University of South Australia | Future Industries Institute

Proteomics and Mass Spectrometry Imaging on gynaecological cancer tissue

Prof Peter Hoffmann

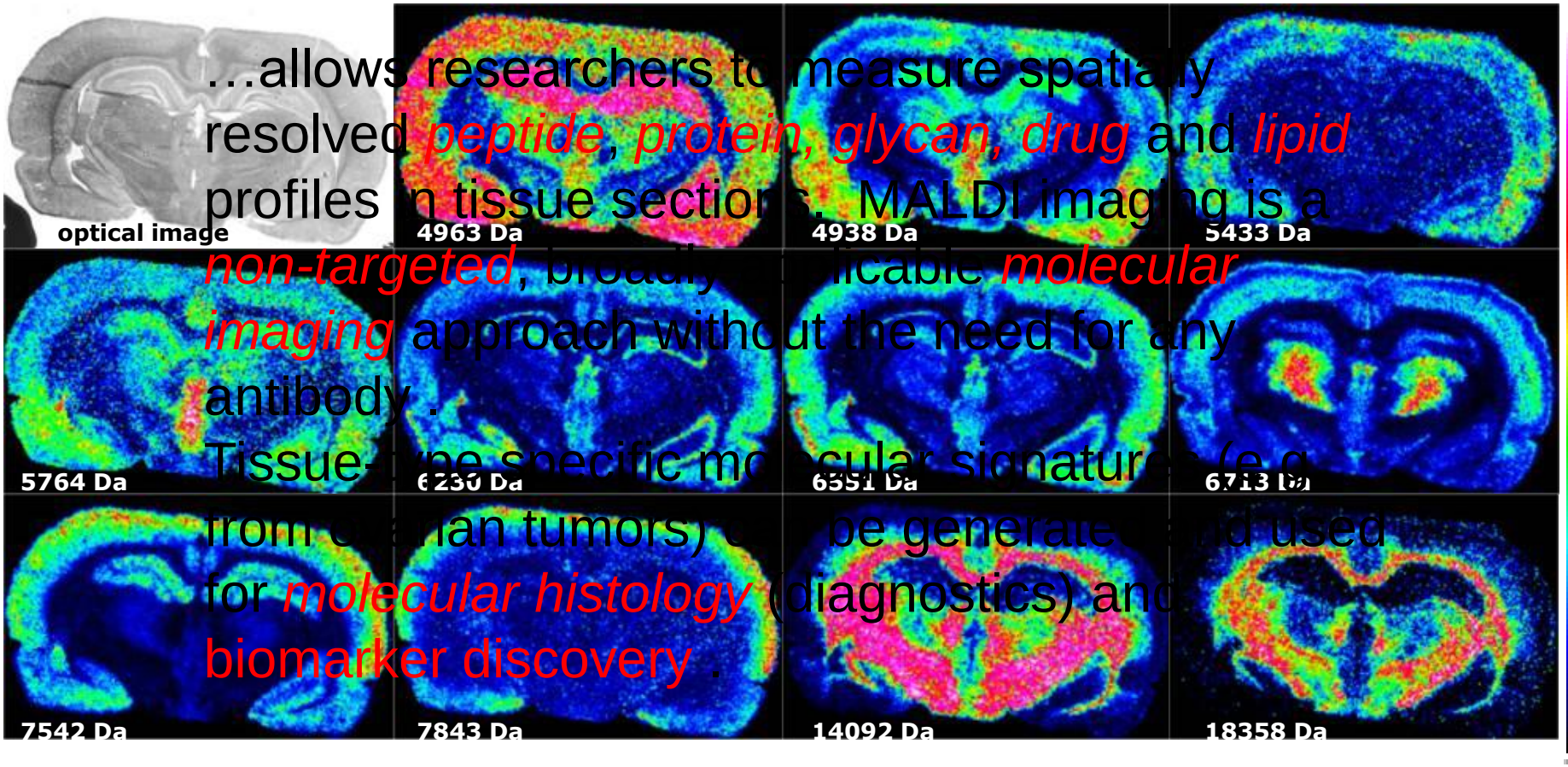
ANZGOG 20th of March 2019

Introduction

- Mass Spectrometry Imaging analysis of Endometrial cancer FFPE tissue to distinguish from the primary tumour if the patient will have lymph node metastasis

- N-Glycan MALDI Imaging Mass Spectrometry on FFPE Ovarian Cancer Tissue changes between stage 1 and stage 3

MALDI Imaging …



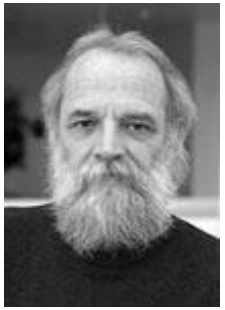
…allows researchers to measure spatially resolved *peptide*, *protein*, *glycan*, *drug* and *lipid* profiles in tissue sections. MALDI imaging is a *non-targeted*, broadly applicable *molecular imaging* approach without the need for any antibody.

Tissue-type specific molecular signatures (e.g. from human tumors) can be generated and used for *molecular histology* (diagnostics) and *biomarker discovery*.

MALDI-ToF Mass spectrometry

Koichi Tanaka

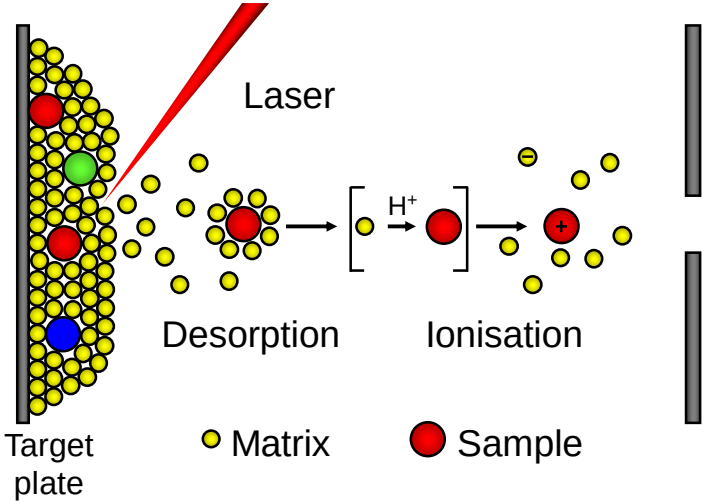
Franz Hillenkamp



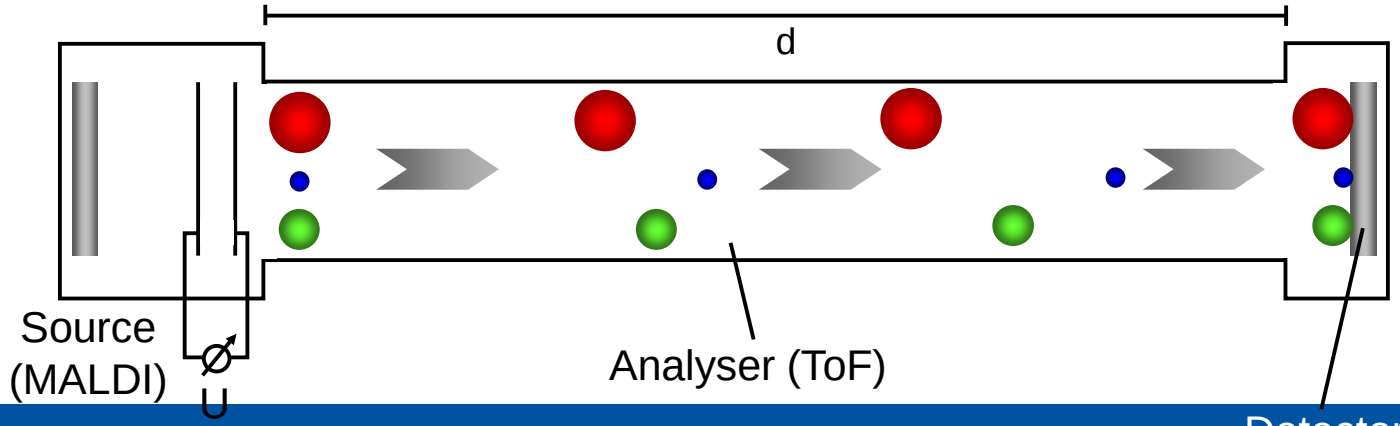
Michael Karas

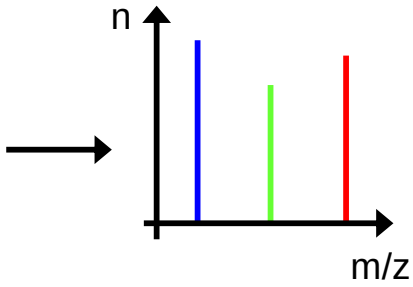
Nobel price in chemistry, 2002

"for the development of methods for identification and structure analyses of *biological macromolecules*"



MALDI-Ionisation

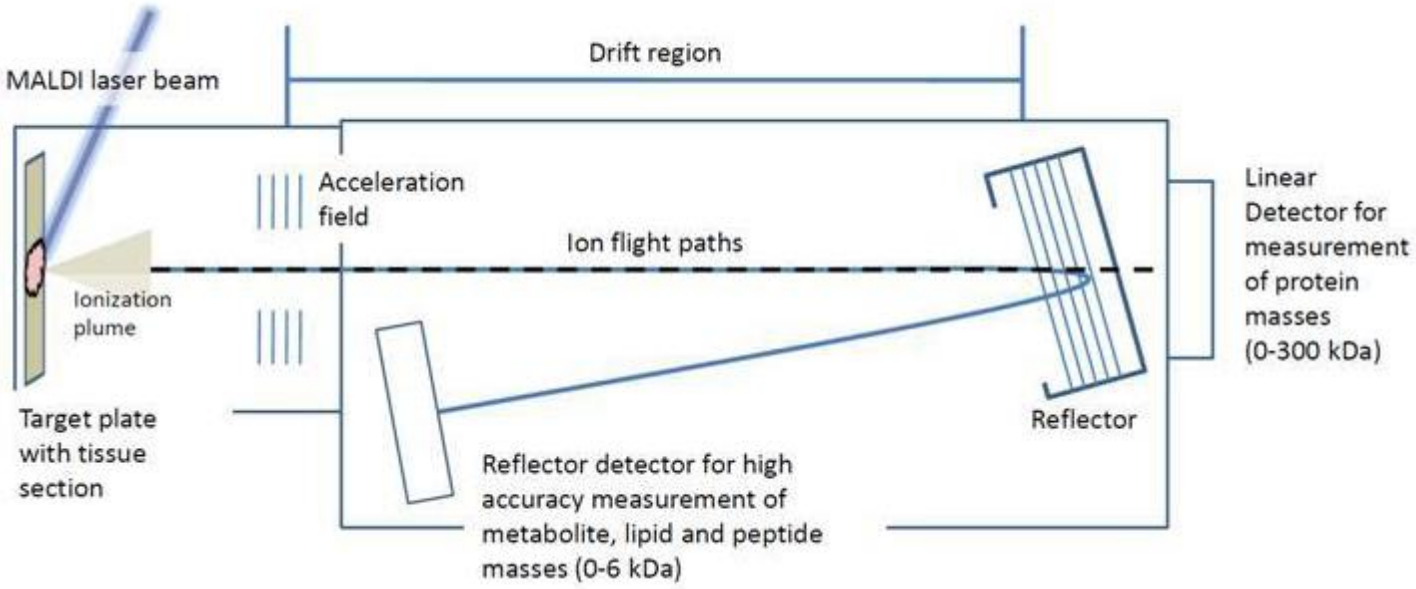




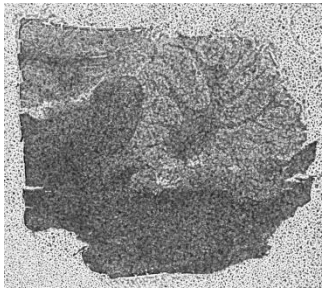
Mass spectrum

$$\frac{m}{z} = t^2\left(\frac{2eU}{d^2}\right)$$

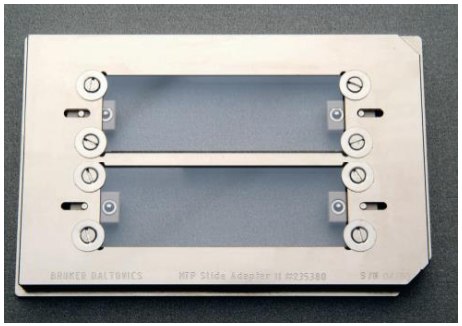
MALDI TOF/TOF MS



MALDI Imaging workflow

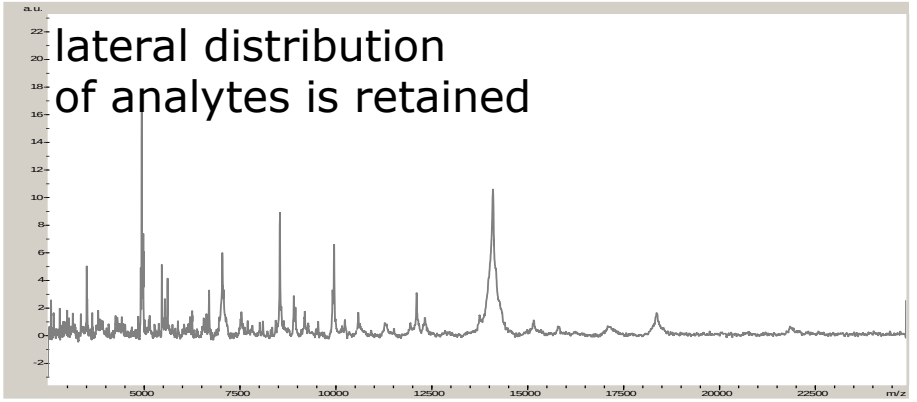


Matrix coated tissue section

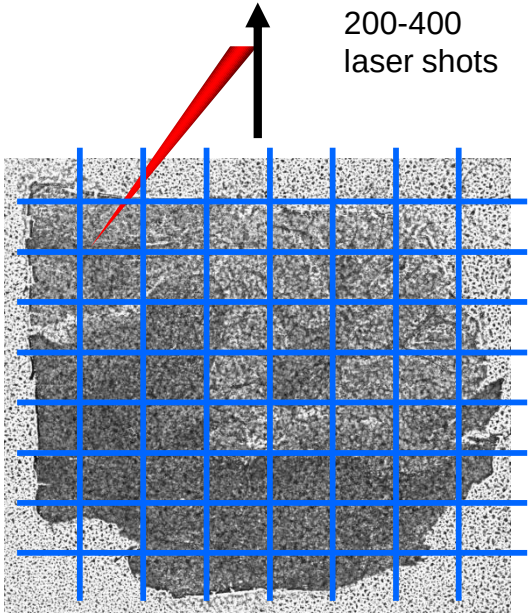


MALDI target adapter for slides

MALDI TOF/TOF MS

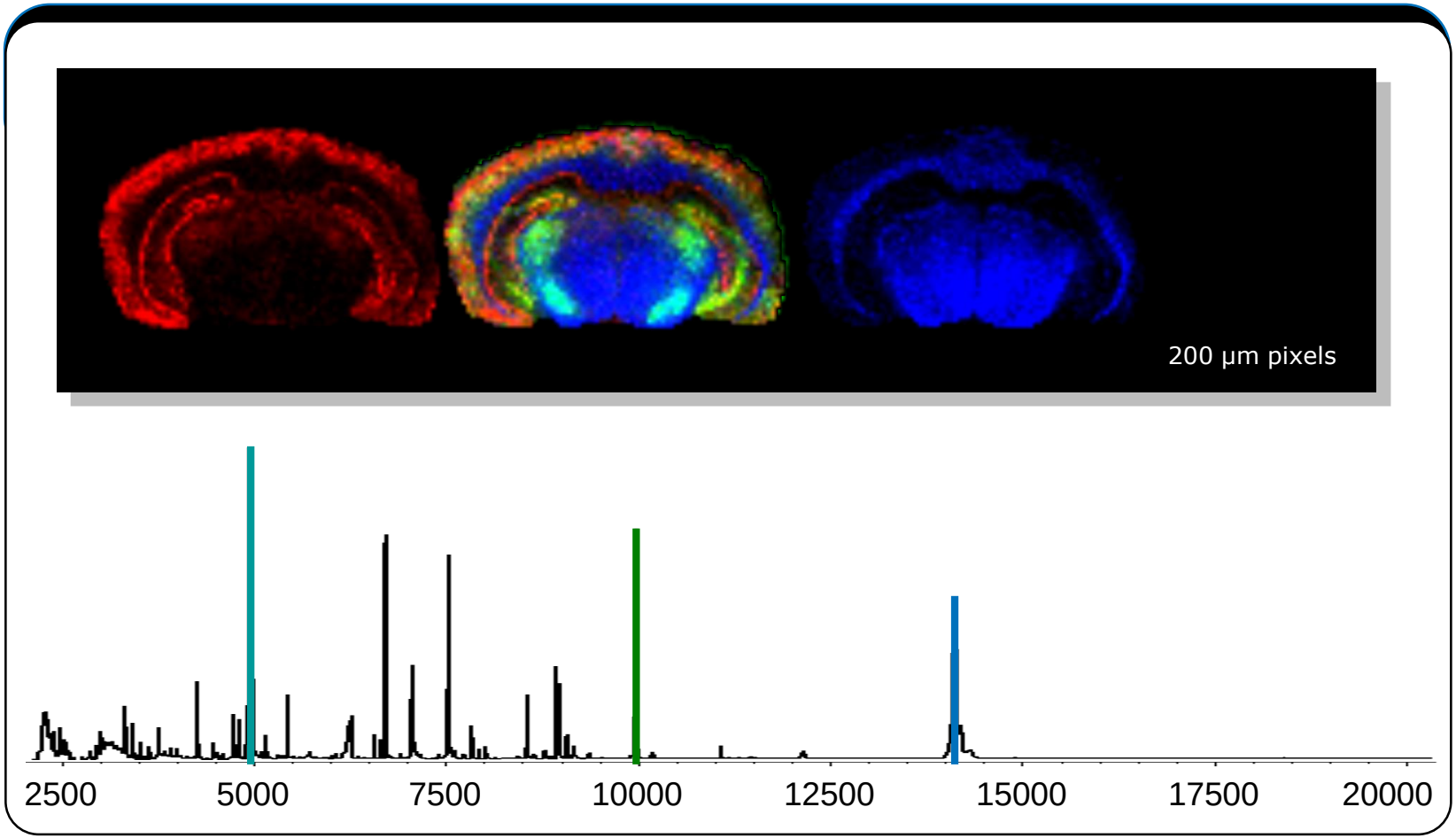


200-400
laser shots

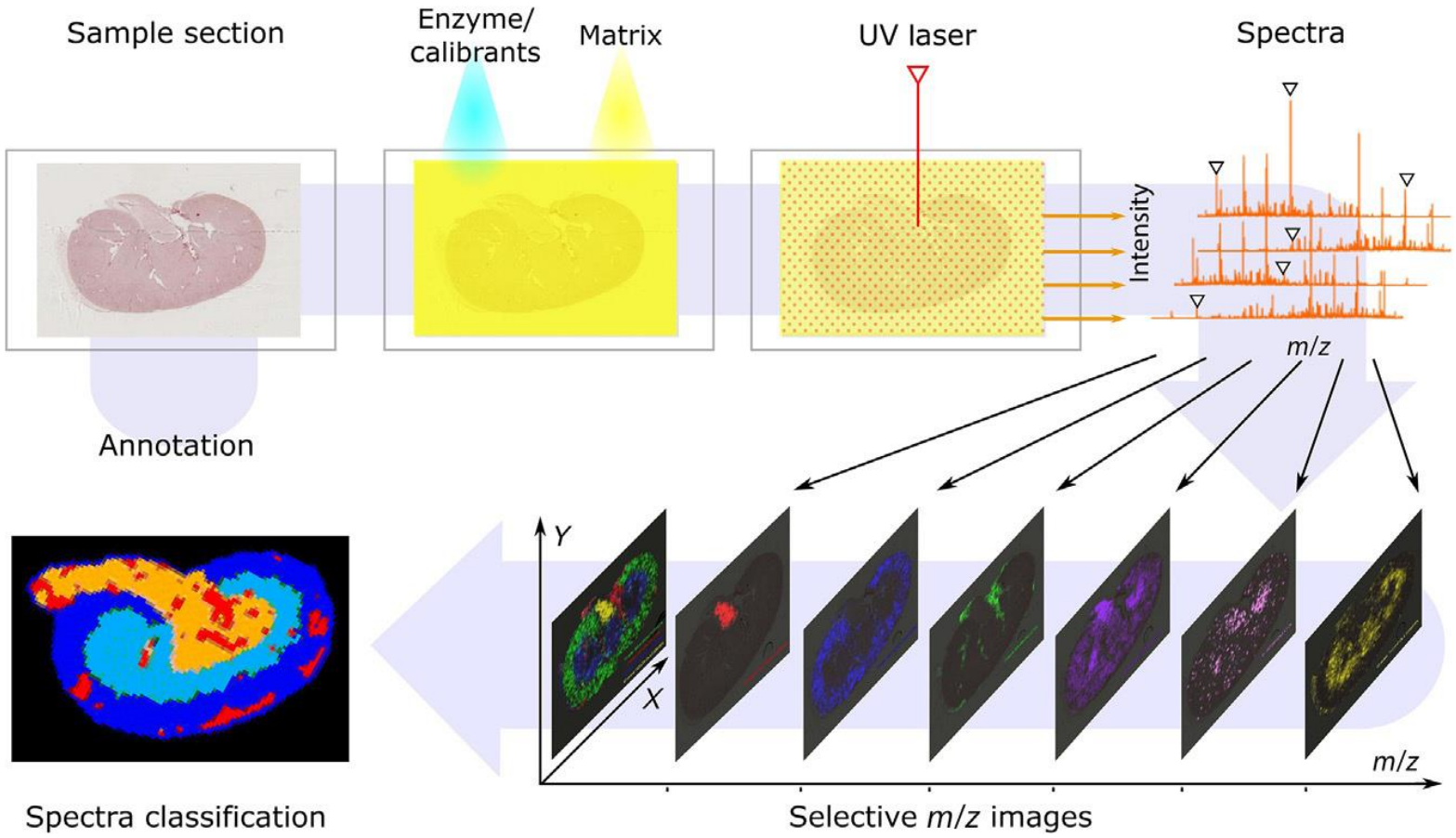


Raster size ~30-300 µm
(=lateral resolution)

MALDI Imaging – easy multiplexing

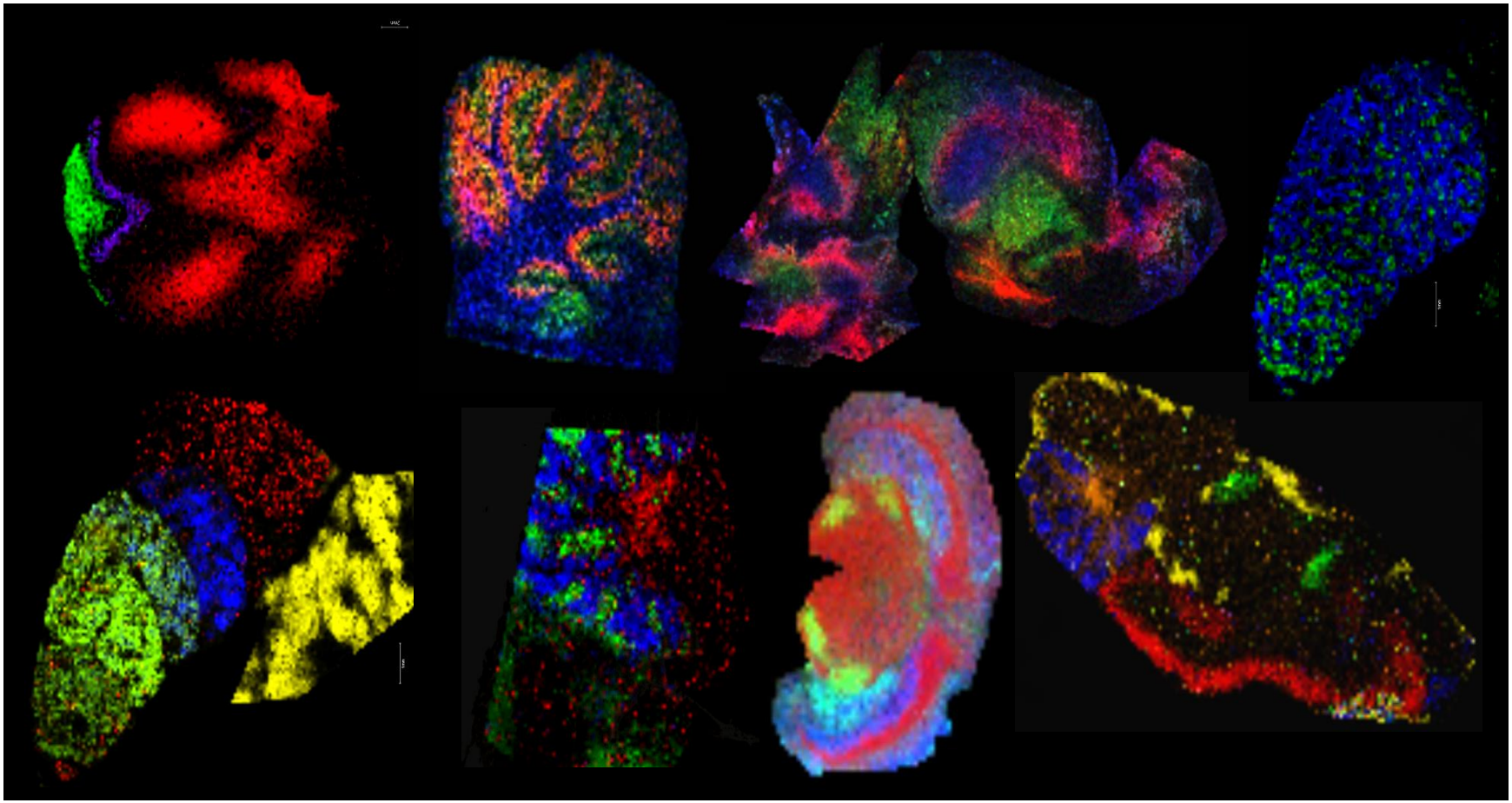


Mass Spectrometry Imaging (MSI)



G. Arentz, P. Mittal, C. Zhang, Y.-Y. Ho, M. Briggs, L. Winderbaum, M.K. Hoffmann, P. Hoffmann: *Applications of Mass Spectrometry Imaging to Cancer*. 01/2017; , DOI:10.1016/bs.acr.2016.11.002

Molecular Histology by MALDI Imaging MS



Visual Microscopy by MALDI Imaging MS

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“Mass spectrometry Imaging analysis of Endometrial cancer”

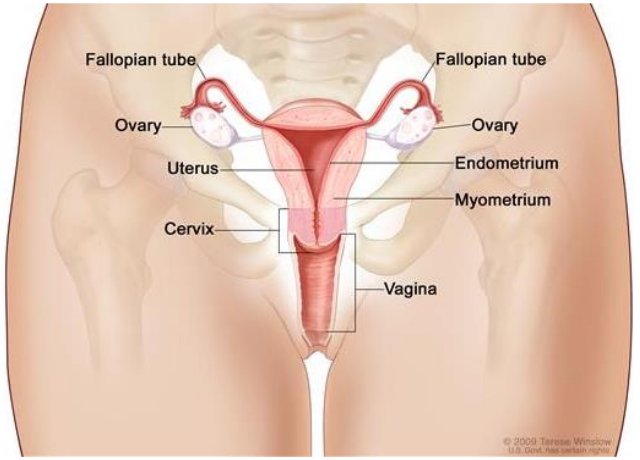
Parul Mittal

Peter Hoffmann, Manuela Klingler-Hoffmann, Martin K. Oehler

Clinical relevance

- Endometrial cancer (EC) is the most frequent malignant tumour occurring in the female reproductive system
- Affects approximately every 1/75 Australian women by the age of 75
- 5 year survival rate

Stage I	~ 85%	No Metastasis
Stage II	~ 75%	
Stage III	~ 45%	Metastasis
Stage IV	~ 25%	

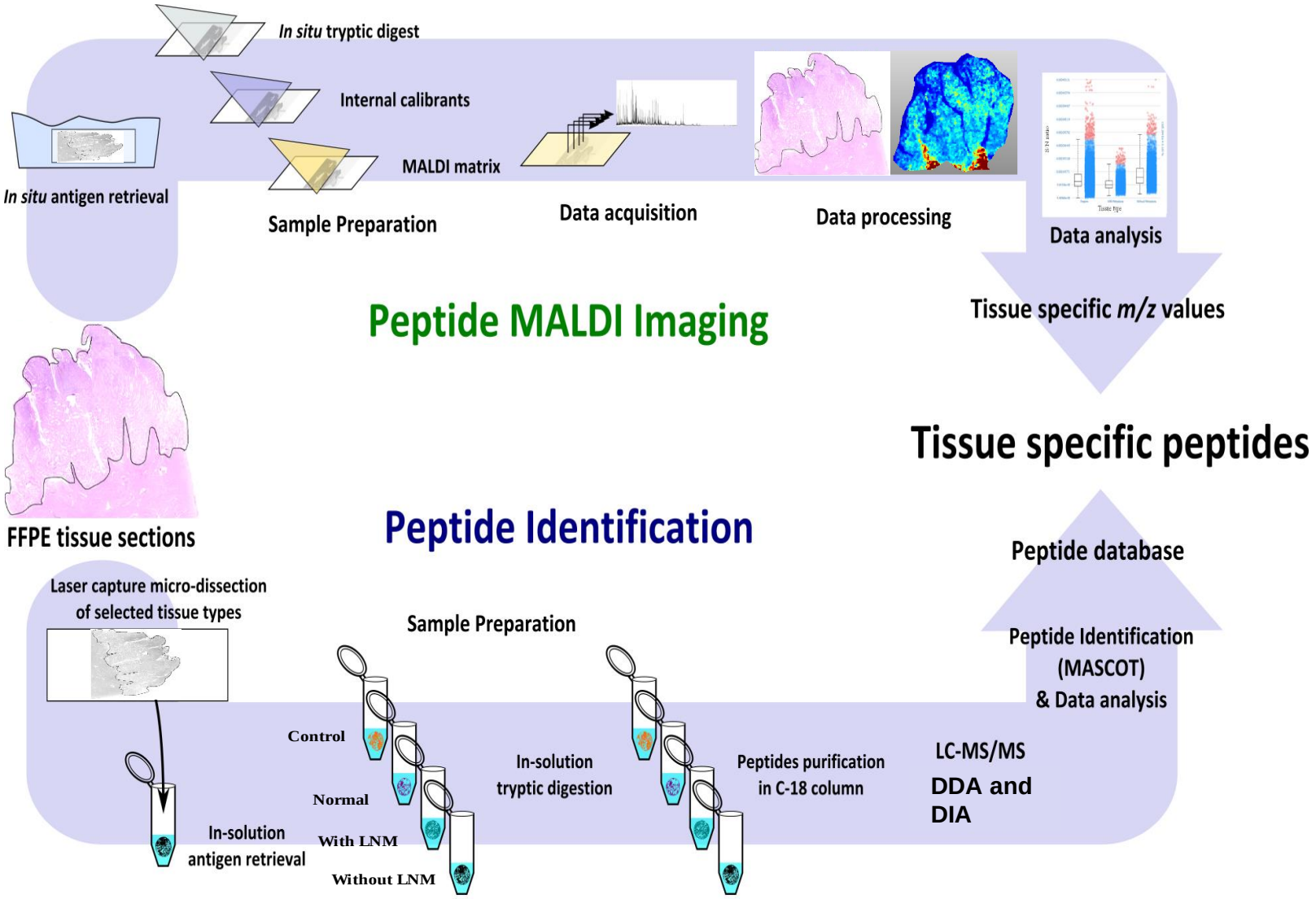


- Most patients with endometrial cancer routinely undergo a lymph node dissection as part of the cancer operation and postoperative lower extremity (only 10% will have lymph node metastasis)
- Could Lymphadenectomy be safely omitted for low risk EC patients?

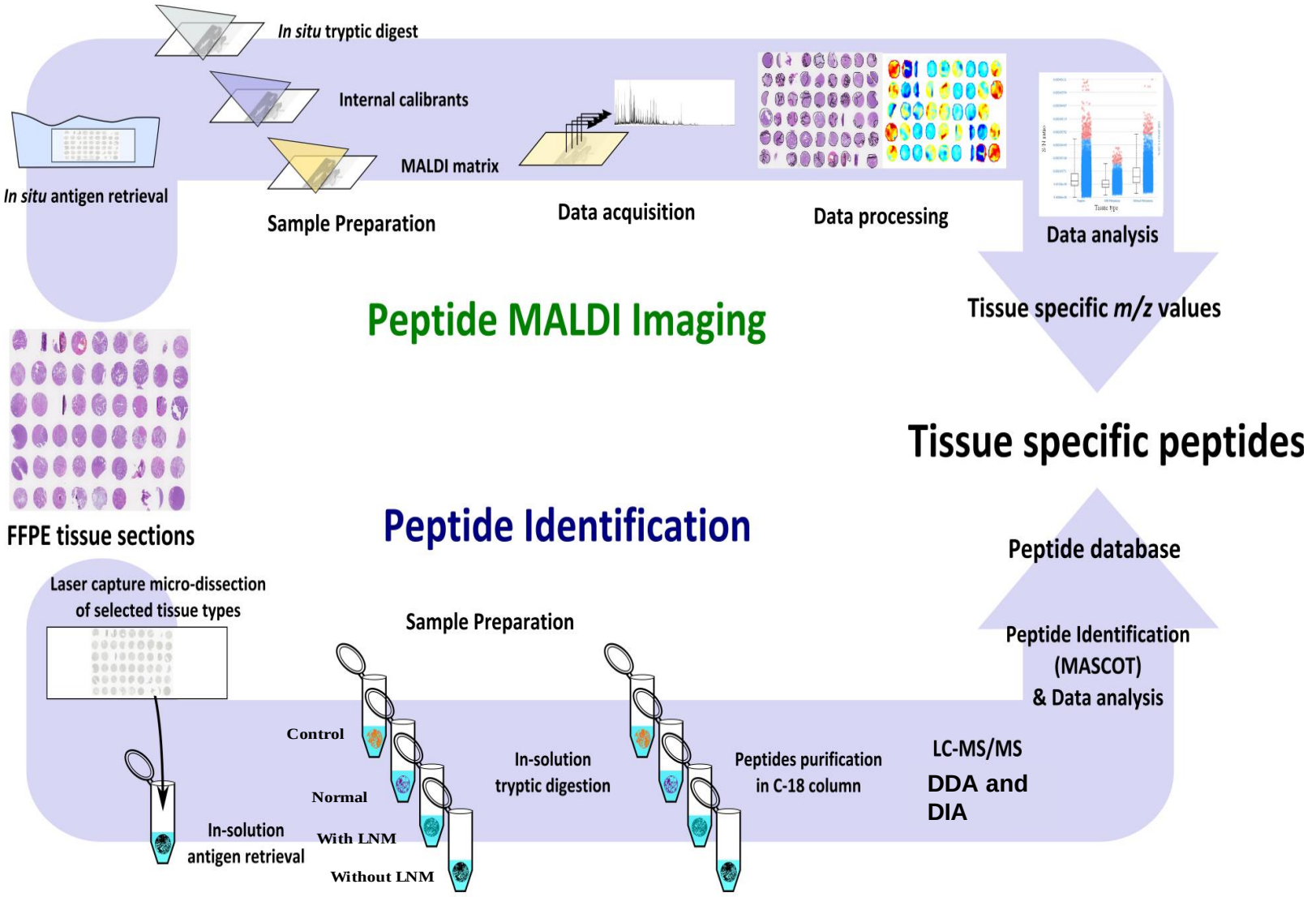
The hunt for a molecular signature

- What if we would find a molecular signature in the primary tumour which can be used to predict the metastatic potential of the tumour?
- This would change the treatment regime for patients with endometrial cancer
- Overtreatment would be prevented

MALD Imaging of FFPE tissue sections



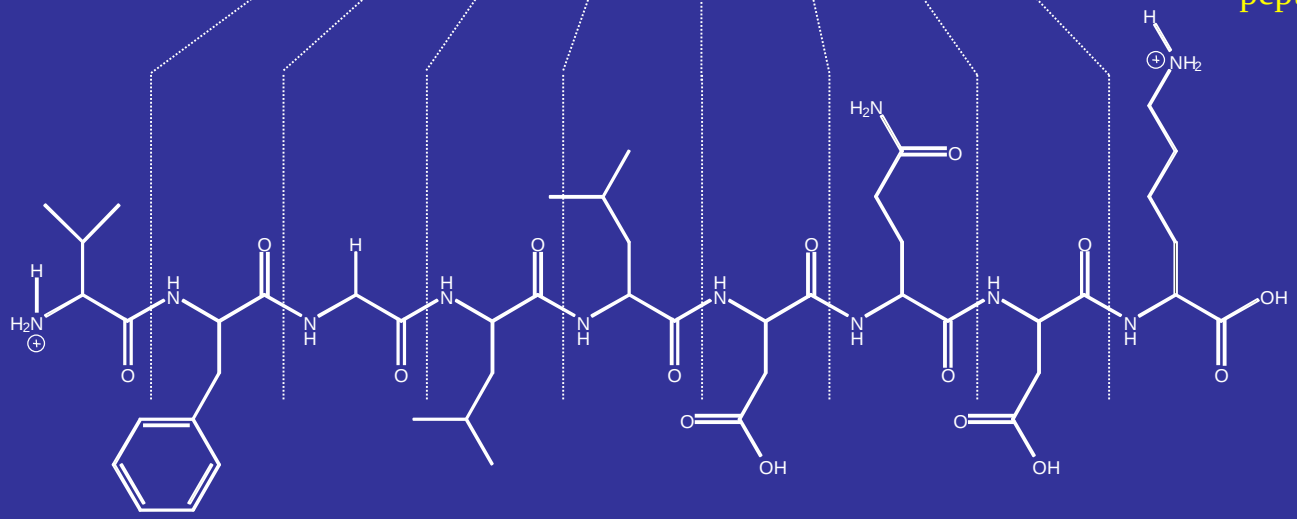
MALDI Imaging of FFPE TMA's



LC-MS/MS Identification

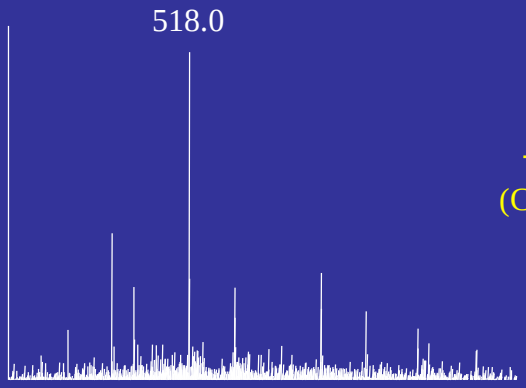
Val Phe Gly Lxx Lxx Asp Glu Asp Lys

Neutral mass of peptide is 1034 Da.



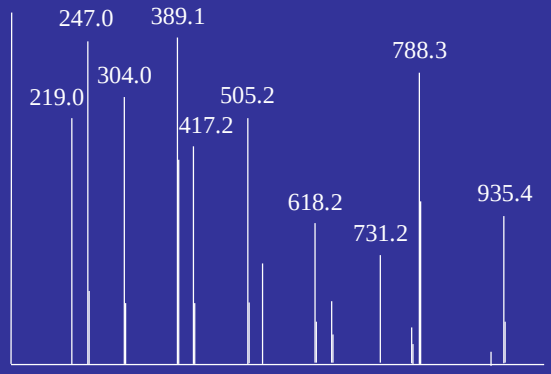
The protonated mass of the peptide, i.e., [M+2H]++ is 1036 Da.

MS Spectrum

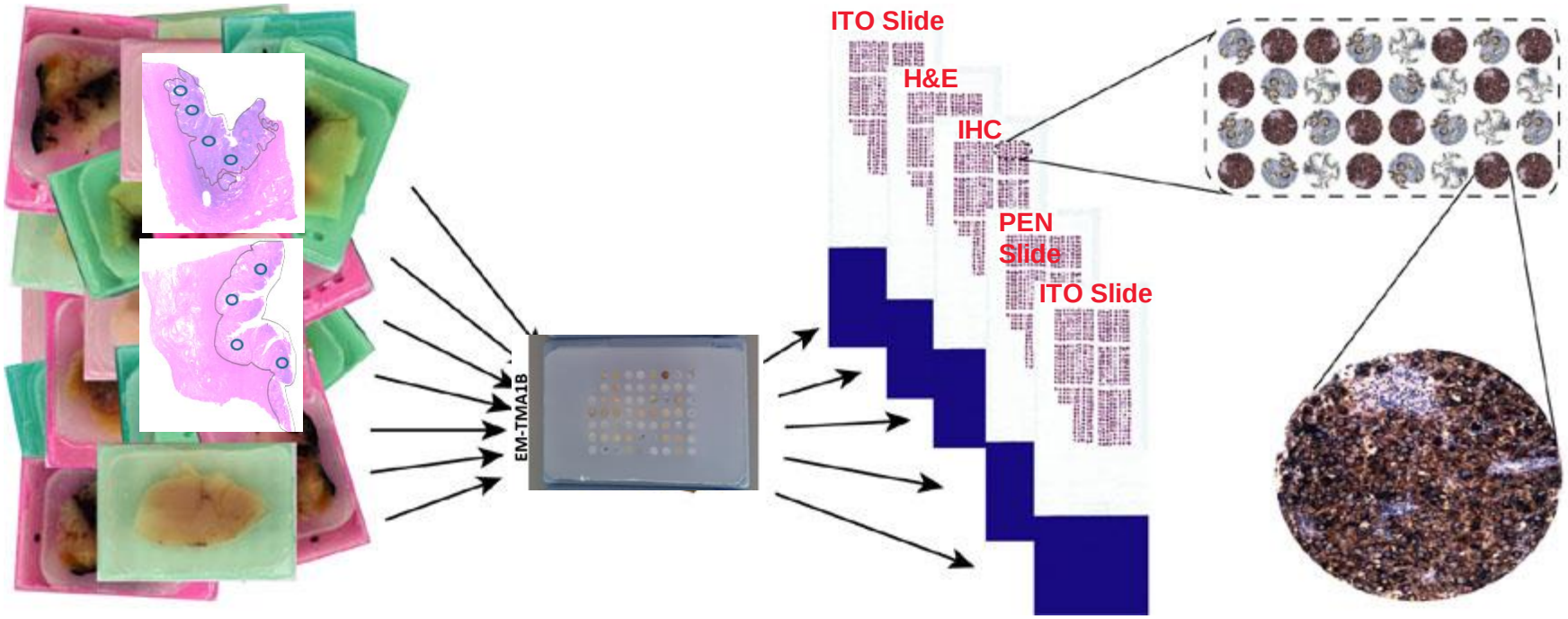


MS/MS Spectrum

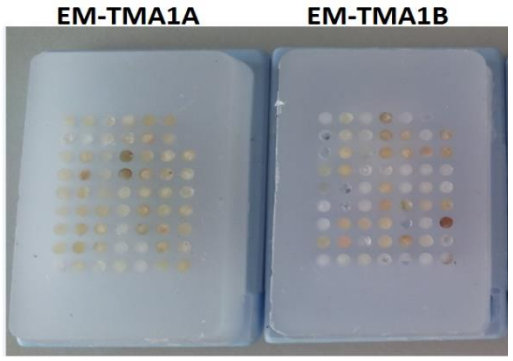
(CID of peptide ion *m/z* 518.0)



TMA construction



EC patient samples (n=57)

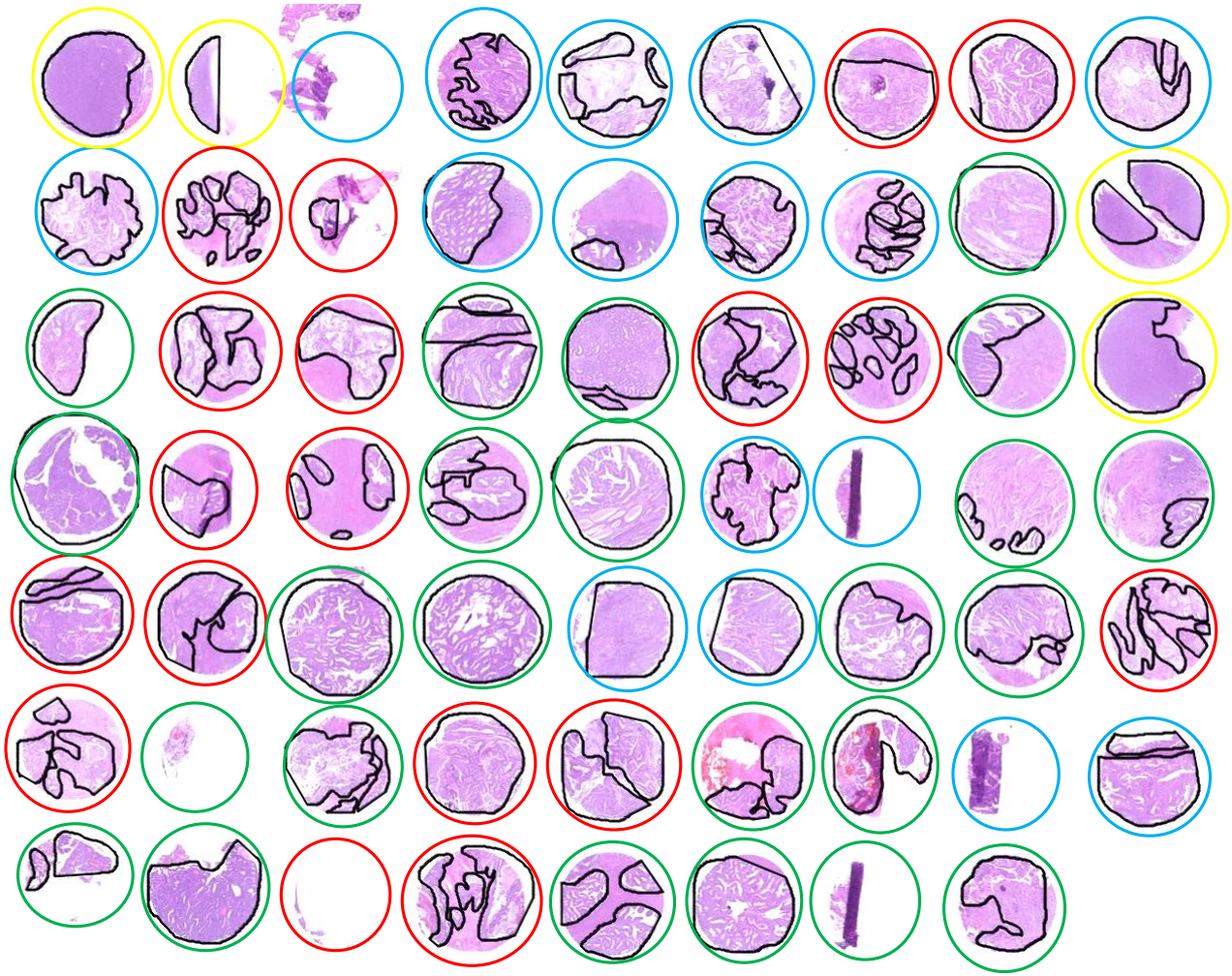


n = 16 with LNM

n = 27 without LNM

n = 14

H&E stained annotated TMA1



Black: Annotated tumour region
Red: With Metastasis
Green: Without Metastasis
Blue: Excluded

Data Acquisition and Analysis

- MALDI MSI data was acquired on an ultrafleXtreme MALDI-TOF/TOF instrument
- Peak groups (intact mass, intensity) were generated from the MALDI-MSI data and then ranked using a CCA (Canonical Correlation Analysis) based method for their ability to distinguish between the primary carcinomas with and without LNM (**Lyron Winderbaum**)
- A classification accuracy of 38 out of 43 patients (88.4%) was achieved

Proteomics 2016, 16, 1731–1735 DOI 10.1002/pmic.201500451

TECHNICAL BRIEF

Classification of MALDI-MS imaging data of tissue microarrays using canonical correlation analysis-based variable selection

Lyron Winderbaum, Inge Koch, Parul Mittal and Peter Hoffmann

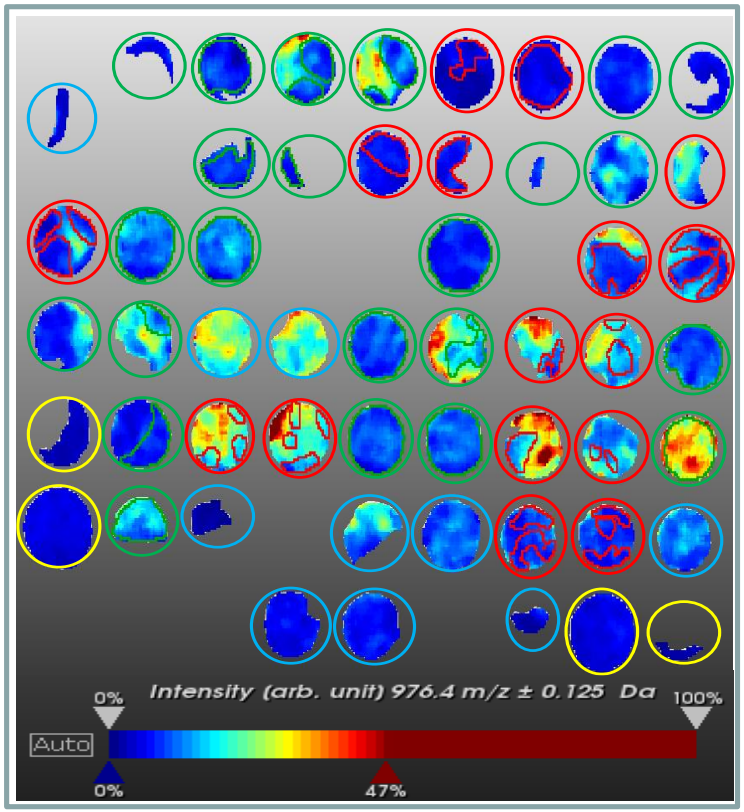
The University of Adelaide, Adelaide, SA, Australia

Top ranked *m/z* peak groups as ranked by the CCA

Ranked *m/z* values by CCA	Protein Accession Name
967.417	PLEC_HUMAN
1157.667	CPNE1_human
1198.667	ACTA_HUMAN
802.417	no match
975.417	RL11_human
1242.667	HS90B_human
1161.667	ACTA_HUMAN
1167.667	HNRPM_human
1612.917	CLH1_human
1032.667	RS9_human
1027.667	PGM1_human
941.417	CPNE3_human
976.417	ACTA_HUMAN
1406.667	SAMP_human
1138.667	EF2_human
1115.417	EIF3E_human
944.417	H2A1D_human
857.417	No match
1905.917	HSPB1_human
915.417	COCOA1_human

Mittal et al. 2016, Proteomics

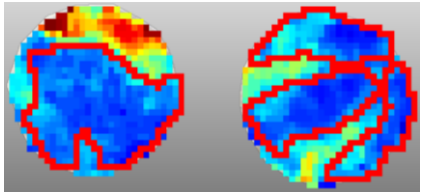
P. Mittal, M. Klingler-Hoffmann, G. Arentz, L. Winderbaum, N.A. Lokman, C. Zhang, L. Anderson, J. Scurry, Y. Leung, C.J.R. Stewart, J. Carter, G. Kaur, M.K. Oehler, P. Hoffmann, Lymph node metastasis of primary endometrial cancers: Associated proteins revealed by MALDI imaging, Proteomics, 2016 Jun;16(11-12):1793-1801. doi:10.1002/pmic.201500455.



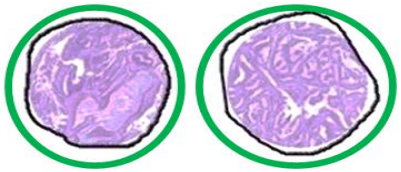
H&E stain

With LNM

Ion intensity images

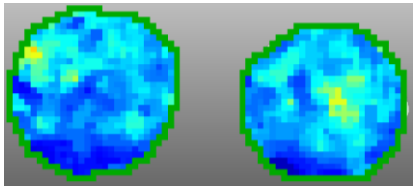


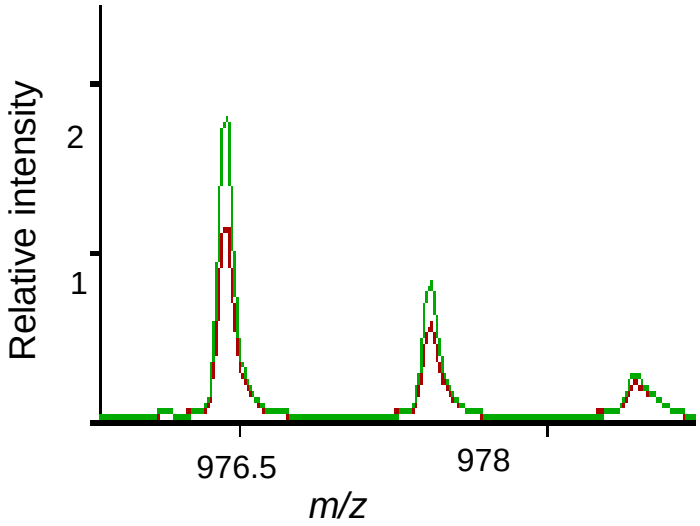
H&E stain



Without LNM

Ion intensity images

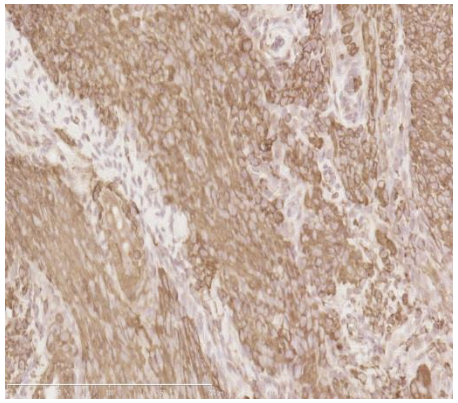




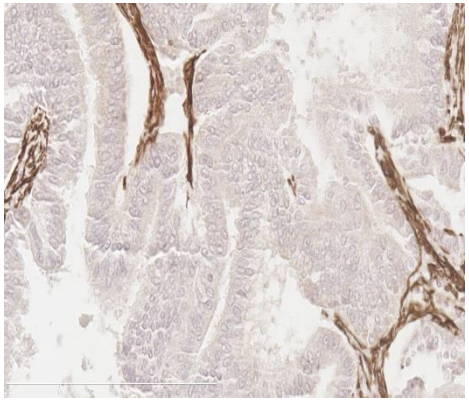
Mittal et al. 2016, Proteomics

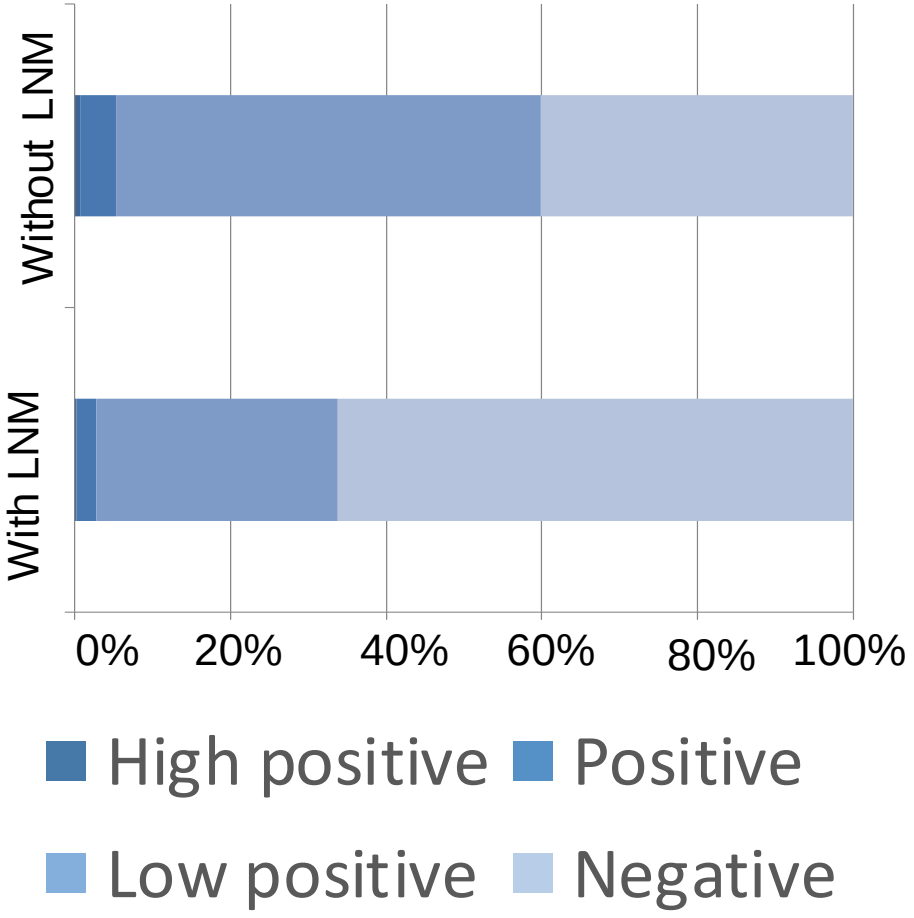
Validation by Immunohistochemistry

Without LNM



With LNM





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Proteomics 2016, 16, 1793–1801 DOI 10.1002/pmic.201500455

RESEARCH ARTICLE

Lymph node metastasis of primary endometrial cancers: Associated proteins revealed by MALDI imaging

Parul Mittal[1,2*], Manuela Klingler-Hoffmann[1,2*], Georgia Arentz[1,2], Lyron Winderbaum[1,2], Noor A Lokman[1,8], Chao Zhang[1,2], Lyndal Anderson[3], James Scurry[4], Yee Leung[5], Colin JR Stewart[5], Jonathan Carter[6], Gurjeet Kaur[7], Martin K. Oehler[8,9] and Peter Hoffmann[1,2**]

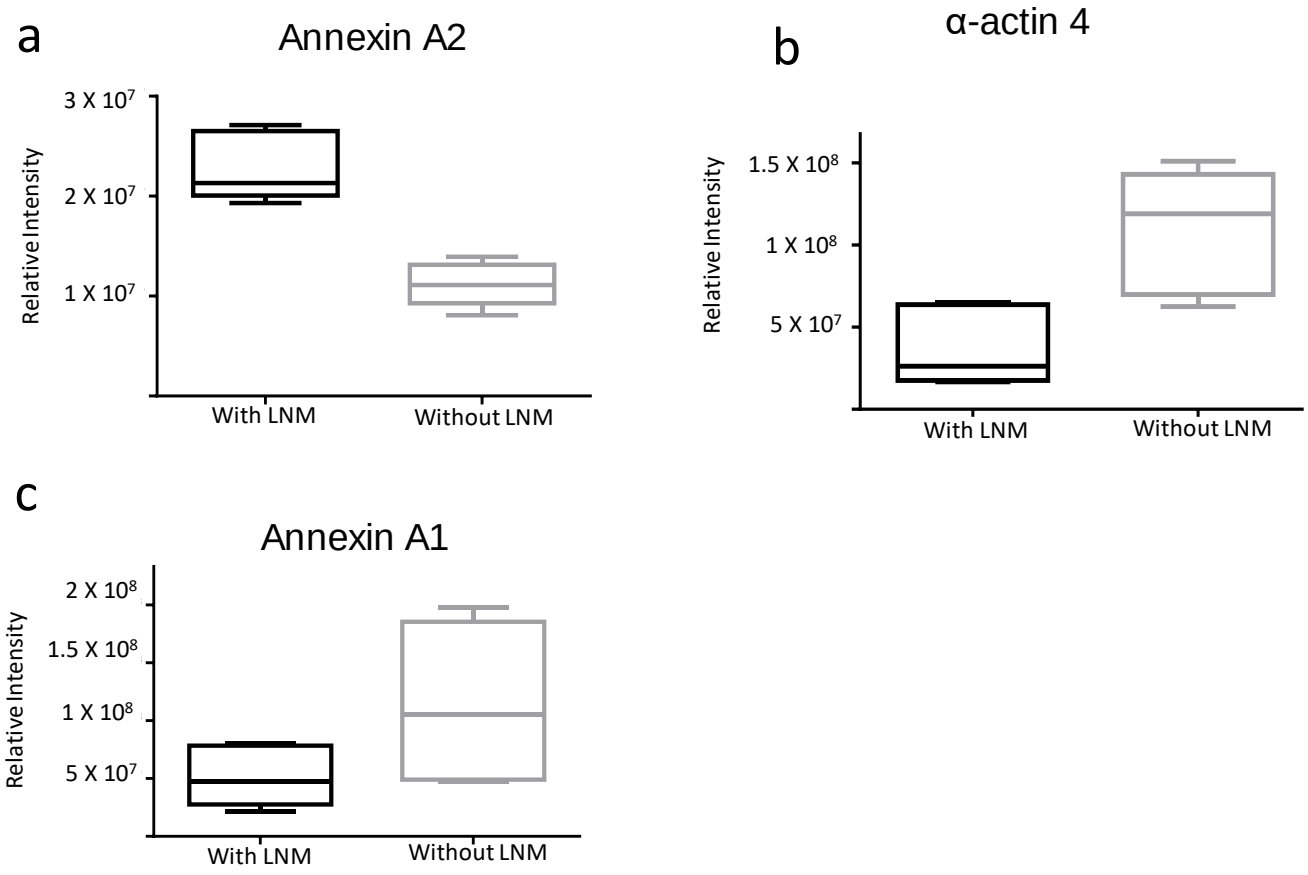
TCGA

- The Cancer Genome Atlas, established in 2005
- High throughput data for genomic and proteomic analysis
- Databank contained 514 EC patients, with RNA sequencing data available for 333 patients (n=54 with LNM and n=279 without LNM)
- From the analysis, we have chosen eleven differentially expressed genes: six most significant up- and five down-regulated genes
- Following extensive literature review the list has grown to 60 target proteins, which might be differentially expressed in primary tumours with and without LNM

- Using traditional form of LC-MS/MS, we were able to detect 23 of these proteins in EC tissue cohort (n=5 with LNM and n=5 without LNM)

- Using targeted LC-MS/MS, we confirmed the differential expression of 5 of the proteins (more than 2 peptides and p value < 0.05)
    - Annexin A2
    - Annexin A1
    - Alpha actinin 4
    - EGFR
    - ERBB2

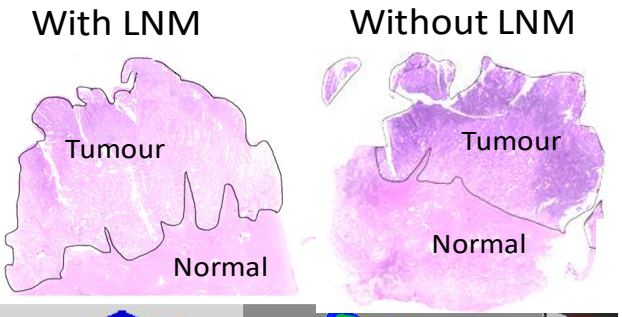
Relative quantification by targeted LC-MS/MS



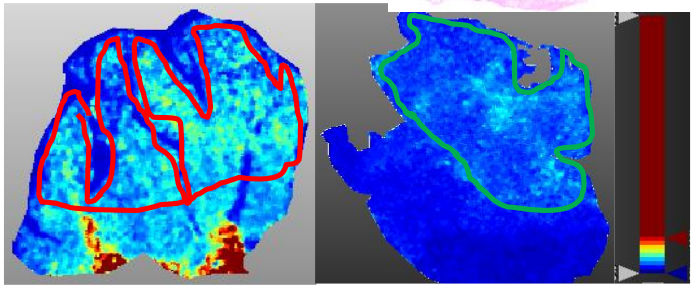
P. Mittal, M. Klingler-Hoffmann, G. Arentz, L.J. Winderbaum, G. Kaur, M.K. Oehler, P. Hoffmann, Annexin A2 and alpha actinin 4 expression correlates with metastatic potential of primary endometrial cancer, Biochimica et Biophysica Acta (BBA), **2017** Jul;1865 (7):846-857

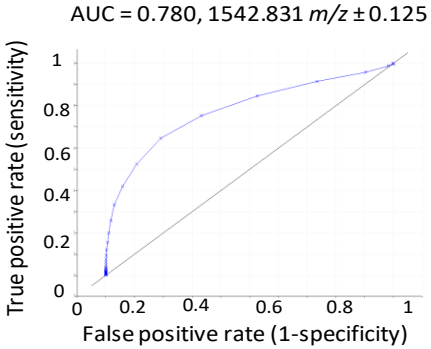
Spatial localization by MALDI MSI

- The tissue localization of peptides from the differentially expressed proteins were visualized using MALDI MSI in whole tissue sections (n=5 with LNM and n=5 without LNM) as well as TMAs (n=16 with LNM and n=27 without LNM)

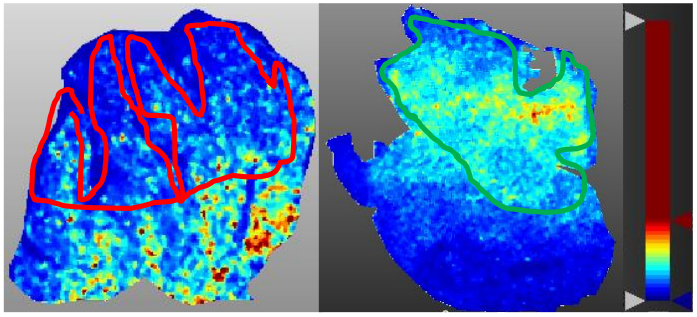


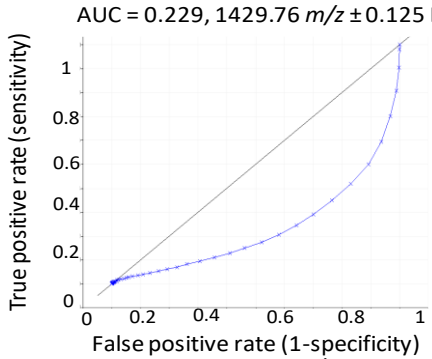
Annexin A2



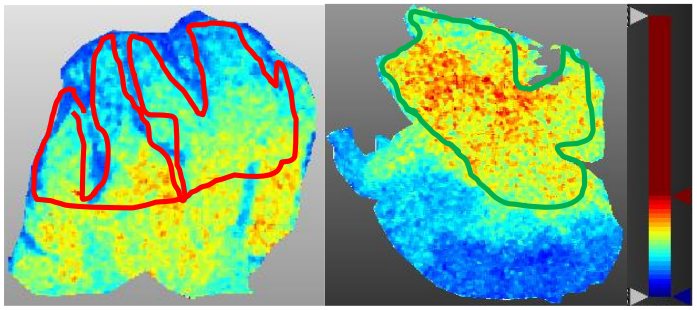


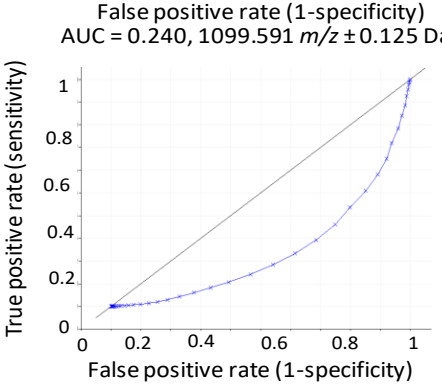
α-actin 4





Annexin A1



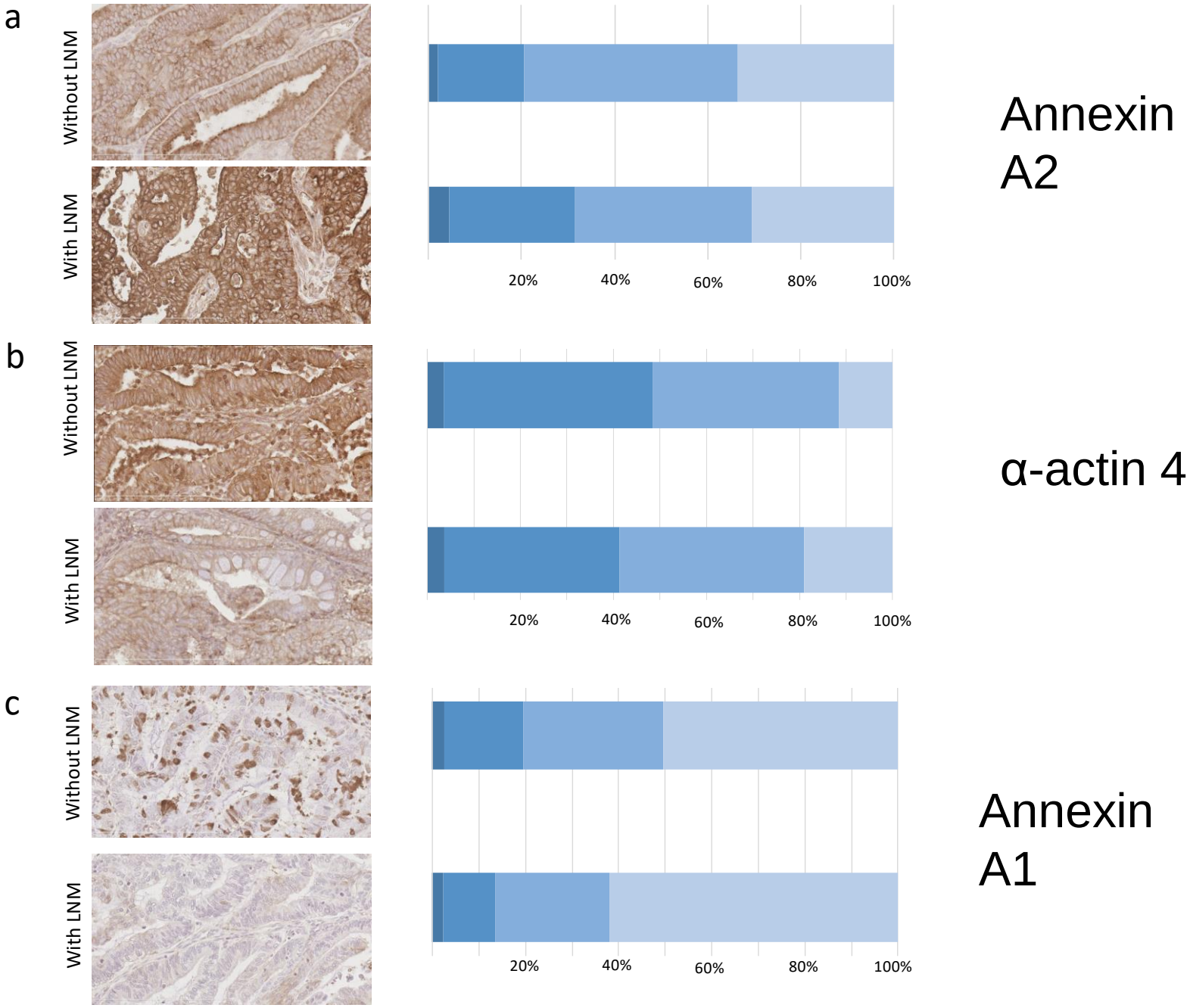


P. Mittal, M. Klingler-Hoffmann, G. Arentz, L.J. Winderbaum, G. Kaur, M.K. Oehler, P. Hoffmann, Annexin A2 and alpha actinin 4 expression correlates with metastatic potential of primary endometrial cancer, Biochimica et Biophysica Acta (BBA), Submitted for publication on 15/06/2016

Validation by Immunohistochemistry

- The proteins identified were further validated by immunohistochemistry

- This allowed the simultaneous analysis of 43 patients (n=16 with LNM and n=27 without LNM) of which 39 had not been analysed by relative quantification LC-MS/MS.



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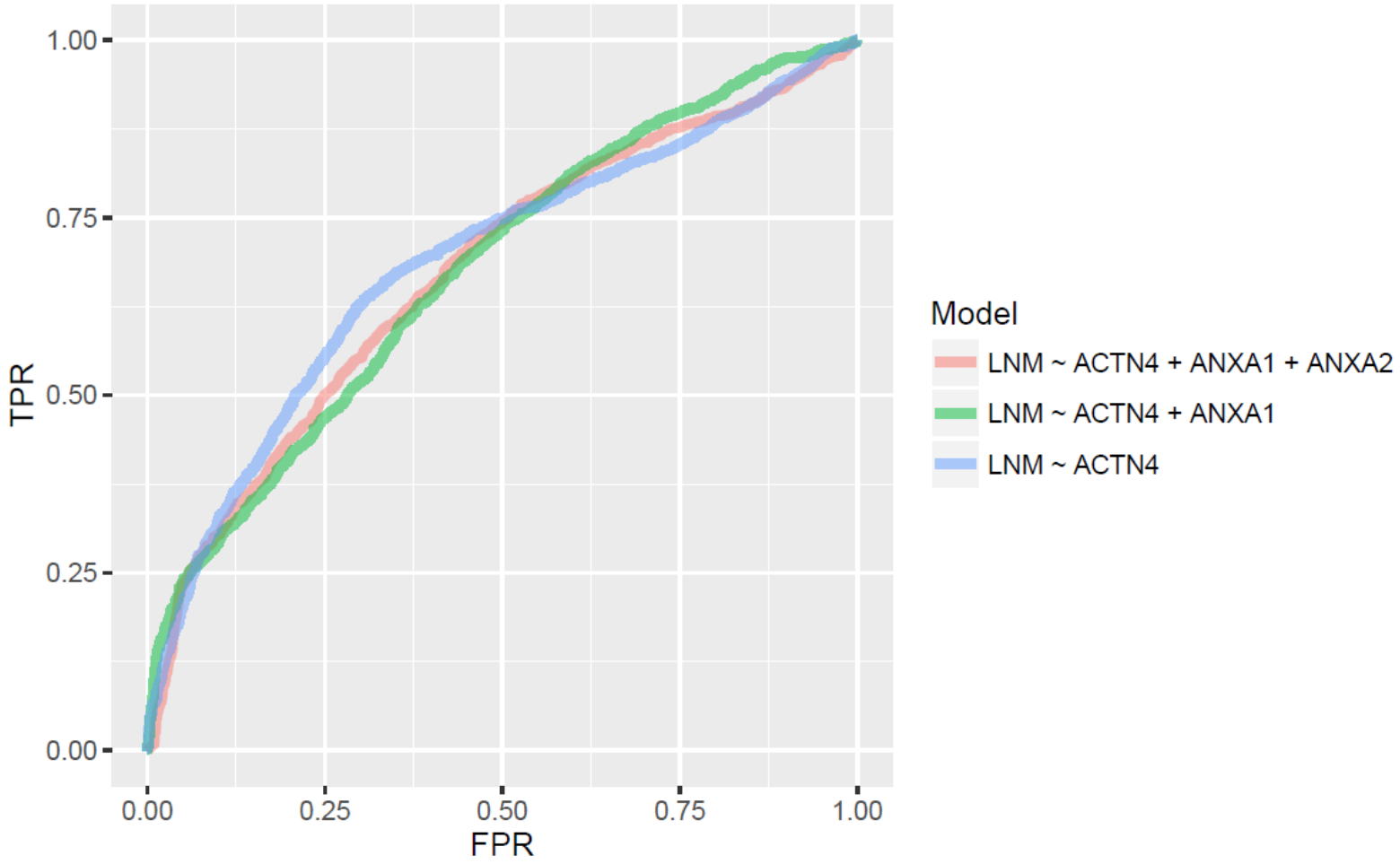
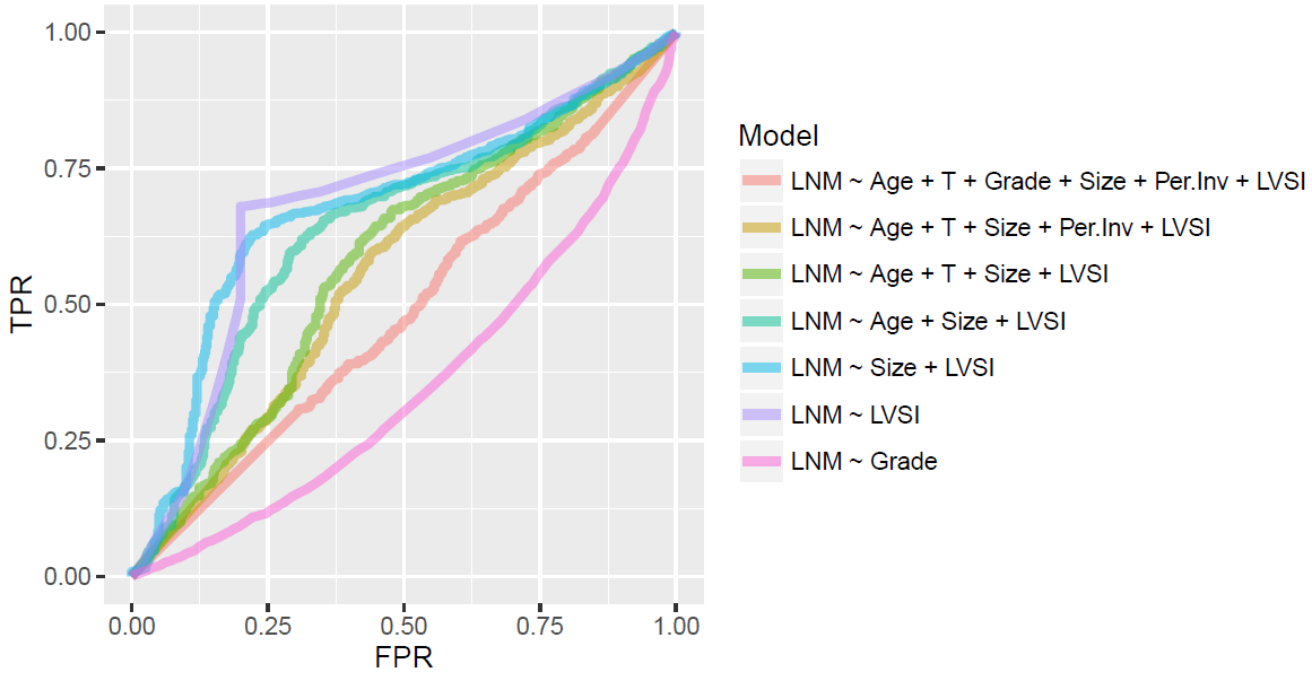
Logistic Regression Model to fit IHC results



Nomogram using clinical variables

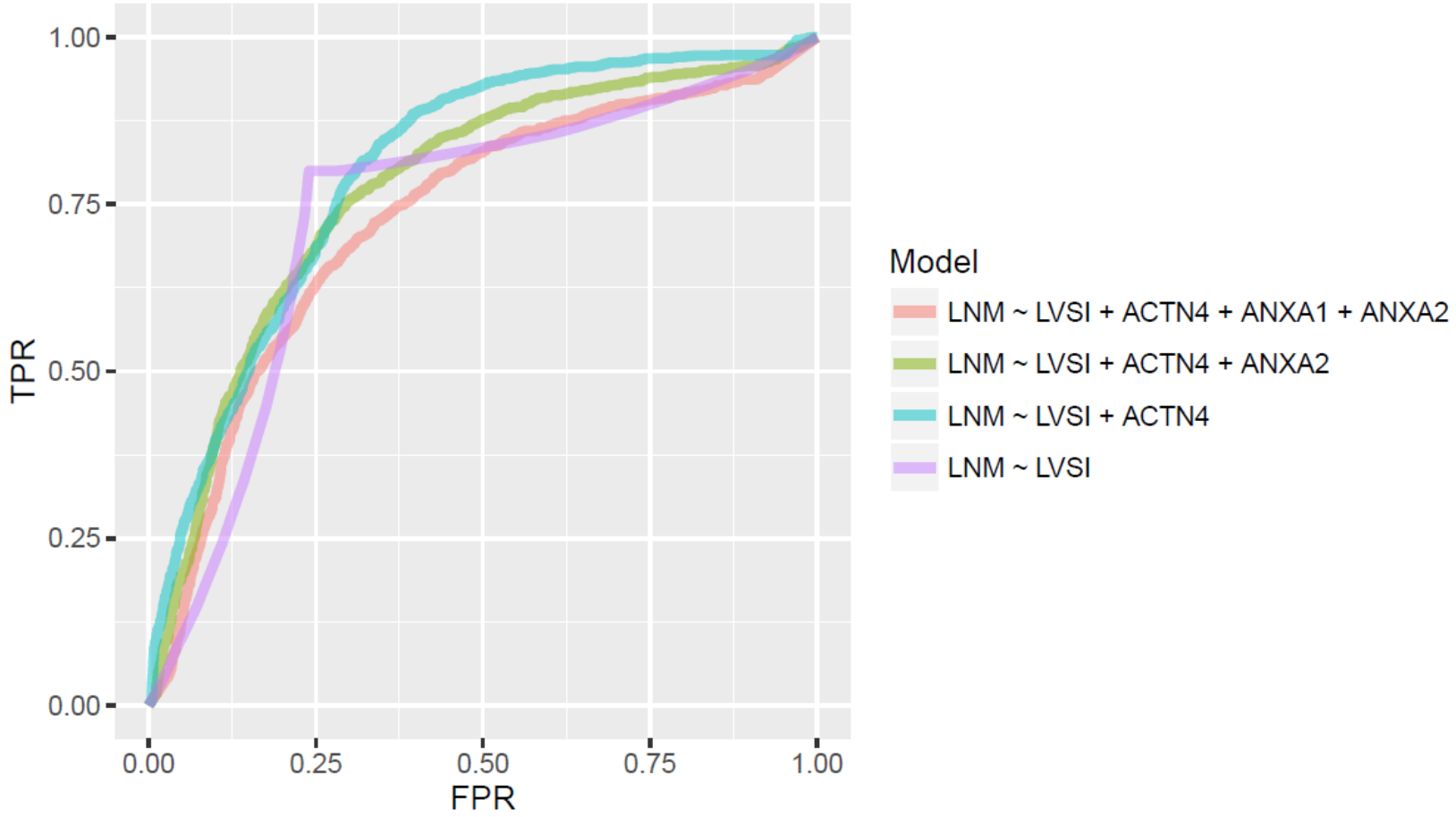
- From the 43 patients analysed, we had 35 patients for which all the following clinical variables have been recorded: age, tumour grade, tumour size, tumour extent, and lympho-vascular space invasion (LVSI)
- Fitting a logistic regression model based on all these clinical variables and iteratively removing the least significant of them
- Resulted in only one significant clinical variable: LVSI, and this agrees with recently published results

Model to fit clinical variables

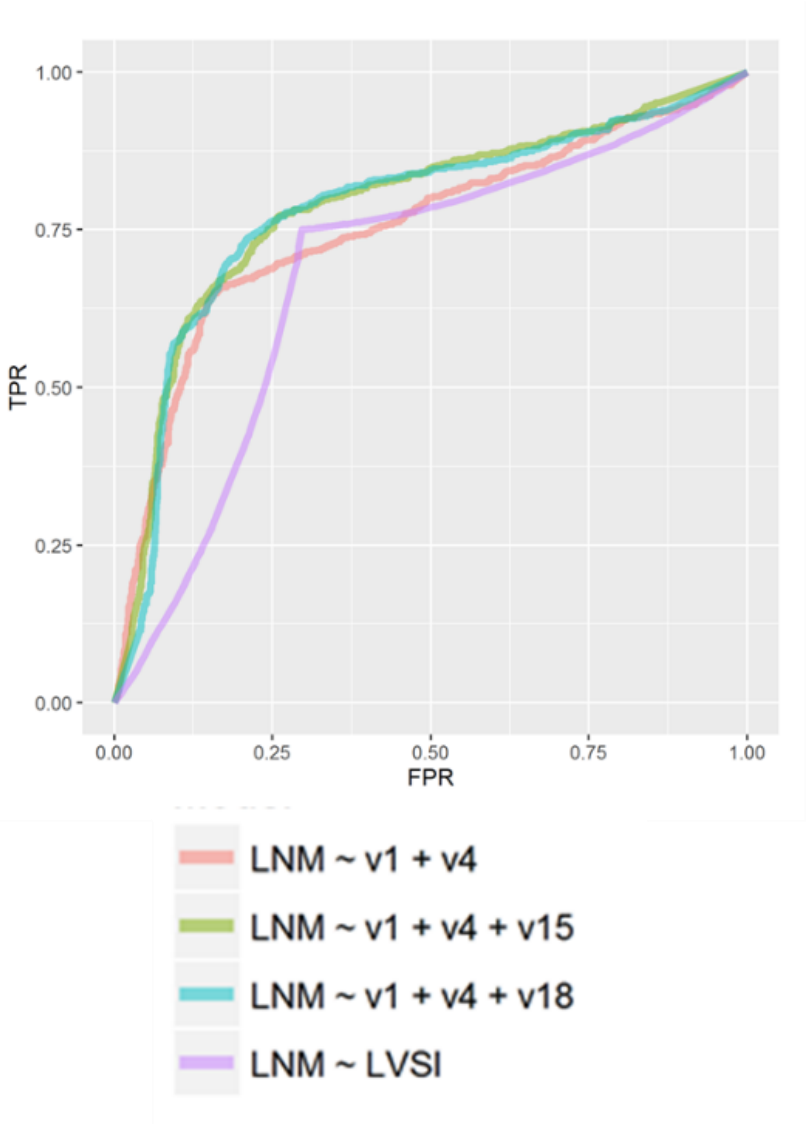


Model	AUC
LNM ~ Age + T + Grade + Size + Per.Inv + LVSI	0.49
LNM ~ Age + T + Size + Per.Inv + LVSI	0.56
LNM ~ Age + T + Size + LVSI	0.58
LNM ~ Age + Size + LVSI	0.64
LNM ~ Size + LVSI	0.68
LNM ~ LVSI	0.68
LNM ~ Grade	0.35

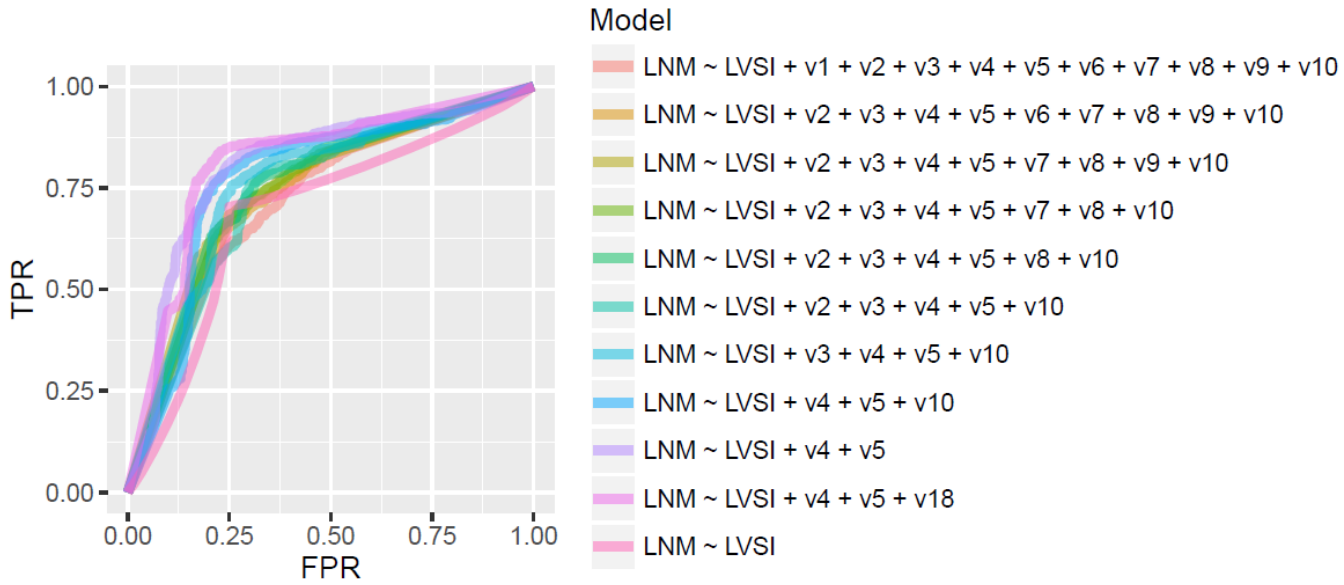
Model to fit LVSI and IHC results



Model to fit MALDI-MSI variables alone



Adding MALDI-MSI Variables with LVSI



Model	AUC
LNM ~ LVSI + v1 + v2 + v3 + v4 + v5 + v6 + v7 + v8 + v9 + v10	0.72
LNM ~ LVSI + v2 + v3 + v4 + v5 + v6 + v7 + v8 + v9 + v10	0.74
LNM ~ LVSI + v2 + v3 + v4 + v5 + v7 + v8 + v9 + v10	0.75
LNM ~ LVSI + v2 + v3 + v4 + v5 + v7 + v8 + v10	0.74
LNM ~ LVSI + v2 + v3 + v4 + v5 + v8 + v10	0.75
LNM ~ LVSI + v2 + v3 + v4 + v5 + v10	0.74
LNM ~ LVSI + v3 + v4 + v5 + v10	0.76
LNM ~ LVSI + v4 + v5 + v10	0.77
LNM ~ LVSI + v4 + v5	0.81
LNM ~ LVSI + v4 + v5 + v18	0.82
LNM ~ LVSI	0.69

Nomogram for Endometrial Cancer

Table 3
Evaluation of postoperative and preoperative models.

Model	Number of patients	Observed LNM probability	Predicted LNM probability (%)	AUC	E_{aver} (%)	E_{max} (%)	FN rate	Patients assigned to the low-risk group
Kamura *et al.* 1999 [4]	316	41/316 (13.0%)	8.4	0.72	4.7	7.5%	NA	NA
French nomogram [3,8]	447	84/447 (18.8%)	11.3	0.78	7.7	15.3	10/206 (4.9%)	206/447 (46.1%)
Milam *et al.* 2012 [5]	283	28/283 (9.9%)	9.3	0.73	3.2	26.1	0/24 (0%)	24/283 (8.4%)
Zhang *et al.* 2012 [18]	285	46/285 (16.1%)	NA	NA	NA	NA	2/67 (3.0%)	67/285 (23.5%)
GOG criteria [6]	330	39/330 (11.8%)	12.0	0.76	2.0	8.3	0/18 (0%)	18/330 (5.4%)
Mayo clinic nomogram [7]	182	28/182 (15.4%)	21.9	0.75	5.5	23.0	1/39 (2.6%)	39/182 (21.4%)
Akbayir *et al.* [17]	229	36/229 (15.7%)	NA	NA	NA	NA	3/86 (3.5%)	86/229 (37.6%)
KGOG model [9]	160	27/160 (16.9%)	13.1	0.76	3.6	4.8	3/68 (4.4%)	68/160 (42.5%)
Lee *et al.* 2010 [10]	201	41/201 (20.4%)	20.2	0.66	5.6	64.2	5/54 (9.2%)	54/201 (26.9%)
Todo *et al.* 2007 [11,19]	184	38/184 (20.7%)	19.2	0.66	6.7	16.3	4/52 (7.7%)	52/184 (28.3%)

Abbreviations: AUC, area under the receiver operating characteristic curve; LNM, lymph node metastasis, NA, not assessable; E, difference in predicted and calibrated probabilities between calibration and AUC; E_{max}, maximal error; E_{aver}, average error; FN, false negative.

LNM: LVSI + v4 + v5 + v18 0.82

LNM: LVSI + v4 + v5 0.81

unisa.edu.au

Summary

- LVSI information not available when women are diagnosed with EC as they have only small biopsy taken
- Would be great to have a model to guide the surgeons whether to remove the lymph nodes before the surgery.
- MALDI MSI in combination with LVSI performs better to predict LNM from primary tumours for endometrial cancer than anything else available

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N-Glycan MALDI Imaging Mass Spectrometry on FFPE Ovarian Cancer Tissue

Matthew T. Briggs

Peter Hoffmann, Nicki Packer, Martin Oehler

Ovarian Cancer (OC)

The most fatal gynaecological malignancy in adult women

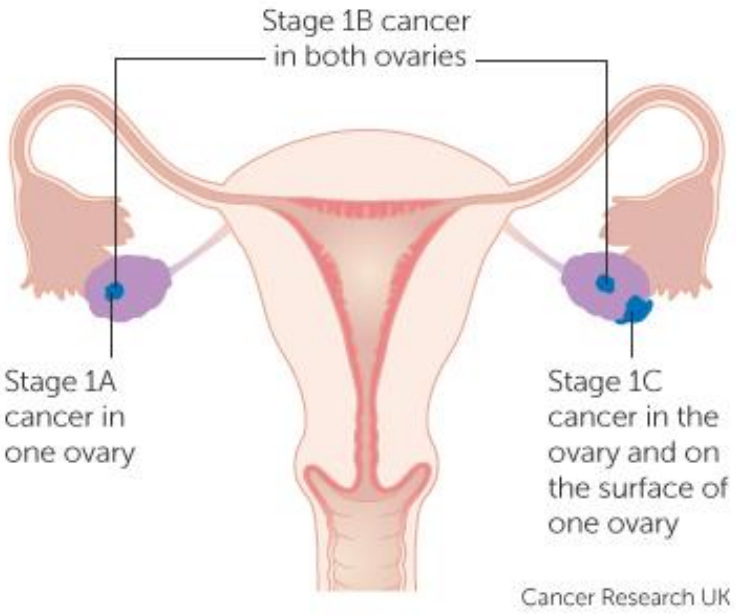
- Asymptomatic in early stages
- Diagnosed in late-stages
- Leads to metastasis

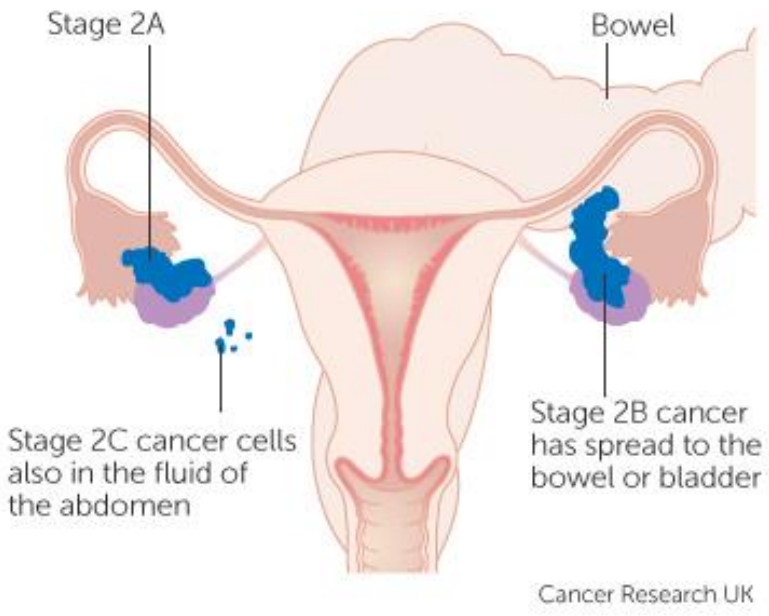
In Australia during 2018, there is estimated to be

- 1,613 new cases diagnosed
- 1,069 deaths estimated

https://ovarian-cancer.canceraustralia.gov.au/statistics

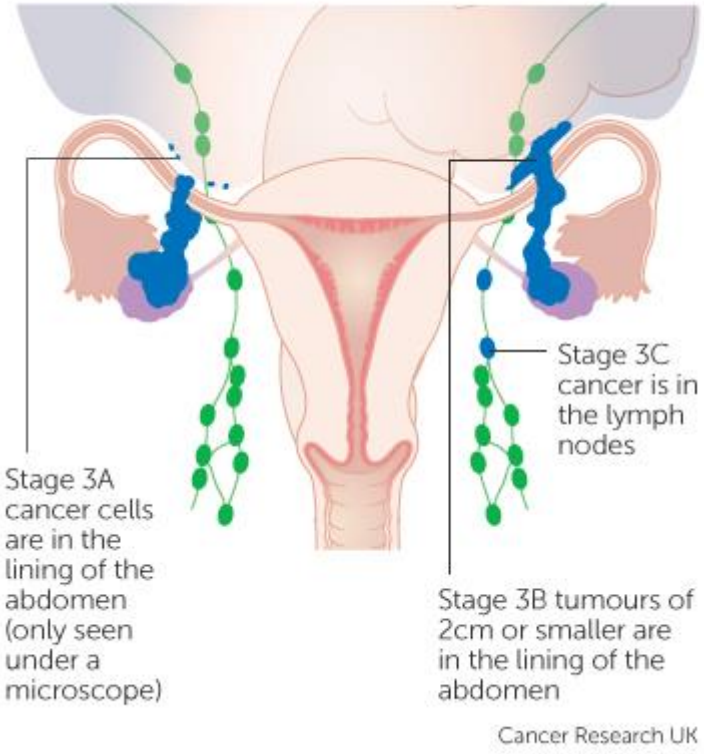
FIGO Stages

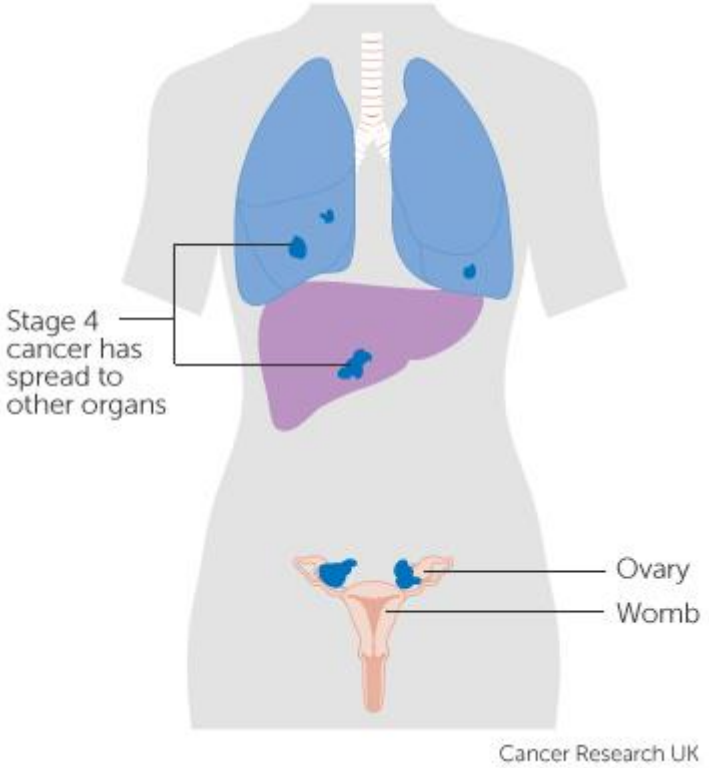




http://www.newkidscenter.com/Ovarian-Cancer-Staging.html

FIGO Stages





http://www.newkidscenter.com/Ovarian-Cancer-Staging.html

From Proteomics to Glycomics

- Matrix-assisted laser desorption ionization (MALDI)-mass spectrometry imaging (MSI)
- Formalin-fixed paraffin embedded (FFPE) tissue = high sample number and long term storage
- Introduction of **glycosylation** research

Glycosylation

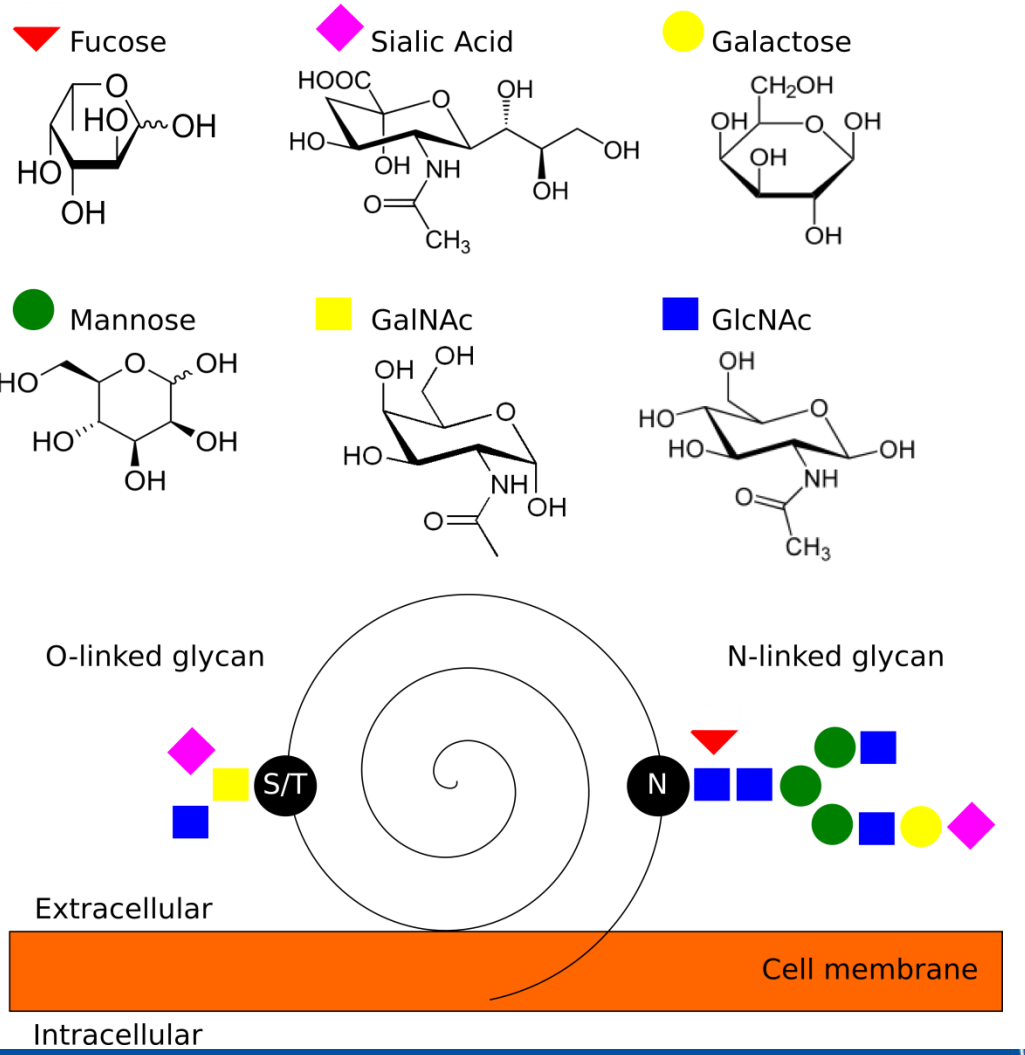
There are two main types of glycosylation:

- *N*-linked glycosylation (attached to asparagine residues)
- *O*-linked glycosylation (attached to threonine or serine residues)

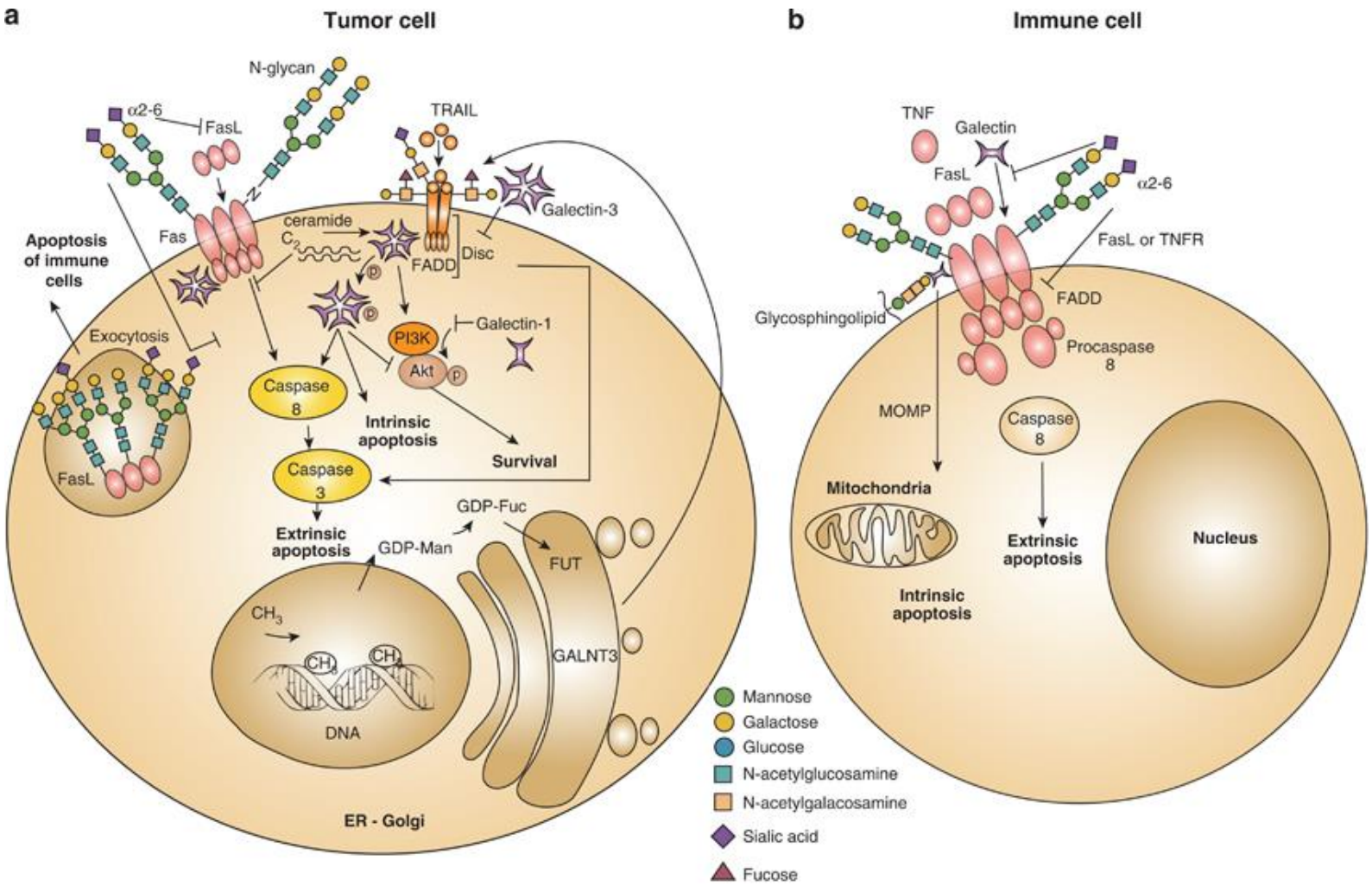
N-linked glycosylation is the most common with 90% of glycoproteins presenting *N*-glycans

N-glycan structures have been observed to be altered in the tumour microenvironment, which contributes to metastasis

Protein Glycosylation

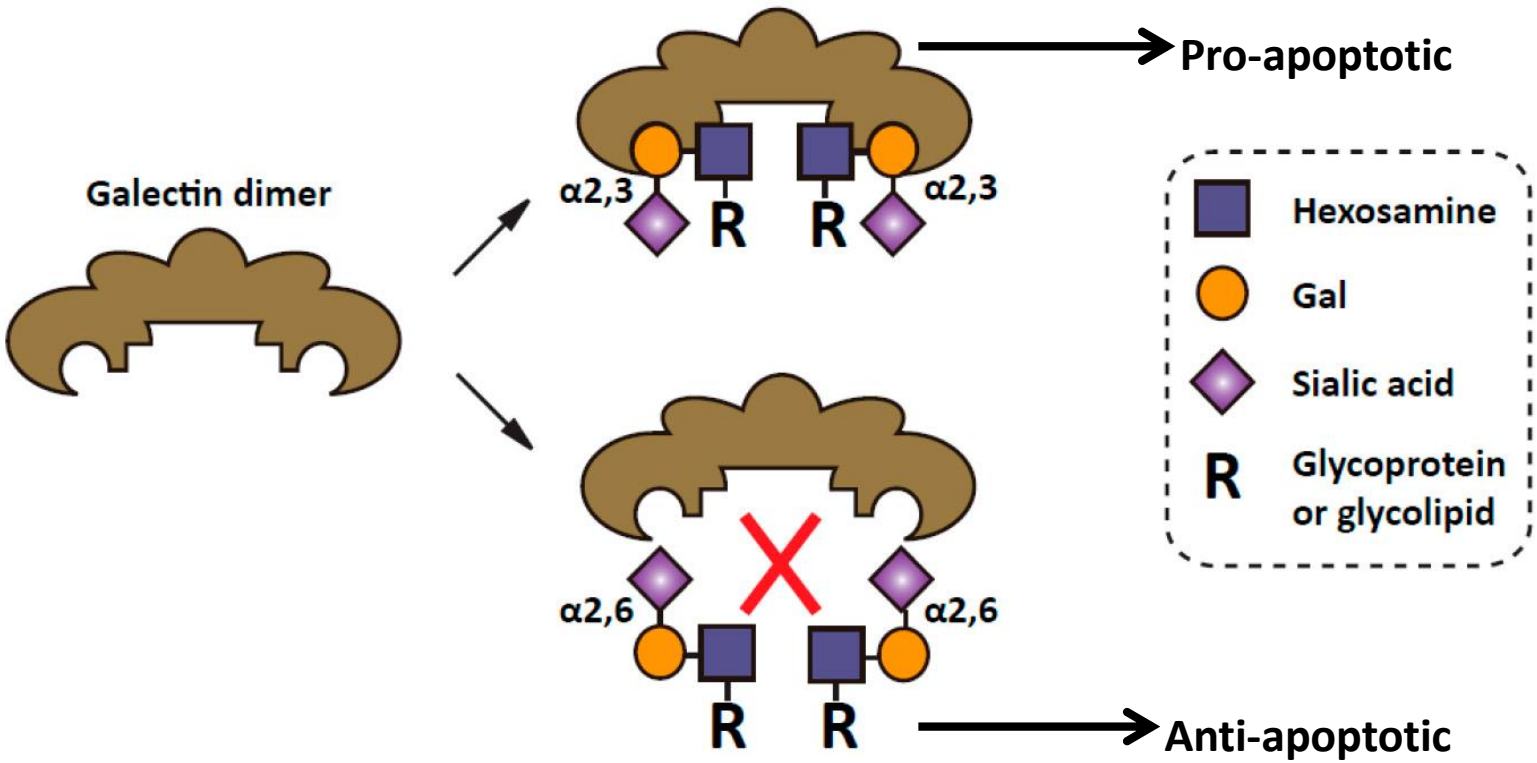


Glycosylation in apoptotic pathways



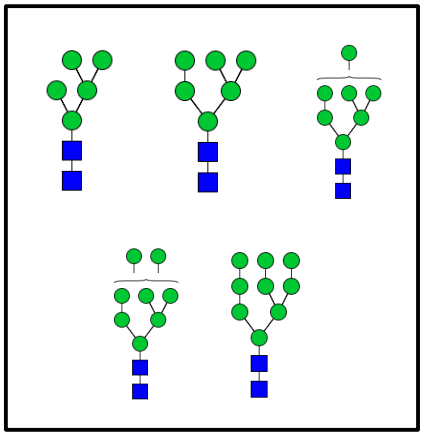
Cell Death and Differentiation (2013) **20,** 976–986; doi:10.1038/cdd.2013.50

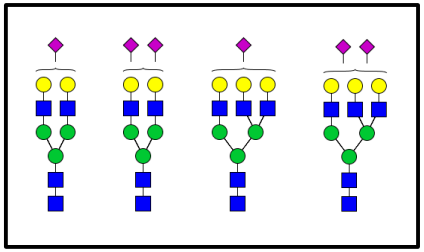
α2-6 Sialylation prevents apoptosis

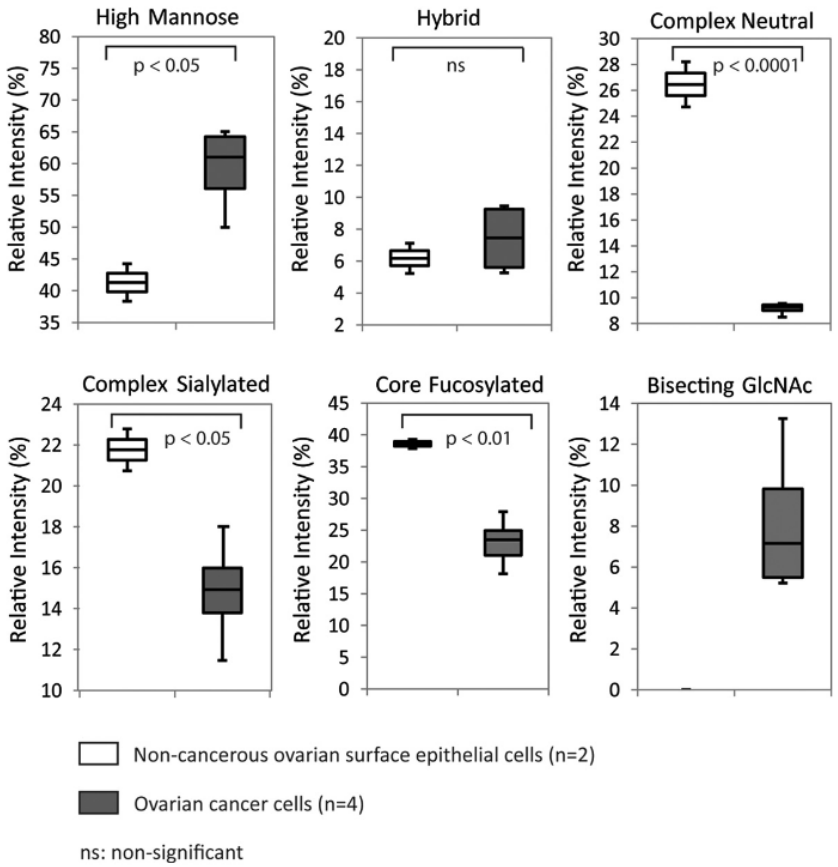


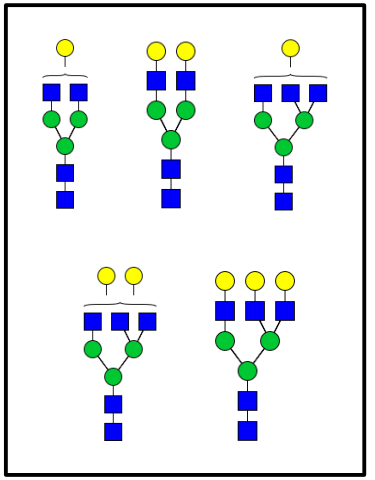
Molecules ***2015, 20, 7509-7527; doi:10.3390/molecules20057509***

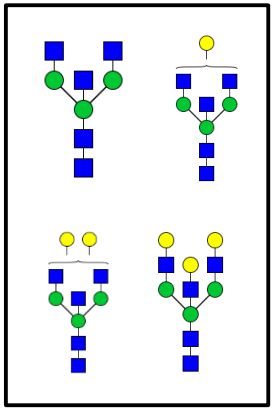
Ovarian Cell Lines: Healthy vs. Cancerous











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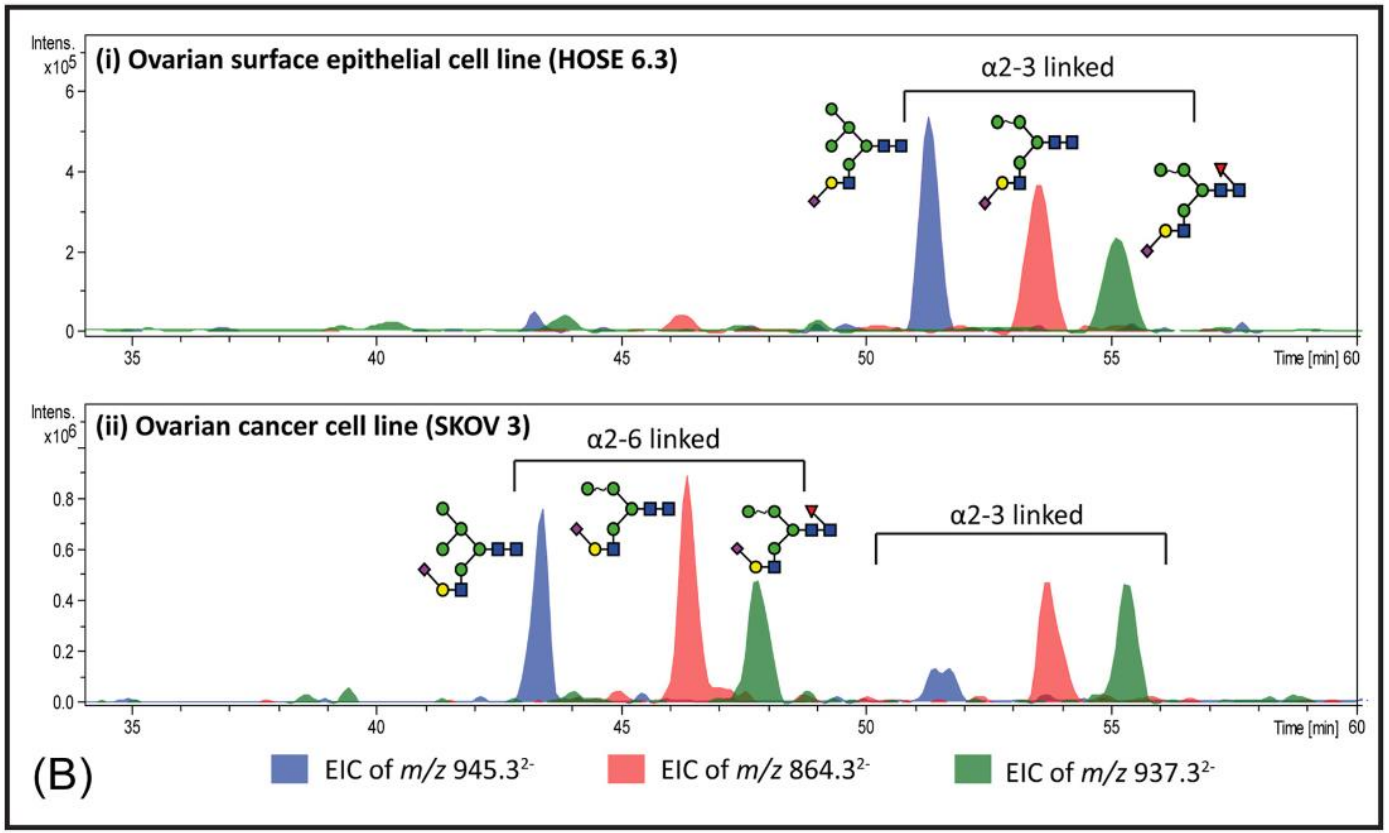
Anugraham M, Jacob F, Nixdorf S, Everest-Dass AV, Heinzelmann-Schwarz V, Packer NH. *Specific glycosylation of membrane proteins in epithelial ovarian cancer cell lines: glycan structures reflect gene expression and DNA methylation status*. Mol Cell Proteomics. 2014 Sep;13(9):2213-32. doi: 10.1074/mcp.M113.037085.

Ovarian Cell Lines: Healthy vs. Cancerous

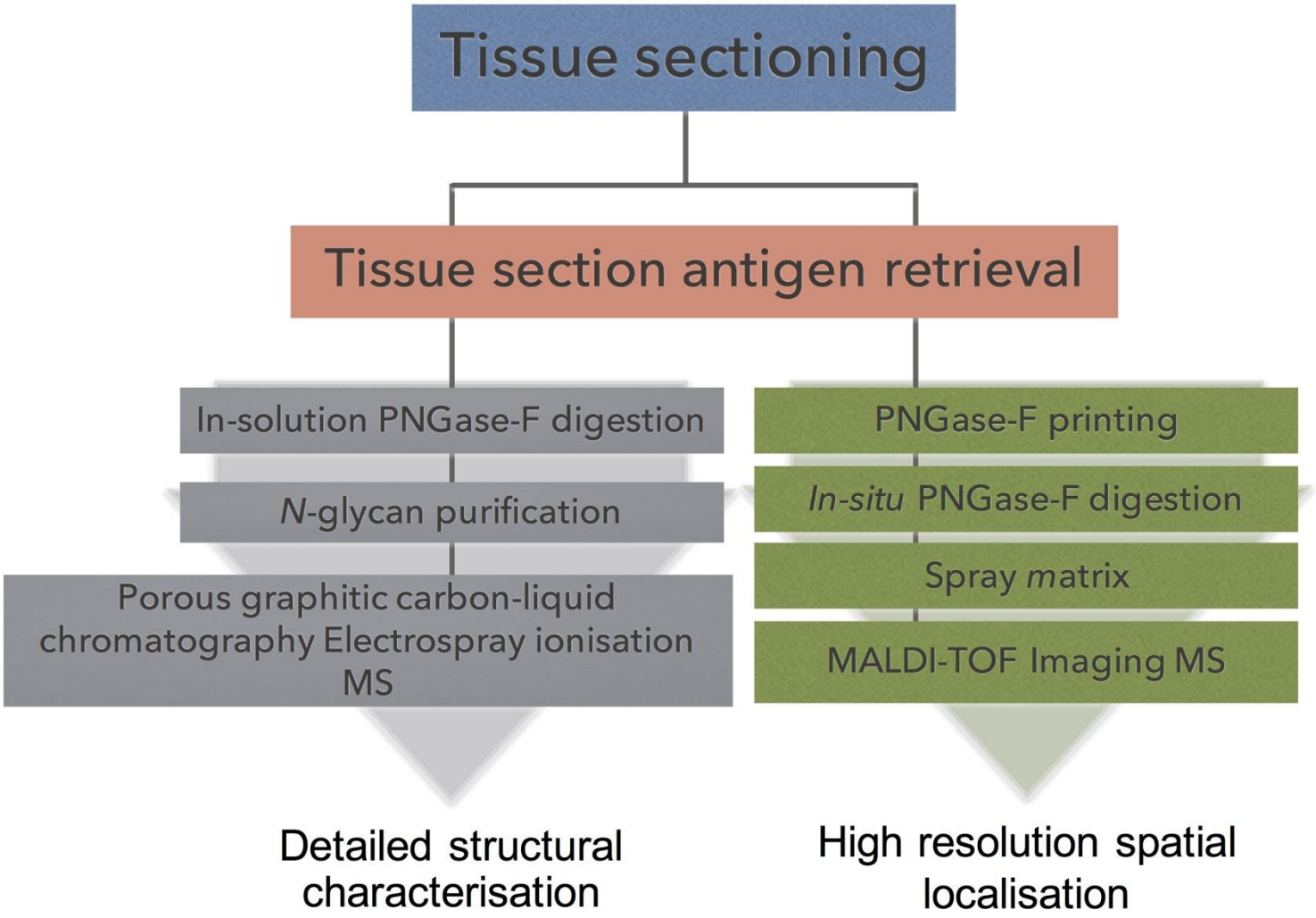


Anugraham M, Jacob F, Nixdorf S, Everest-Dass AV, Heinzelmann-Schwarz V, Packer NH. *Specific glycosylation of membrane proteins in epithelial ovarian cancer cell lines: glycan structures reflect gene expression and DNA methylation status*. Mol Cell Proteomics. 2014 Sep;13(9):2213-32. doi: 10.1074/mcp.M113.037085.

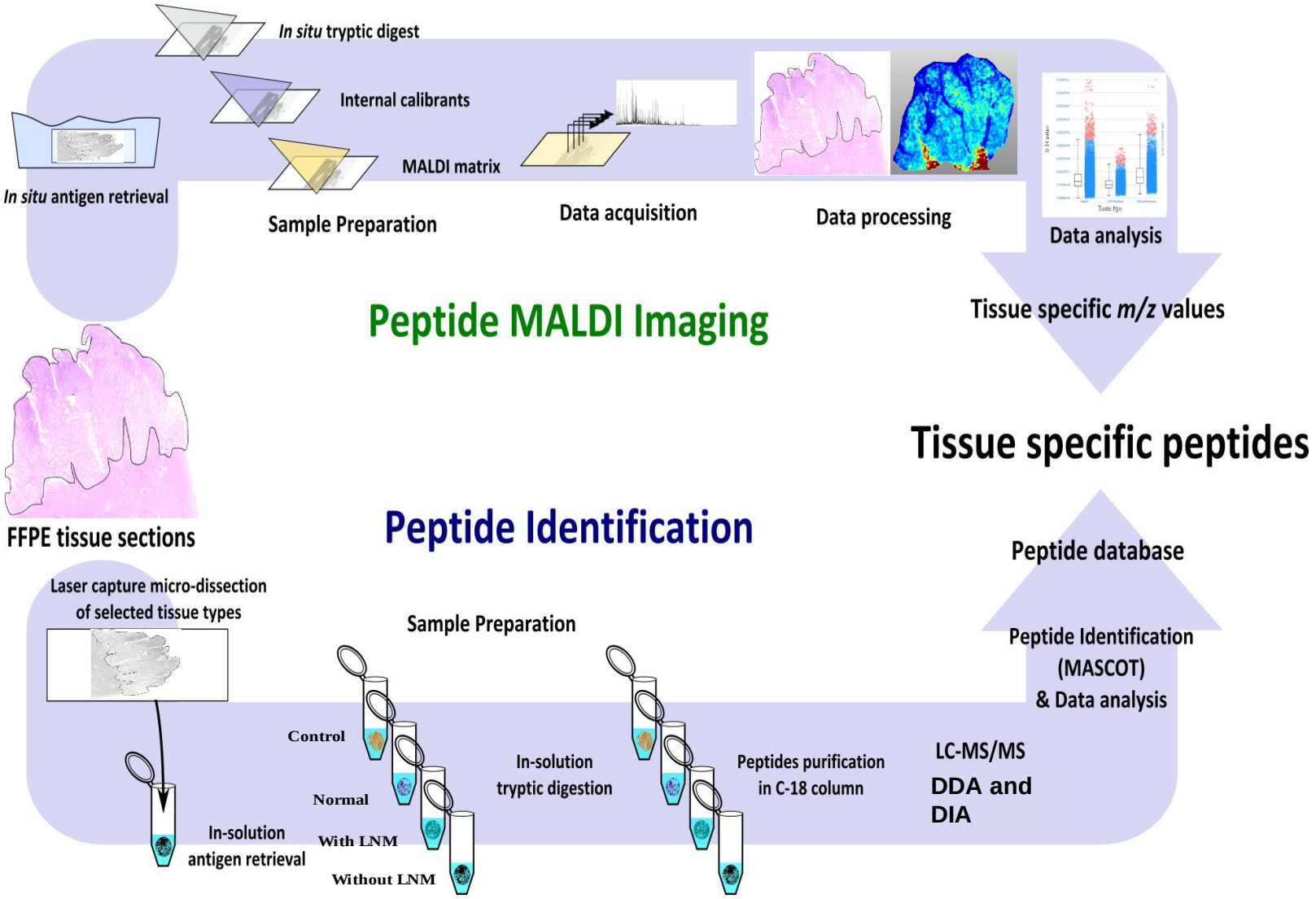
Questions

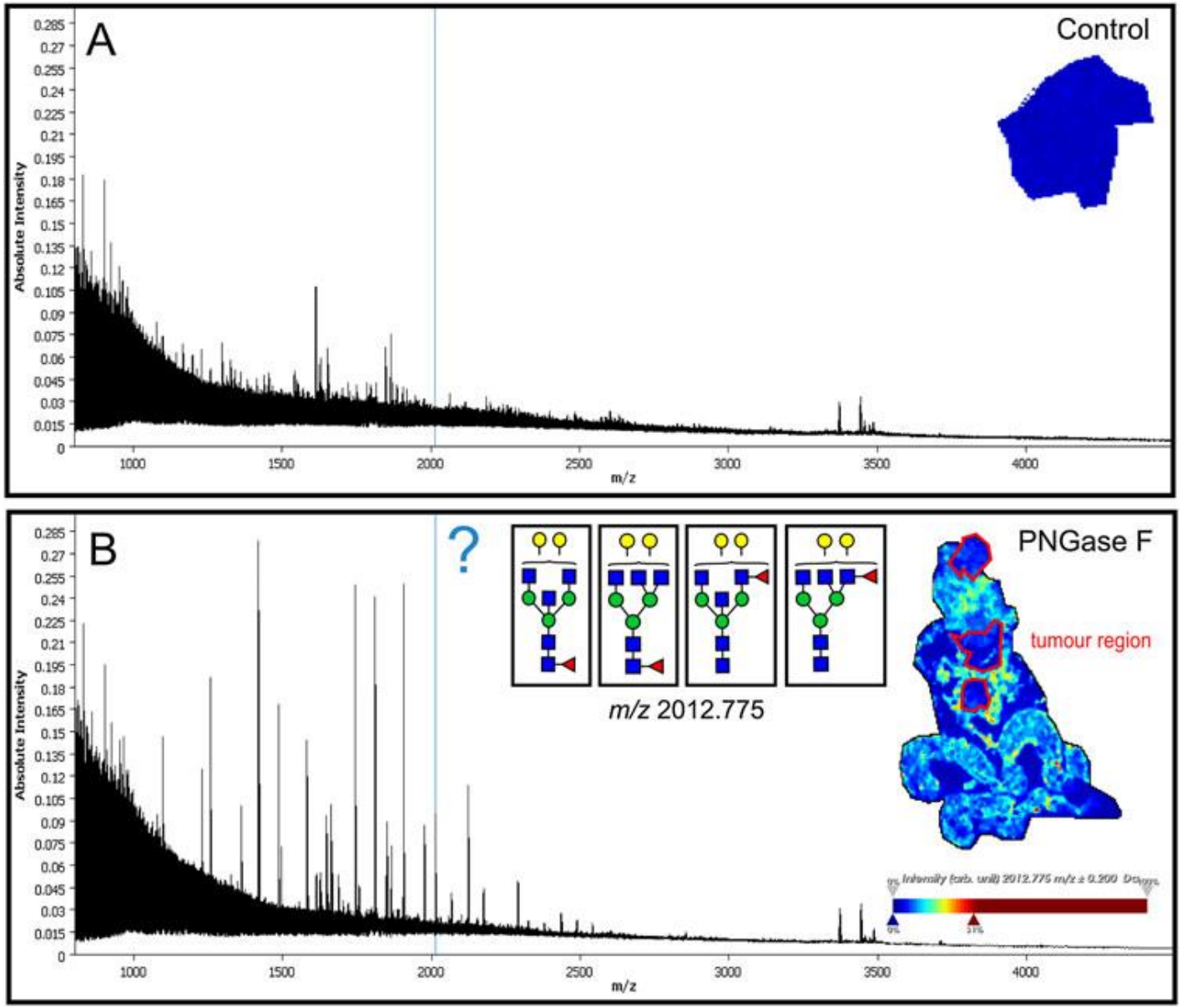
- Can the tumour, stroma and adipose regions of late-stage ovarian cancer tissue sections be differentiated based on the *N*-glycosylation pattern alone?

Workflow of *N*-glycan Imaging

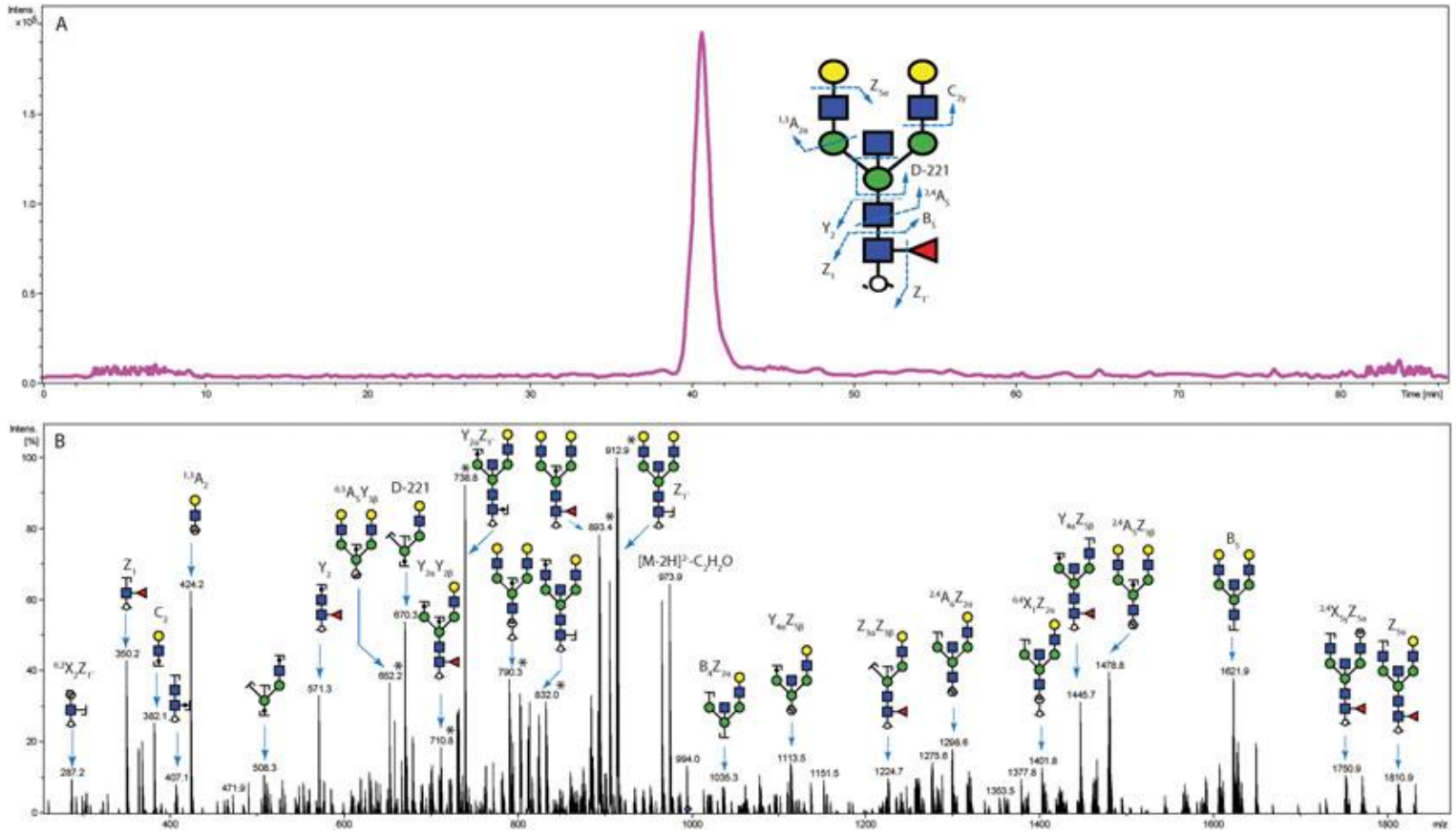


MALD Imaging of FFPE tissue sections



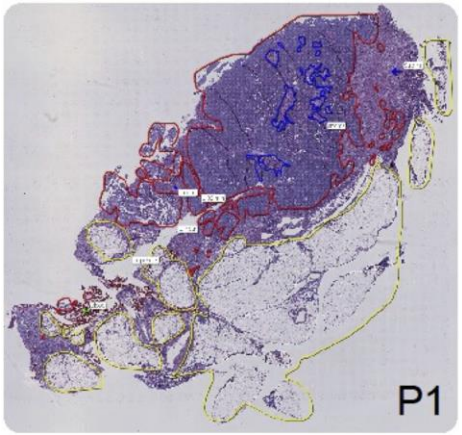

A
Control
Absolute Intensity
m/z
B
?
PNGase F
tumour region
m/z 2012.775
Absolute Intensity
m/z

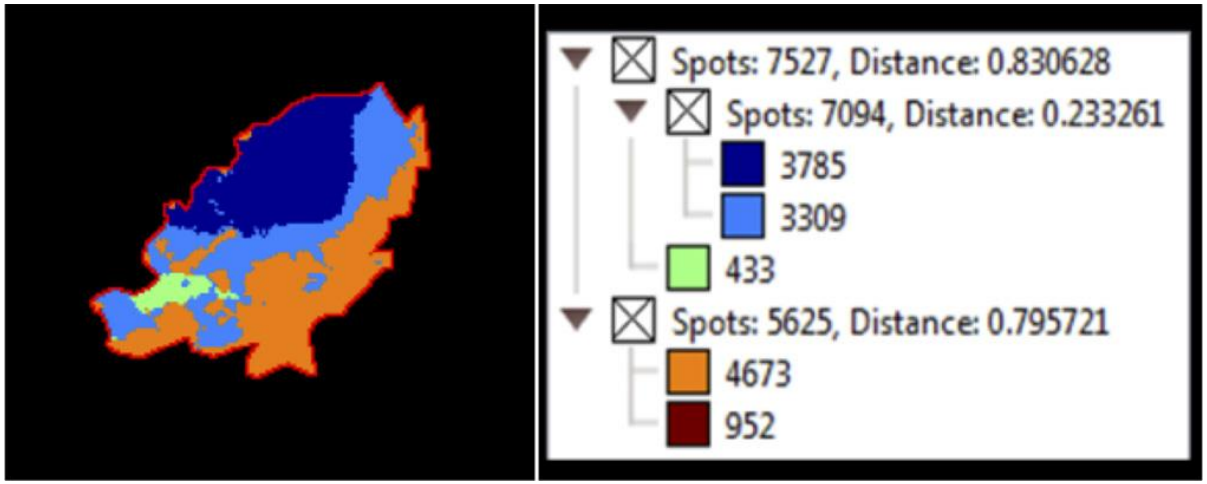
LC-MS/MS: Bisecting *N*-Glycan



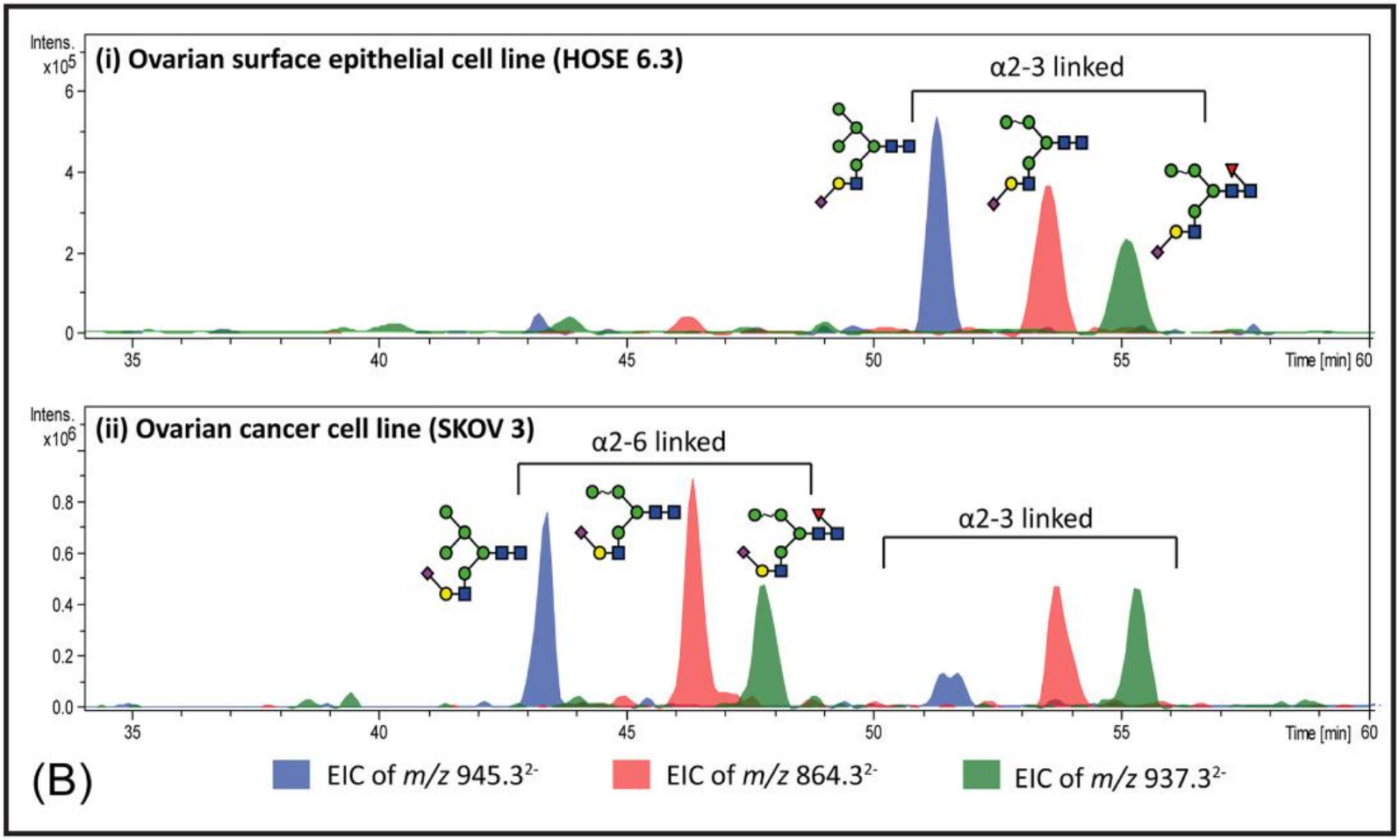
Another Stage IIIC Ovarian Cancer Patient: Segmentation Map

- Tumour
- Stroma
- Adipose



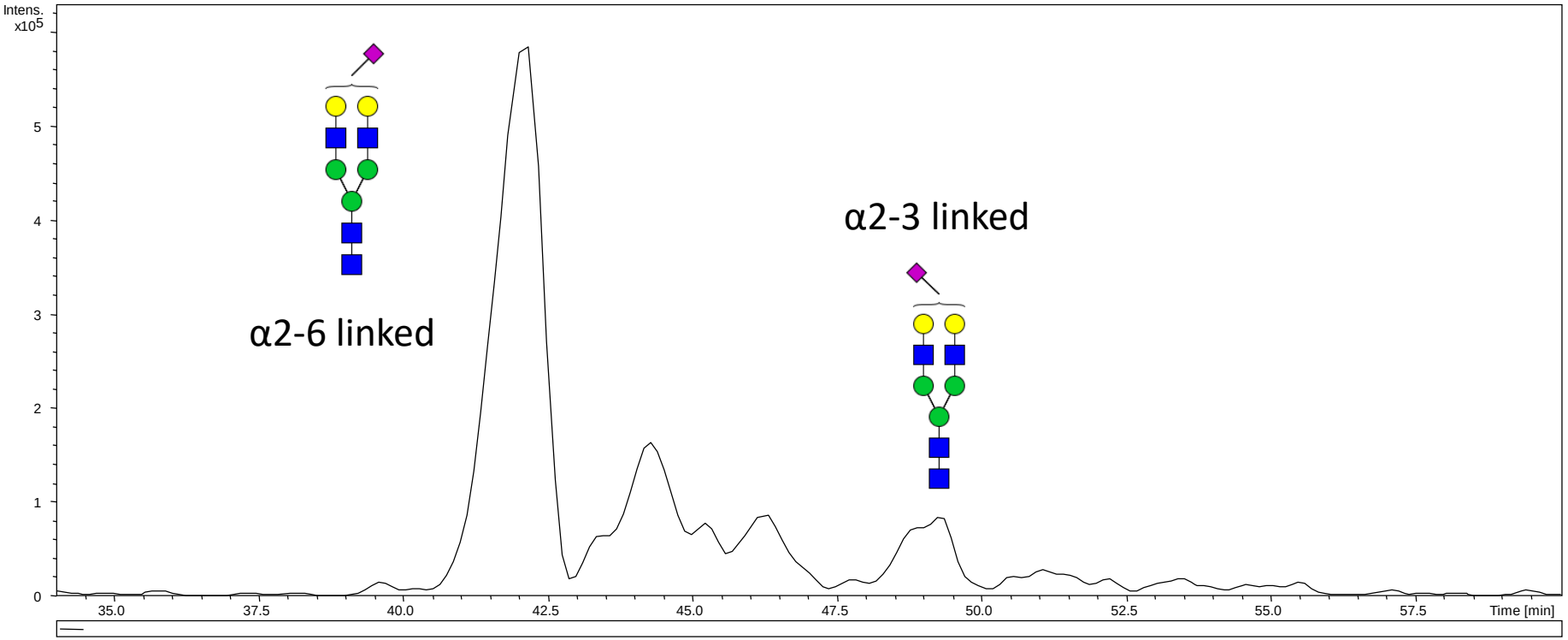


Sialylation in ovarian cell lines



Mol Cell Proteomics. 2014 Sep;13(9):2213-32. doi: 10.1074/mcp.M113.037085.

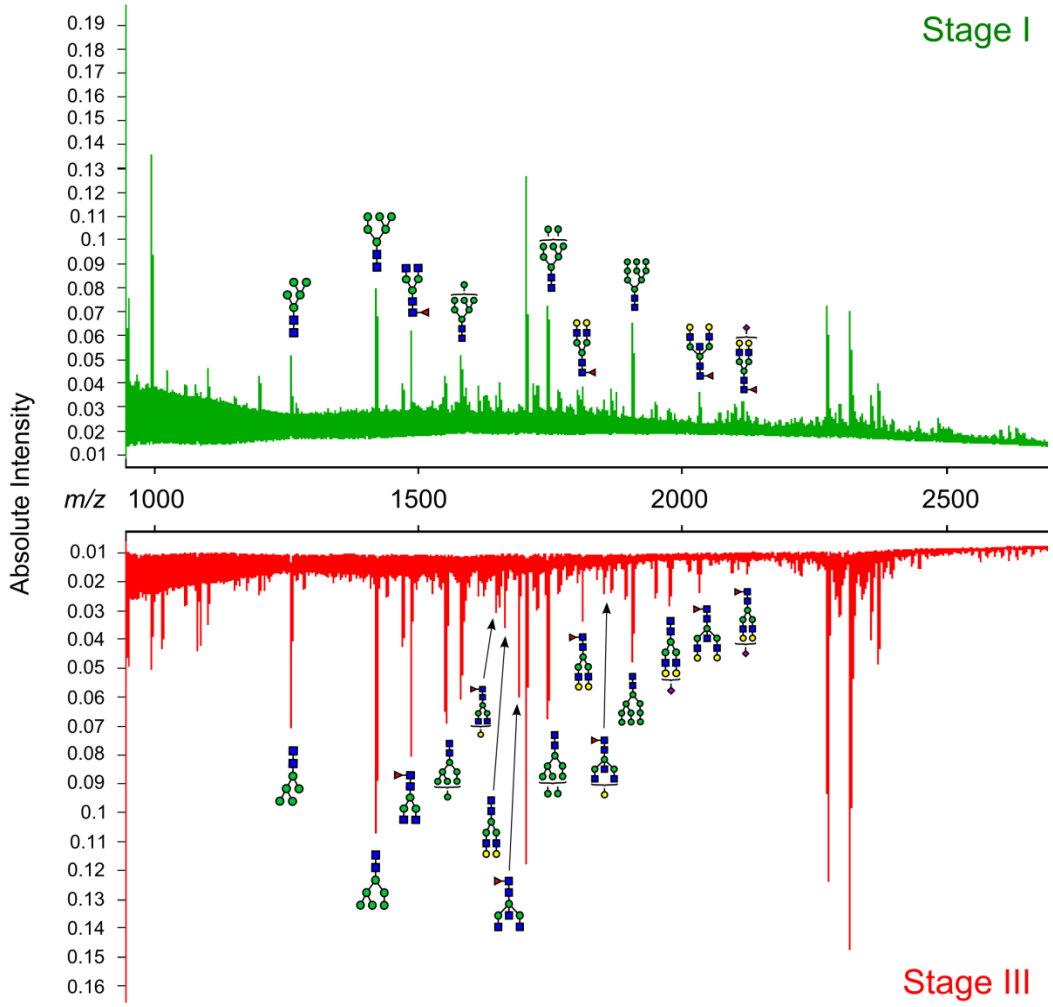
Sialic acid linkages from tumor tissue

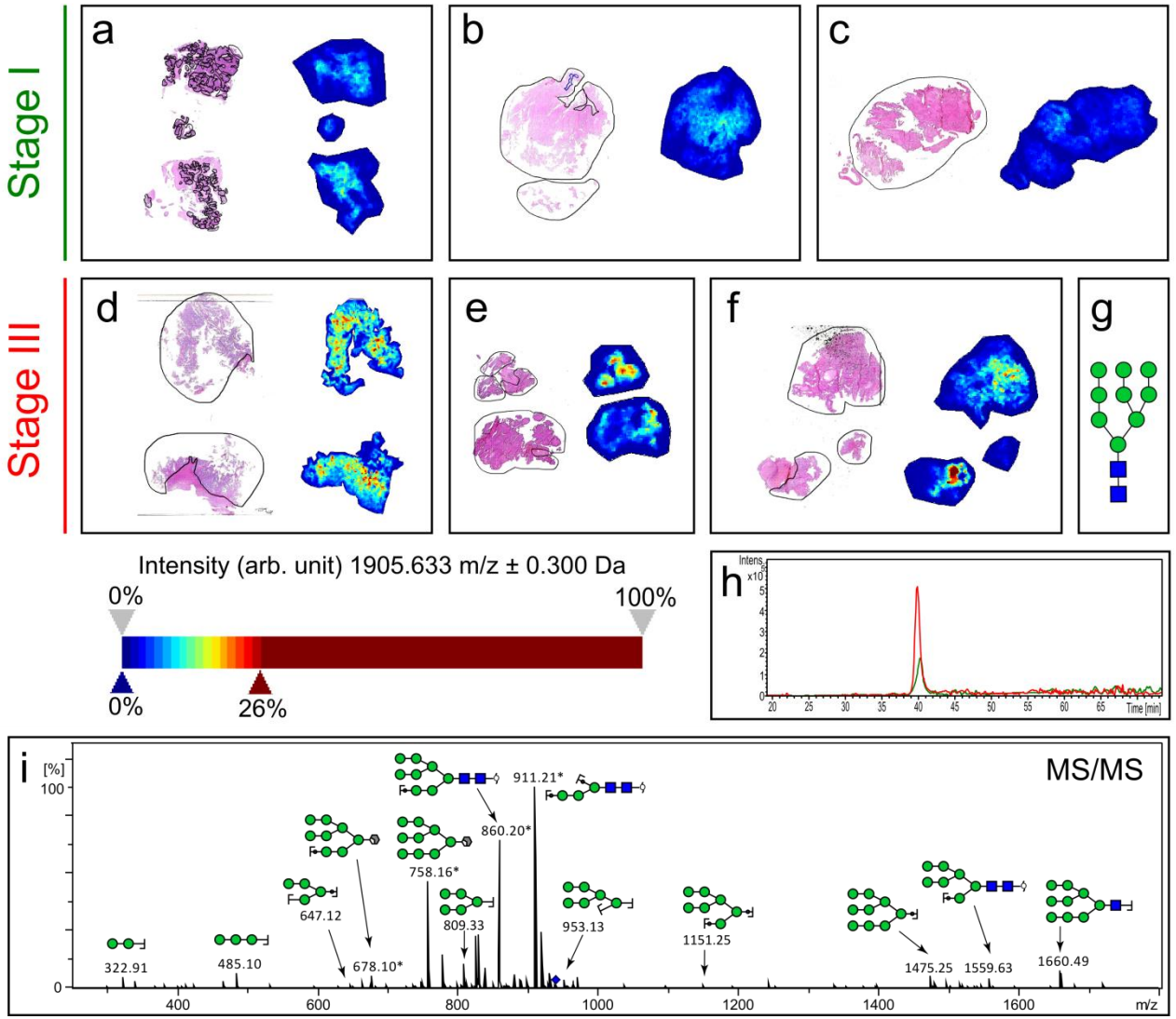


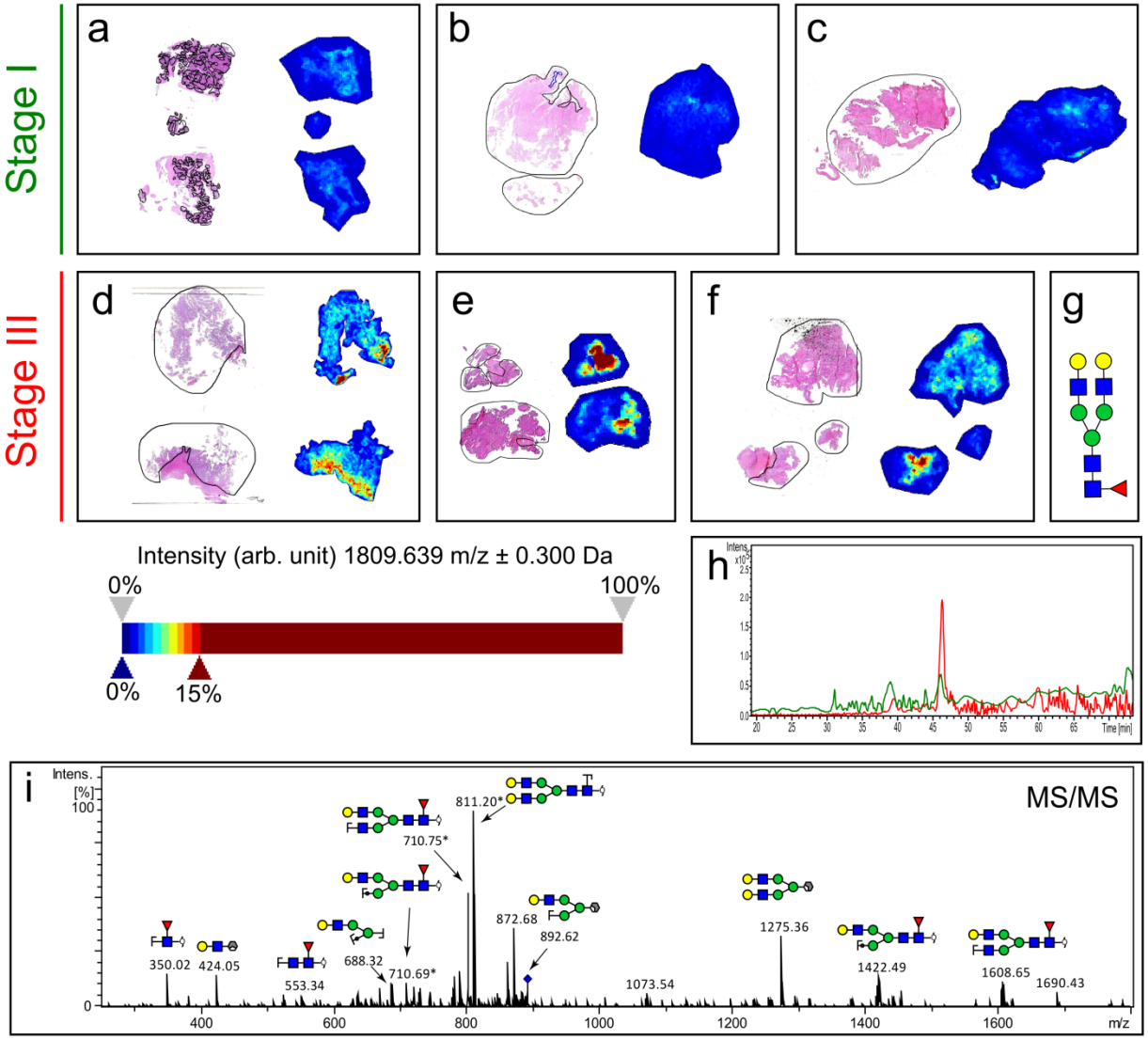
Questions

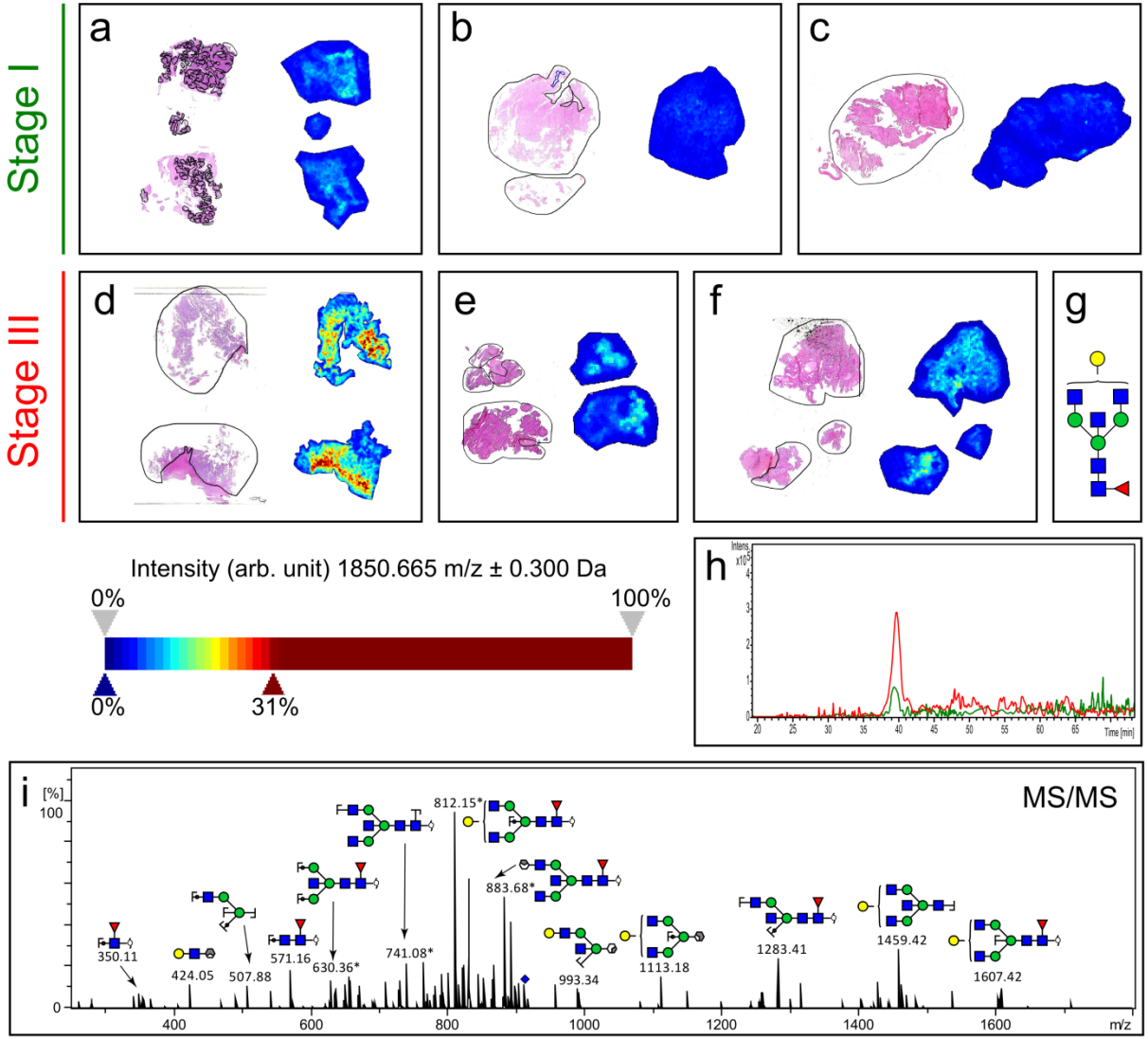
- Can early- and late-stage ovarian cancer tissue sections be differentiated based on the *N*-glycosylation pattern alone?

MALDI Sum Spectra: Stage I vs. Stage III




Stage I
Stage III
a
b
c
d
e
f
g
Intensity (arb. unit) 1905.633 m/z ± 0.300 Da
0%
100%
0%
26%
h
Time [min]
i
MS/MS
[%]
100
0
322.91
485.10
647.12
678.10*
758.16*
809.33
860.20*
911.21*
953.13
1151.25
1475.25
1559.63
1660.49
400
600
800
1000
1200
1400
1600
m/z


Stage I
Stage III
a
b
c
d
e
f
g
Intensity (arb. unit) 1809.639 m/z ± 0.300 Da
0%
100%
0%
15%
h
i
Intens.
[%]
100
0
MS/MS
811.20*
710.75*
710.69*
872.68
892.62
688.32
553.34
350.02
424.05
1073.54
1275.36
1422.49
1608.65
1690.43
400
600
800
1000
1200
1400
1600
m/z


Stage I
Stage III
a
b
c
d
e
f
g
h
i
Intensity (arb. unit) 1850.665 m/z ± 0.300 Da
0%
100%
0%
31%
MS/MS
[%]
100
0
350.11
424.05
507.88
571.16
630.36*
741.08*
812.15*
883.68*
993.34
1113.18
1283.41
1459.42
1607.42
400
600
800
1000
1200
1400
1600
m/z

Summary

Increased *N*-glycans in late-stage tumour regions relative to early-stage tumour regions:

- High mannose
- Complex/fucosylated
- Bisecting

However, a larger cohort of patients (tissue microarray) is required

Overall Summary

Overall, our group has successfully:

- Established a *N*-glycan MALDI-MSI workflow with complimentary LC-MS/MS of consecutive sections
- Applied our *N*-glycan MALDI-MSI workflow to FFPE ovarian cancer to answer clinical questions
- Discovered *N*-glycan differences between early- and late-stages ovarian cancer patients that could lead to the potential discovery of a novel diagnostic marker

MALDI Imaging on *N*-glycans

Gustafsson OJ**, Briggs MT**, Condina MR, Winderbaum LJ, Pelzing M, McColl SR, Everest-Dass AV, Packer NH, **Hoffmann P.** MALDI imaging mass spectrometry of N-linked glycans released from formalin-fixed murine kidney. Anal & Bioanal Chem., **2015**, 407, 2127-2139

M.T. Briggs, J.S. Kuliwaba, D. Muratovic, A.V. Everest-Dass, N.H. Packer, D.M. Findlay, **Peter Hoffmann**. MALDI mass spectrometry imaging of N-glycans on tibial cartilage and subchondral bone proteins in knee osteoarthritis. Proteomics, **2016**, 16(11-12):1736-41.

A.V. Everest-Dass*, **M.T. Briggs***, K. Gurjeet, M.K. Oehler, **P. Hoffmann***, N.H. Packer*. N-glycan MALDI imaging mass spectrometry on formalin-fixed paraffin-embedded tissues enables the delineation of ovarian cancer tissues. MCP, **2016**, 15(9): 3003-3016.

M.T. Briggs, Y.Y. Ho, G. Kaur, M.K. Oehler, A.V. Everest-Dass, N.H. Packer, **P. Hoffmann**. 2N-glycan matrix-assisted laser desorption/ionization mass spectrometry imaging protocol for formalin-fixed paraffin-embedded tissues. *Rapid Communication in Mass Spectrometry*. **2017** May 30;31(10):825-841.

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- **Matthew Briggs**
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- Michelle Turvey
- Mitch Acland
- Brooke Dilmetz

Royal Adelaide Hospital

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- Dr. Carmela Riccardelli

Macquarie Univervisty

- **Prof. Nicolle Packer**
- **Dr. Arun Dass**

University Sains, Malaysia

- **Prof Gurjeet Kaur**