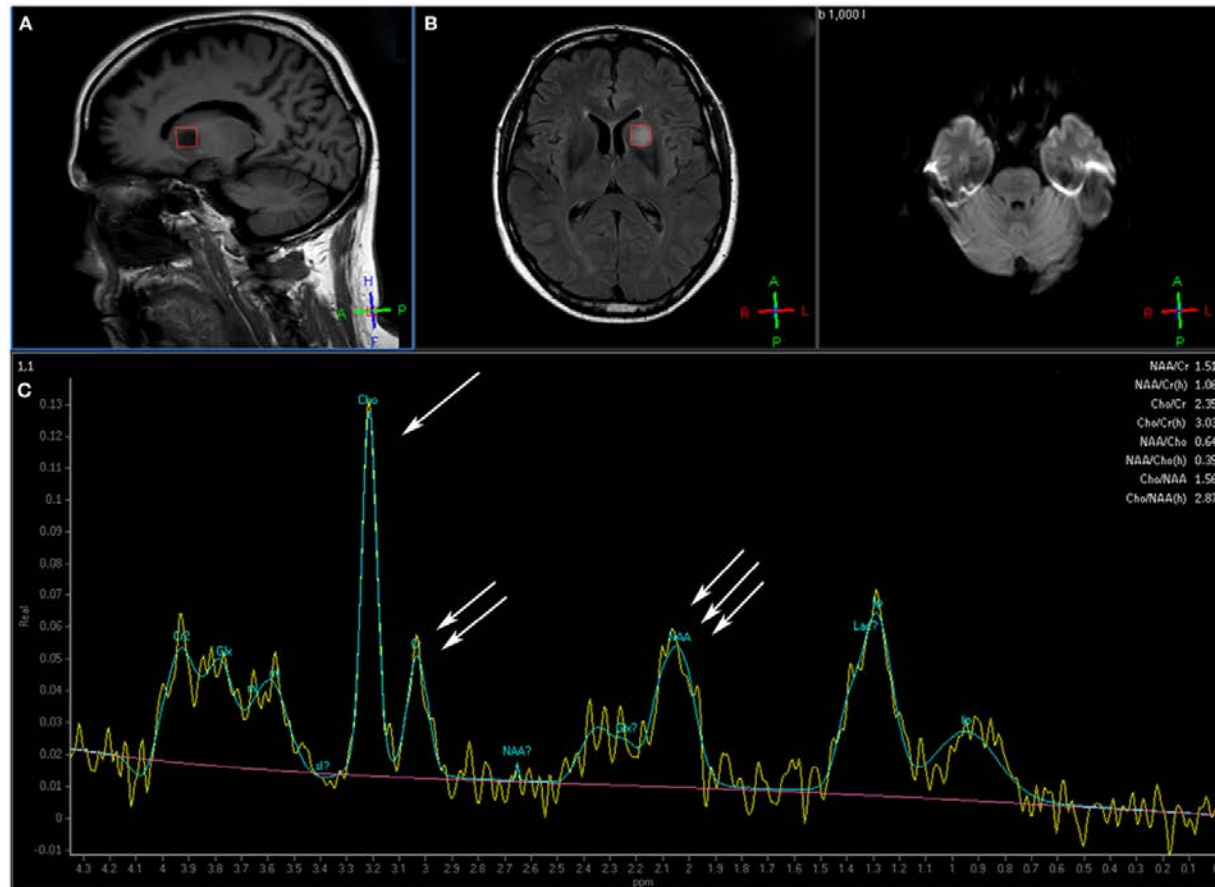
- Theoretical advantages
 - Simple to obtain sample
 - Direct evidence of altered processes, cf evidence of potential processes
 - Includes information from tumour and host
 - Eg. why do some clear CTCs
 - May be predictive for behaviour

Search for biomarkers

- In its simplest form, allows detection of a range of metabolites. Where these differ from normal tissue, a potential biomarker exist.
- Many metabolites correlate with cancer presence – few are in clinical practice as tumour markers.
- Examples
 - Prostate ca – urine
 - Gastric and colon ca – early diagnosis
 - GBM – classifying aggressive phenotype, searching for novel targets

Clinical examples - imaging

- FDG-PET
- MRI with spectroscopy



Tissue samples

- Analyses have identified numerous metabolites that are altered
 - vary between tumour types
 - but also inconsistent within a single tumour type
- Clinical correlation
 - NMR on FNA of breast cancer could predict grade, receptor status, LVI and lymph node status
 - Prospective trial needed to assess any clinical benefit
- Theoretical benefit of metabolomics based on serum is the assessment of both tumour and host, which is missed in tissue samples

Uses for metabolomics

- Discovery: Identification of biomarkers
- Prognostics
- Predictors
- Monitoring response
- Surveillance or screening

- Target analysis vs. metabolite profiling

Examples

- In geriatric oncology, metabolomic profiles have differentiated fit, unfit and frail elderly patients with EGFR mutant lung cancer
- Breast cancer
 - Prediction of toxicity
 - Early diagnosis
 - Prognostication: prediction of relapse

Detecting relapse

- Asiago *et al.* analysed serial blood samples of women in surveillance following resection of EBC to test if met profiles could be used to predict relapse earlier than conventional methods.
- 257 samples from 56 breast cancer patients
 - 20 patients relapsed (116 samples)
 - 36 no evidence relapse min 2 years (141 samples)
- Combined NMR and MS to detect a panel relevant metabolites that discriminated relapsed and non relapsed patients.
- Developed a prediction model
 - Predicted relapse over 13 months earlier than clinical methods in 55% of relapsing patients
 - Outperformed other serum biomarker CA 27.29 significantly

Prognostication

- In the adjuvant treatment of early breast cancer, many women are treated unnecessarily.
- CMF vs. placebo trial (Bonnodonna)
 - 22% long term survival with no adjuvant therapy
- Standard clinicopathologic risk factors insufficient
- Even with sophisticated GEP, a significant proportion of 'high risk' patients may not need chemotherapy.
 - Paik *et al.* OncotypeDX
- A better biomarker is required

Metabolomic biomarker?

- Residual micrometastatic disease after surgery is cause of relapse, reason for adjuvant therapy.
- Breast cancer cells have altered metabolism, and may also affect local tissue metabolism.
- This remaining population may therefore leave a metabolic signature in the serum.
- Such a signature might offer a stronger prediction of relapse, and need for adjuvant therapy.

Does a signature exist?

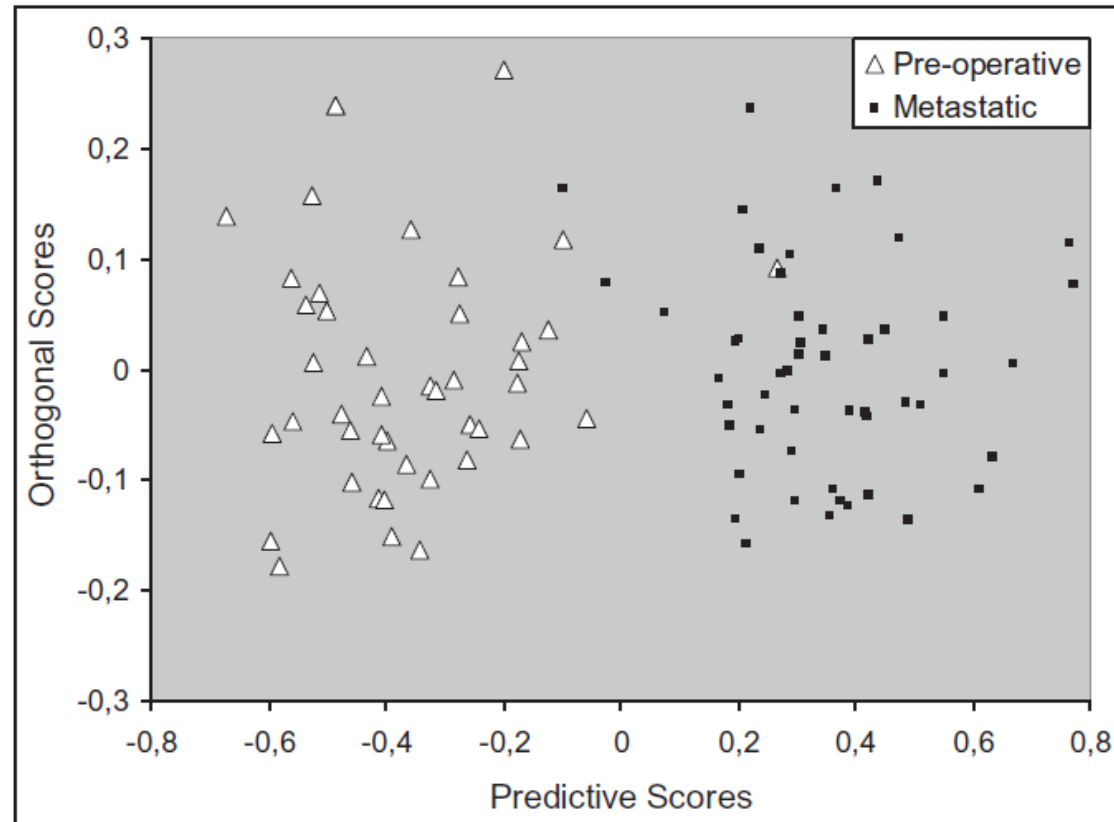
- Several trials have shown that serum metabolomic profiles can differentiate metastatic from early breast cancer.
- These trials use defined groups of early and metastatic patients to examine differences in metabolomic signatures and develop a prediction model.
- Tested on a second set of patients to validate model and assess prediction accuracy.
- Discussion of these trials will illustrate how translational studies in metabolomics may be carried out.

Revealing a fingerprint - Oakman *et al.*

- Aim
 - Detect a difference in metabolic profiles between local and advanced breast cancer
 - Use the profiles to develop a risk score
 - See if this changed with resection
- 44 early breast cancer patients
 - Serum collected pre and postoperatively
- 51 metastatic patient serum samples
- Further 45 patients with post operative sample to use as a validation series

Revealing a fingerprint

- Generate spectra
 - Different sequences
- Analyse spectra and employ data reduction techniques to allow simple comparison
- Orthogonal Projection to Latent Structure (OPLS)
 - Converts each complex spectrum to a single point on a 2D scatter plot
 - near complete separation of the early and metastatic groups



Revealing a fingerprint

- A clear metastatic metabolic fingerprint
- Can be used to classify the patients – predict early or late
 - Support vector machines method
 - Prediction ability, assessed with double cross validation, showed that most patients were appropriately assigned, with a discrimination sensitivity 75%, specificity 69% and predictive accuracy 72%

Metabolomic risk

- Development of a metabolomic risk score for early patients
 - Reduce fingerprint further still to a single number
 - Related to the inverse distance from the metastatic cluster
- High metabolomic risk score in the correlated strongly with misclassification as metastatic
- Analysis repeated on the post op set
 - 86% of high preop met risk moved to low met risk with tumour removal
- Results confirmed with validation set

Study features

- A metastatic fingerprint was identified, which was discriminatory
- Fewer post op patients were classified as high risk than by standard clinicopathological characteristics
 - Gene expression profiling also classifies more patients as low risk
- True value could only be conclusively assessed with longitudinal clinical follow up over 10 years.
- Clinical data required, and further validation in other groups

Metastatic fingerprint

- Jobard *et al.* compared preoperative sera from women with local disease in situ to sera from women with metastatic disease.
- Training set: 46 preoperative samples and 39 metastatic samples
- Developed a prediction model
- Tested in the validation set: 61 early and 51 metastatic
- Higher discriminating power
- But, no post op samples

Applying to known clinical outcome

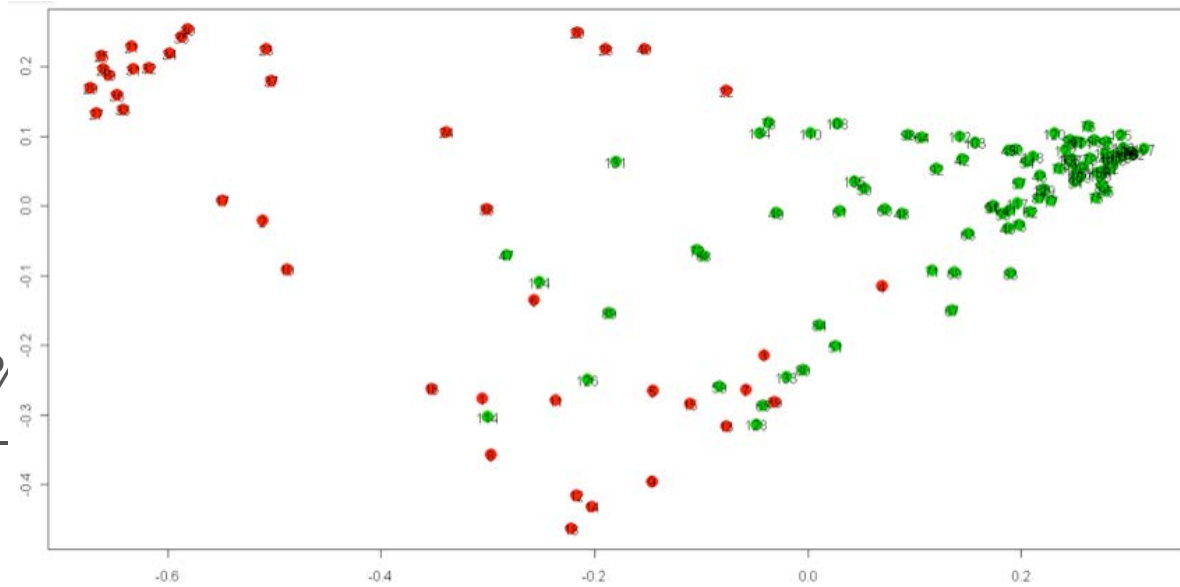
- Comparing to an established risk profile is inherently limiting
 - Imperfect comparator
 - Convenient
 - Proof of concept

- Development of a prognostic risk calculator repeated on post op blood samples of women with 5 years clinical follow up.

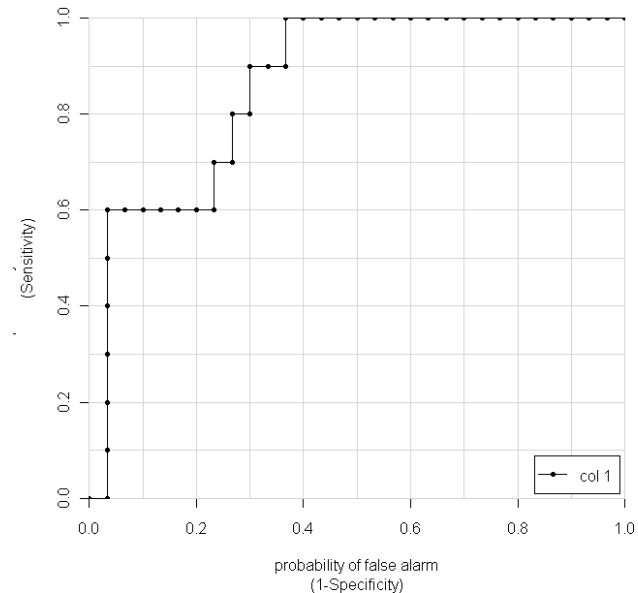
Tenori *et al.*

- Biobank from MSKCC
- 90 metastatic patients
 - Generate the metastatic fingerprint
- 80 early patients
 - Post op serum
 - 5 years clinical follow-up data
 - Training set (40 patients – 10 with relapse)
 - Validation set (40 patients – 11 with relapse)

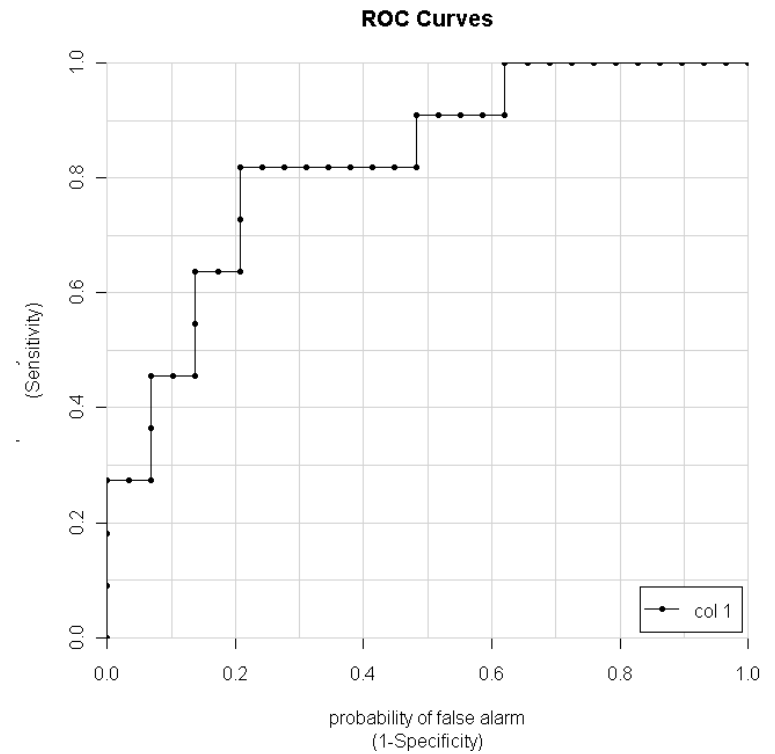
- Spectra obtained (CPMG shown)
- Random Forest classification
 - High accuracy 84-87%
- Risk score generated – risk of relapse
- Scores compared to known clinical outcomes in training set – ROC analysis



ROC Curves



- Cut off for risk score chosen at ≥ 53
 - Sensitivity 90%
 - Specificity 67%
 - Accuracy 73%
- Then applied to the spectra of the validation set, blind to clinical outcome
 - AUC 0.824
 - Sensitivity 82%
 - Specificity 72%
 - Predictive accuracy 75%



Trial limitations

- Small numbers
- Only ER negative disease included in the relapse-free cohort
 - ER status could not be determined from spectra
 - This is in contrast to other studies
 - Repeating the study with only ER- pts gave similar results
- Variable time from surgery to blood sample
- Chemotherapy in 79%
 - Potential confounder, esp Her2+ disease.

Metabolites

Study	Higher in metastatic BC	Lower in MBC
Oakman	Phenylalanine, glucose, proline, lysine, N-acetyl cysteine	Lipids
Jobard	Phenylalanine, glutamate, N-acetyl cysteine, mannose, pyruvate, glycerol, acetoacetate, lipids (NS)	Histidine, alanine (NS), betaine(NS)
Tenori	Glucose, lactate, tyrosine, lipids	Histidine
Asiago	Tyrosine (NS), lactate (NS)	Histidine formate proline choline, N-acetyl glycine, ketone body

Oakman C, *et al.* Ann Oncol. 2011 Jun;22(6):1295-301.

Jobard E, *et al.* Cancer Lett. 2014 Feb 1;343(1):33-41

Tenori L, *et al.* Mol Oncol. 2014 Aug 10. pii: S1574-7891(14)00167-7

Asiago VM, *et al.* Cancer Res. 2010 Nov 1;70(21):8309-18..

NS = non significant

Concerns

- A complex algorithm is required to convert spectra into an easily comparable form.
- It is unknown if it is picking up the optimal set of characteristics, ie capturing the true signature of the cancer.
- Training sets required for each new cohort.
- Applicability and generalisability remain a long way off.

Metabolomics - challenges

- Many things keep metabolomics in the experimental phase.
- Difficulties in sample handling.
- Intra- and inter-patient variability.
- No standard reference that can take all background variables into account – hence need for a test set at every location and for every population. May never be achieved.
- Complicated machinery with sophisticated technique required. Unlikely to move into standard hospital or commercial labs.
- Difficult to prove superiority over current risk assessment without long prospective randomised trial, which is unfeasible.

Where does its role lie

- For now, clinical application remains in the proof-of-concept stage.
- Scientific exploration continues to improve understanding of tumour biology.
- Should identification of a reasonable panel of individual metabolites be achieved that can add prognostic or predictive value, then this could be incorporated alongside GEP.
- It may prove complimentary to other liquid biopsy techniques – CTCs, CTDNA.

Final note

While this is an interesting area of research and shows promise for the future, there are currently no approved treatment methodologies based on metabolomics.

Thank you!

References

Nguyen ML, Willows B, Khan R, et al. The potential role of magnetic resonance spectroscopy in image-guided radiotherapy. *Front. Oncol.* 4:91. doi: 10.3389/fonc.2014.00091. Permission for reproduction available under the Creative Commons Attribution License 3.0 (<http://creativecommons.org/licenses/by/3.0/>)

D. Ben Sellem, K. Elbayed, A. Neuville, et al., "Metabolomic Characterization of Ovarian Epithelial Carcinomas by HRMAS-NMR Spectroscopy," *Journal of Oncology*, vol. 2011, Article ID 174019, 9 pages, 2011. doi:10.1155/2011/174019. Permission for reproduction available under the Creative Commons Attribution License 3.0 (<http://creativecommons.org/licenses/by/3.0/>)

Asiago VM, Alvarado LZ, Shanaiah N, et al. Early detection of recurrent breast cancer using metabolite profiling. *Cancer Res.* 2010 Nov 1;70(21):8309-18. doi: 10.1158/0008-5472.CAN-10-1319. Epub 2010 Oct 19.

Oakman C, Tenori L, Claudino WM, et al. Identification of a serum-detectable metabolomic fingerprint potentially correlated with the presence of micrometastatic disease in early breast cancer patients at varying risks of disease relapse by traditional prognostic methods. *Ann Oncol.* 2011 Jun;22(6):1295-301. doi: 10.1093/annonc/mdq606. Epub 2011 Jan 3. Permission for reproduction of figure granted from Oxford University Press, licence no 3515310045157

Jobard E, Pontoizeau C, Blaise BJ et al. A serum nuclear magnetic resonance-based metabolomic signature of advanced metastatic human breast cancer. *Cancer Lett.* 2014 Feb 1;343(1):33-41. doi: 10.1016/j.canlet.2013.09.011. Epub 2013 Sep 14.

Tenori L, Oakman C, Morris PG et al. Serum metabolomic profiles evaluated after surgery may identify patients with oestrogen receptor negative early breast cancer at increased risk of disease recurrence. Results from a retrospective study. *Mol Oncol.* 2014 Aug 10. pii: S1574-7891(14)00167-7. doi: 10.1016/j.molonc.2014.07.012. [Epub ahead of print]

Lisanti MP, Martinez-Outschoorn UE, Sotgia F Oncogenes induce the cancer-associated fibroblast phenotype: metabolic symbiosis and "fibroblast addiction" are new therapeutic targets for drug discovery. *Cell Cycle.* 2013 Sep 1;12(17):2723-32. doi: 10.4161/cc.25695. Epub 2013 Jul 30.

Martinez-Outschoorn U, Sotgia F, Lisanti MP Tumor microenvironment and metabolic synergy in breast cancers: critical importance of mitochondrial fuels and function. *Semin Oncol.* 2014 Apr;41(2):195-216. doi: 10.1053/j.seminoncol.2014.03.002. Epub 2014 Mar 5.

Martins D, Beça FF, Sousa B et al. Loss of caveolin-1 and gain of MCT4 expression in the tumor stroma: key events in the progression from an in situ to an invasive breast carcinoma.