BILIARY TRACT CANCER SERUM PROFILING USING MAGNETIC BEAD-BASED PEPTIDE EXTRACTION AND MALDI-TOF MASS SPECTROMETRY

Stéphane Camuzeaux

Cancer Proteomics Laboratory
Institute for Women's Health
University College London

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Background

- Cholangiocarcinoma and gall bladder cancer are referred to as Biliary Tract Cancer (BTC). Around 1,600 death each year in the UK

- Early diagnosis is key to reducing mortality

- BTC is uniformly fatal unless detected early with the potential for surgical resection and has a dismal prognosis

- The standard for diagnosis is cytological or histological confirmation of malignancy within the biliary stricture; involving invasive procedures

- Need non-invasive alternatives

Background

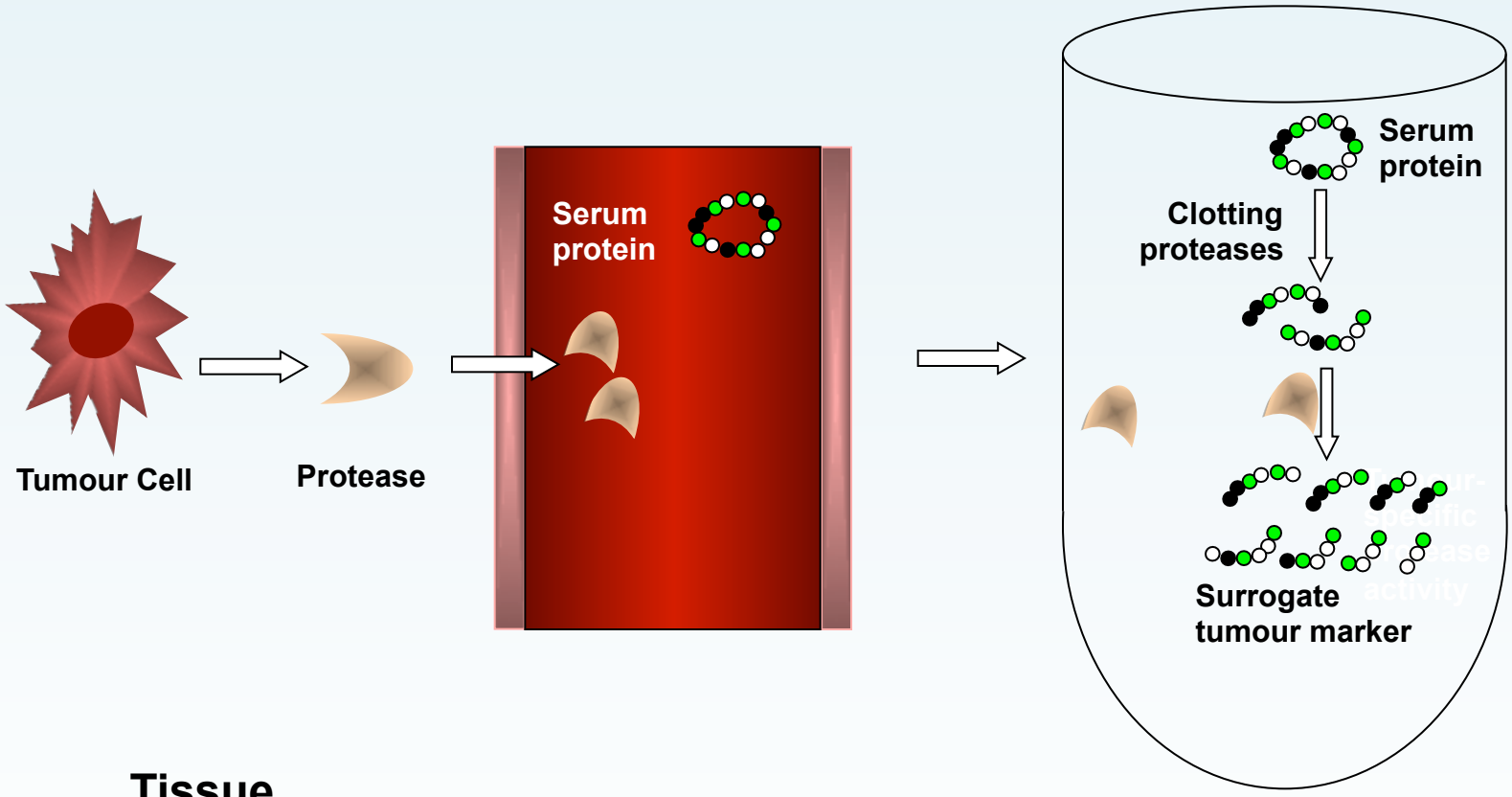
- Most commonly used blood marker is CA19-9 (carbohydrate antigen 19-9/sialylated Lewis (a) antigen)

- However, CA19-9 lacks adequate sensitivity and specificity; it is often elevated in benign conditions (cholangitis and pancreatitis) and undetectable in 7% of the population who are Lewis (a) negative

- Need to find better diagnostic markers for early BTC detection, preferably from human serum

- It has been postulated that the serum peptidome may be a valuable source of diagnostic cancer biomarkers, specifically in relation to the activity of tumour-related exopeptidases

Generation of surrogate tumour markers in the blood

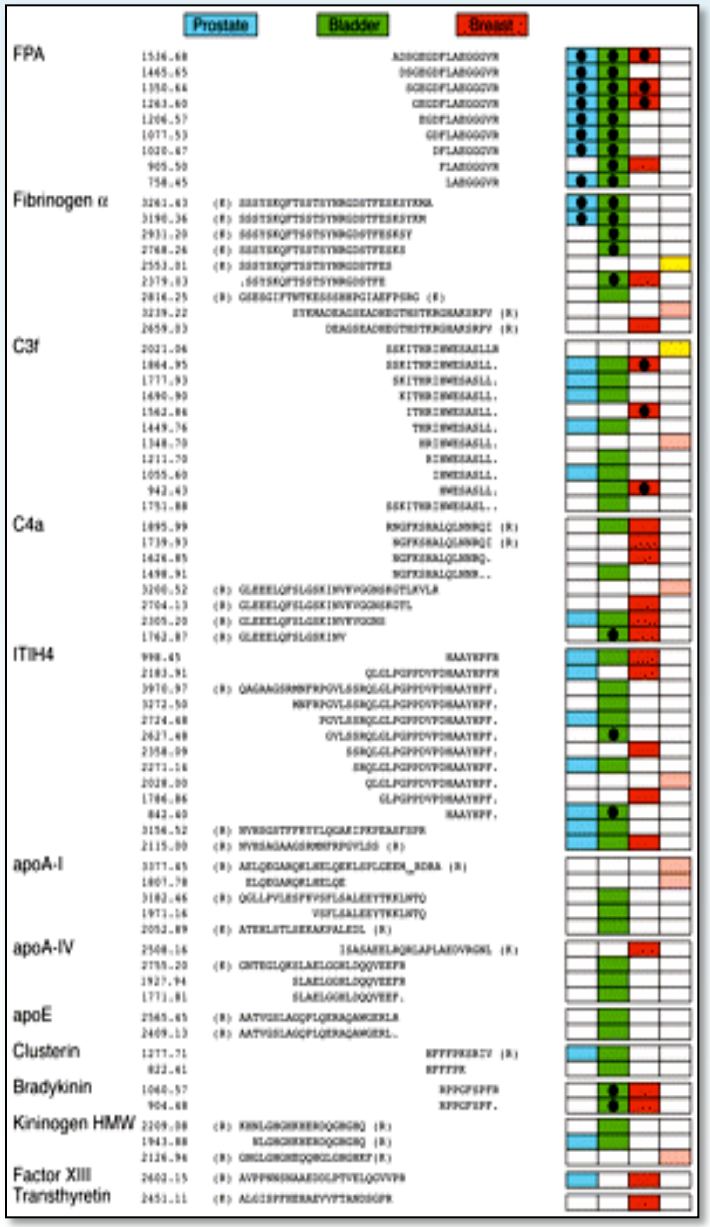


Villanueva *et al.*,2005 JCI

Serum peptidome profiling

- Peptide signatures diagnostic and specific for prostate, breast and bladder cancer were found by MALDI-TOF profiling using C8-coated magnetic beads for peptide extraction

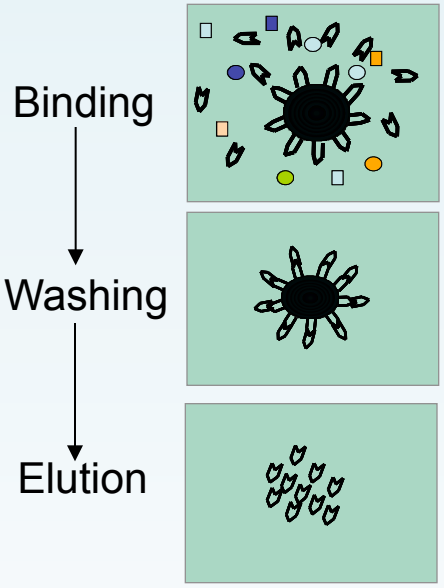
- Ladders of peptides identified as products of abundant serum proteins hypothesised to be generated *ex vivo* (at clotting) by tumour-specific exopeptidase activities – surrogate markers

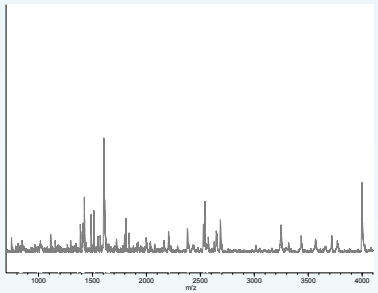


prostate
bladder
breast
decreased ion intensity
increased ion intensity

From Villanueva *et al.*, 2006 JCI

High-throughput semi-automated serum peptide profiling by MALDI-TOF MS





Serum collection from patient → Robotic Handling → Automated mass spectrometry acquisition → Data processing and statistical analysis

C18 magnetic bead extraction

Two independent sets:
-discovery set
-validation set

Biomarker discovery workflow

- Platform reproducibility assessed (intra/inter assay precision) using QC Sigma serum

- Analyse case and control serum samples

- Data analysis to find discriminatory peaks between groups

- Generation of a model

    - Discovery set: training set and test set

- Validation of the model (validation set)

- Identification of peaks of interest

Sample handling

- Blood collected in gel tubes (gel plug)

- Tubes inverted five times

- 60 min clotting at room temperature

- Centrifuged, aliquoted

- Storage at -80°C

- Discovery set: 95 case control serum samples collected from patients diagnosed with BTC, benign biliary strictures and healthy volunteers attending University College London Hospital between 2006 and 2008

- Validation set: 14 BTC and 16 healthy volunteer samples collected in 2009 and 2010

(Healthy control volunteers had no active illnesses and were not on medication)

Platform reproducibility

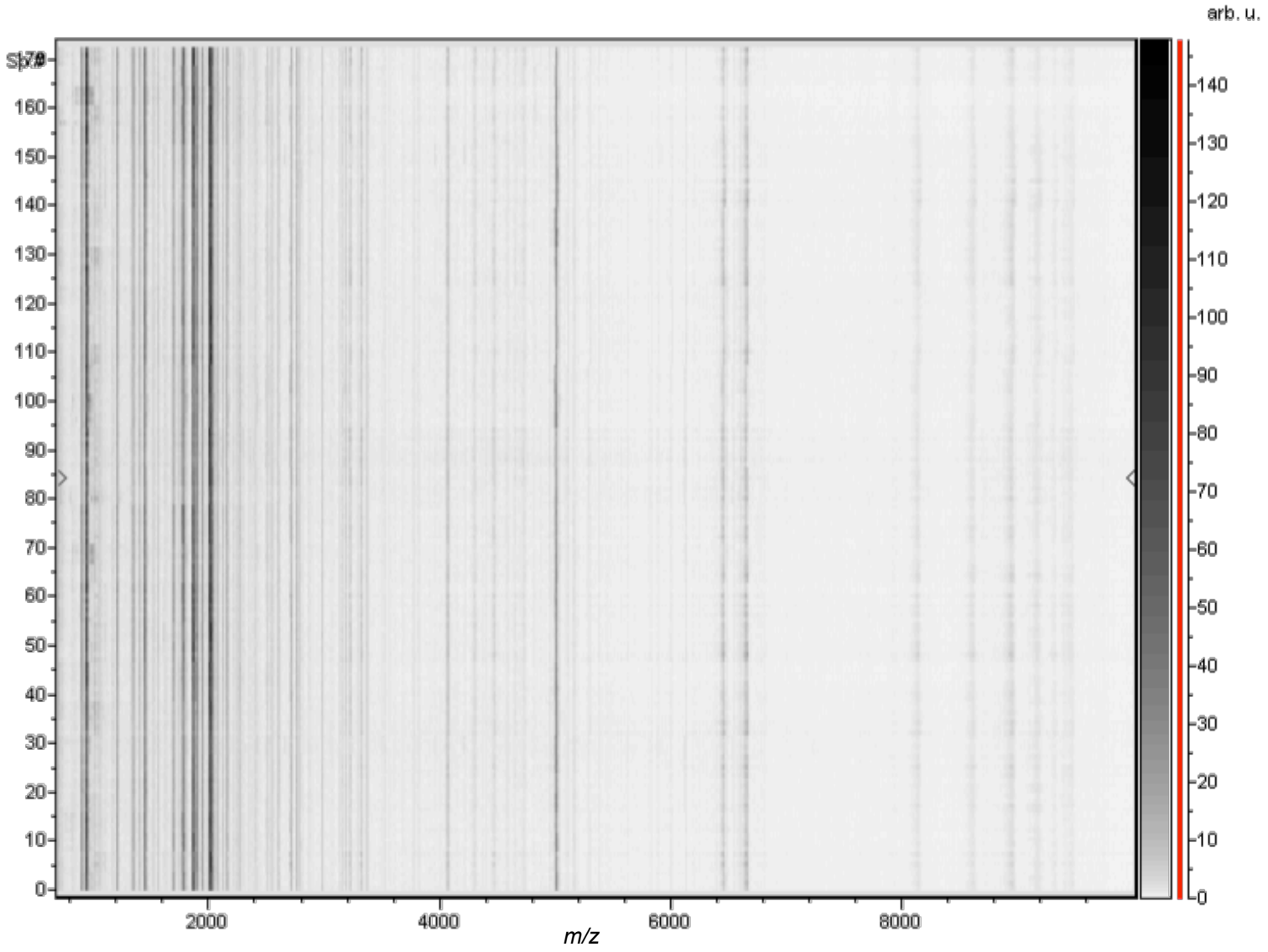
-Three replicate runs, four spotting replicates per sample

-Intra- and inter-assay variation for quality control using Sigma serum:

Discovery set:
-27 Sigma serum across 12 MALDI targets:
-Average intra-assay variation (all peaks; S/N>3): 10.6% +/- 7.2
-Inter-assay variation (all peaks; S/N>3): 12.8% +/- 6.7

Validation set:
-24 Sigma serum across 4 MALDI targets:
-Average intra-assay variation (all peaks; S/N>3): 12.8% +/- 10.0
-Inter-assay variation (all peaks; S/N>3): 14.5% +/- 10.8

Platform reproducibility



Spectral filtering

- 1000 shots acquired for each spotting (up to 12 positions, 100 shots per position)
- At least 3 spotting replicates with 1000 shots per sample
- At least 2 run replicates per sample

Number of samples included after filtering:

	Discovery set	Validation set
number of samples run	95	30
number of spectra acquired	1140	360
number of spectra included after filtering	1079	355
% of spectra included	94.6	98.6
number of spectra included after visual inspection	1069	347
% of spectra included after visual inspection	93.8	96.4
total number of samples included	92	30
% of sample included	96.8	100

3 samples removed from discovery set

Sample sets after filtering

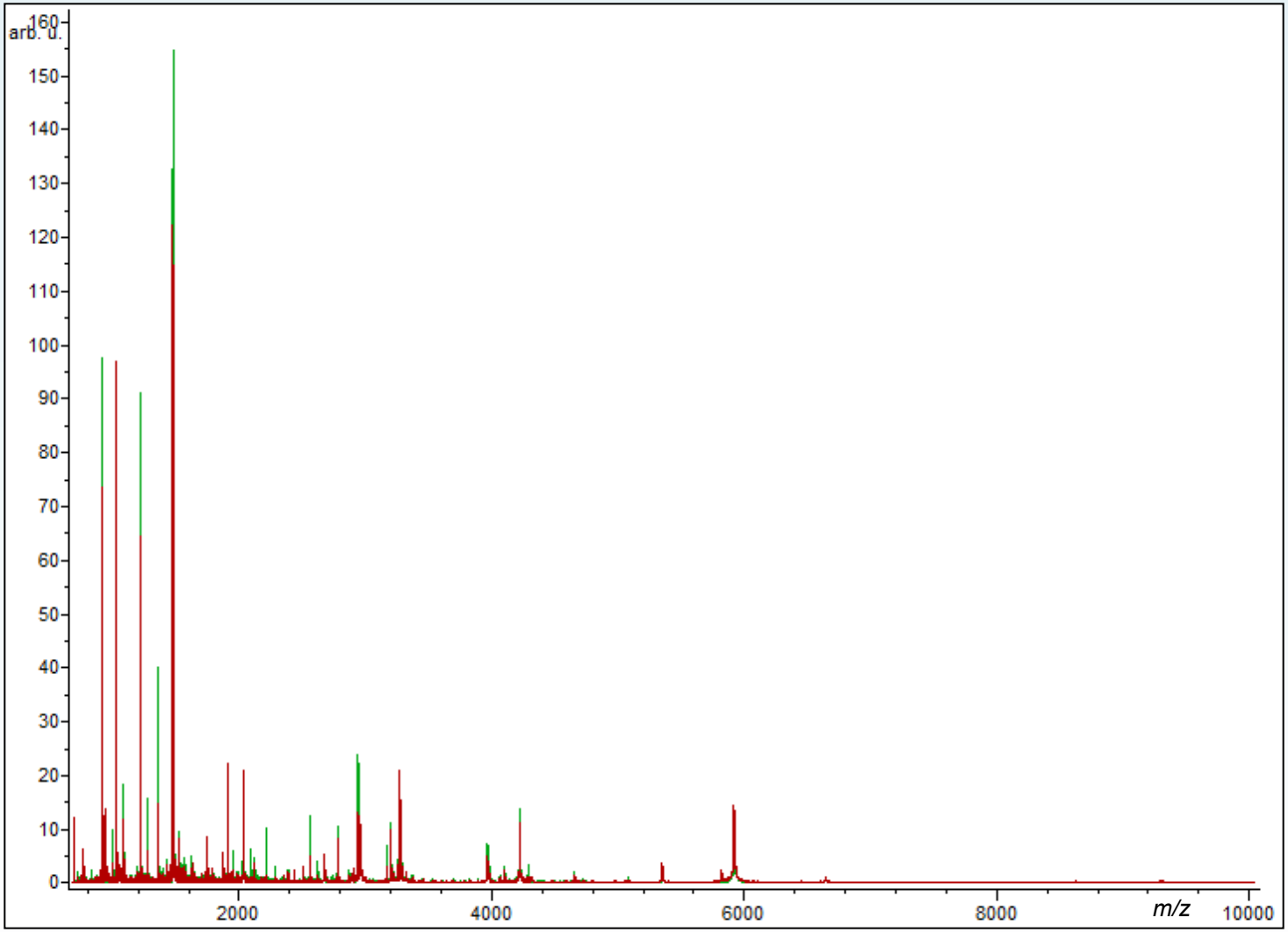
- Discovery set: 92 samples

Group	Patients	Gender	Median age (yrs)	Age range (yrs)	Median bilirubin (g/L)	Median CA19-9 (IU/mL)	CA19-9 >37 IU/ mL	BTC stage <T3	BTC stage ≥T3
BTC	39	15F:24M	67	26-92	40	295	30/39	21/39	18/39
Healthy	22	7F:15M	60	39-78	--	--	--	n/a	n/a
PSC	10	3F:7M	48	22-76	17	17	3/10	n/a	n/a
AIP/IAC	7	7M	63	43-71	12	15	1/4	n/a	n/a
Benign other	14	9F:5M	53	35-74	8	--	--	n/a	n/a

- Validation set: 30 samples

Group	Patients	Gender	Median age (yrs)	Age range (yrs)	Median bilirubin (g/L)	Median CA19-9 (IU/mL)	CA19-9 >37 IU/ mL	BTC stage <T3	BTC stage ≥T3
BTC	14	7F:7M	73	44-90	19	404	11/14	6/14	8/14
Healthy	16	8F:8M	34	23-80	--	--	--	n/a	n/a

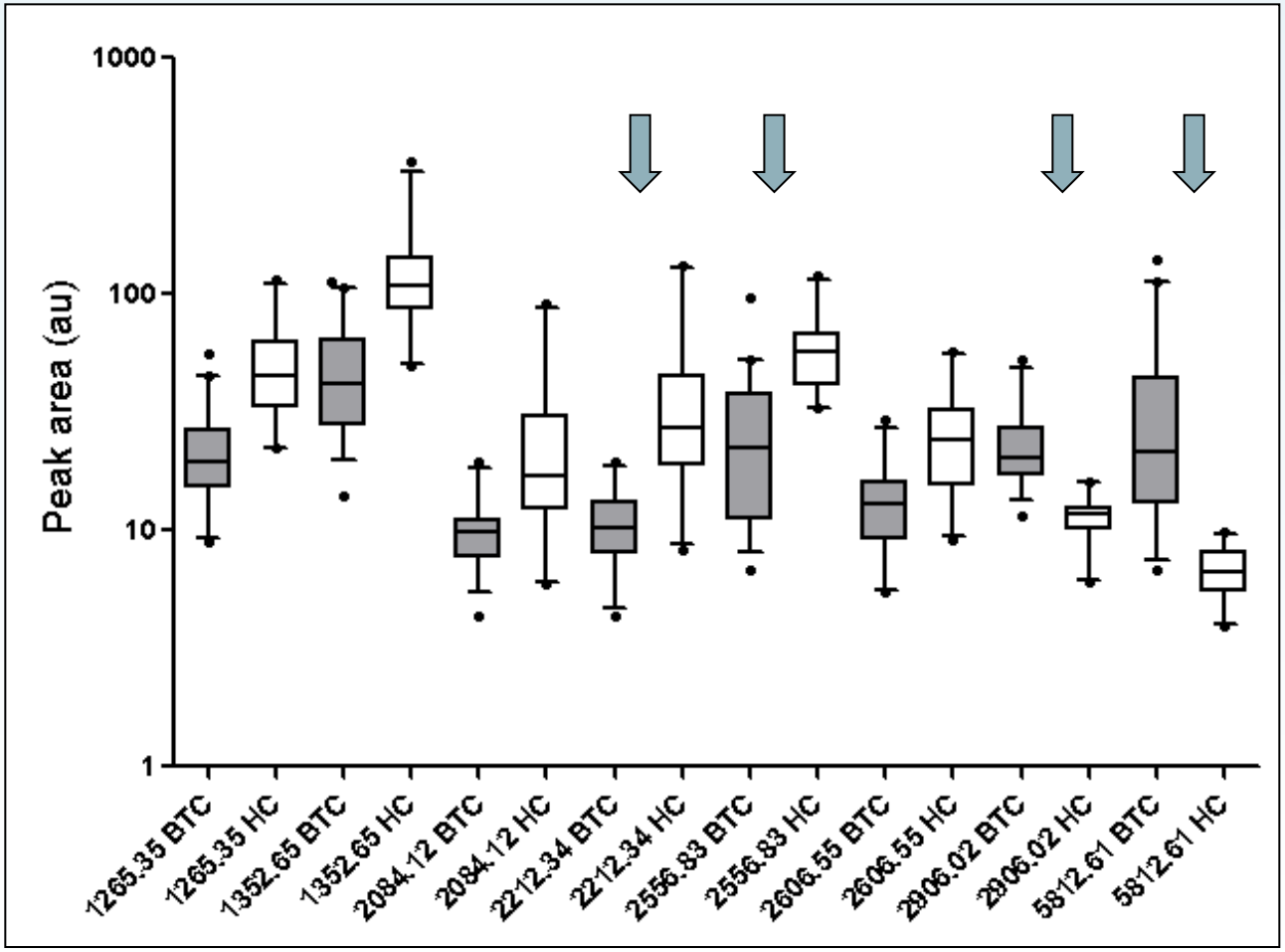
Average MALDI-TOF spectra

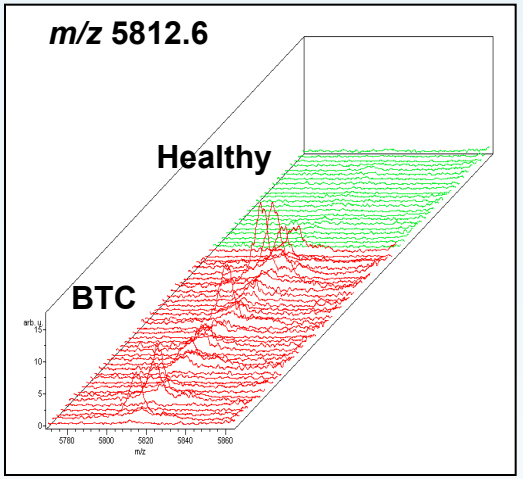


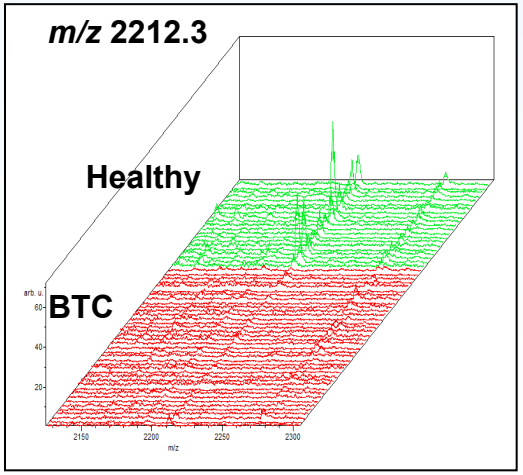
Discovery set: BTC (red) and healthy (green)

Discriminatory peaks in discovery set

- 8 peaks found to significantly discriminate BTC from healthy ($p<0.001$, average fold ≥ 2)
- 5 peaks discriminate BTC from benign group ($p<0.05$, average fold ≥ 1.5); 4 common



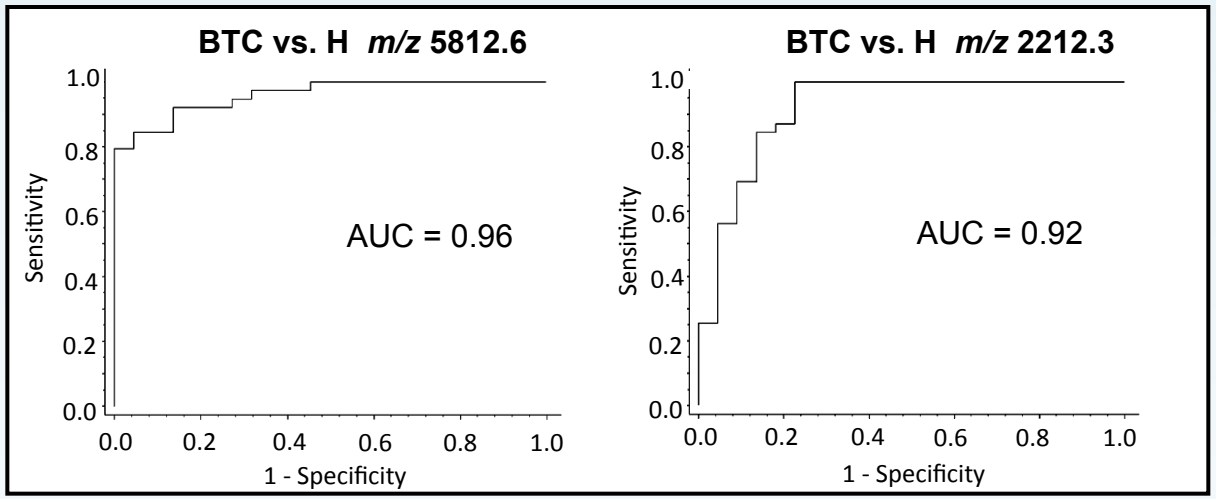




ROC Curve Analysis

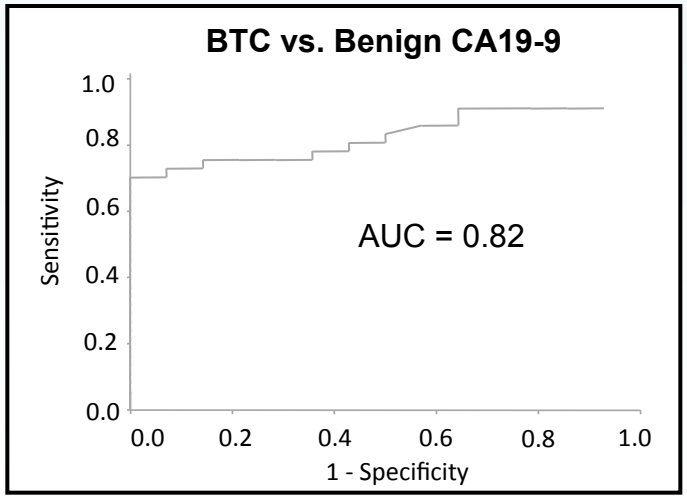
- ROC curve BTC vs. Healthy AUC:

m/z 2606.6 : 0.82
m/z 2084.1 : 0.86
m/z 2556.8 : 0.91
m/z 1265.3 : 0.91
m/z 2212.3 : 0.92
m/z 1352.6 : 0.92
m/z 5812.6 : 0.96
m/z 2906.0 : 0.97



- ROC curves BTC vs. Benign AUC:

m/z 5812.6 : 0.70
m/z 2935.7 : 0.71
m/z 2556.8 : 0.73
m/z 2906.0 : 0.76
m/z 2212.3 : 0.77



Model generation and validation

- 5 permutations of : 75% BTC/Healthy training set; 25% BTC/Healthy test set

- Models generated per permutation:
    - Genetic Algorithms (GA) or Support Vector Machine (SVM)
    - Number of k-Nearest Neighbours (k-NN)
    - Number of peaks

- 20% Leave Out Cross Validation (20% LOCV)

- Best performing model SVM 8 peaks, 3 k-NN, 20% LOCV 95.2% :

    m/z: 1021.7, **1265.3**, **1352.6**, 1364.9, **2556.8**, **2906.0**, 5070.7, 8779.6

- Classification of independent validation set: BTC (n=14) / Healthy (n=16)

Sensitivity = 85.7%; Specificity = 100%; PPV = 100%; NPV = 88.9%

Peak identification

- BTC samples pool and healthy samples pool prepared
- Extraction using C18 magnetic bead
- Extracts split in two for parallel top-down analysis: GeLC-MS/MS / Zip-Tip LC-MS/MS

Av Mass (*m/z*)	Name	Fragment Sequence	Identification
1021.7	Fibrinopeptide A	DFLAEGGGVR	yes; Villanueva et al; Tiss et al
1265.3	Fibrinopeptide A	GEGDFLAEGGGVR	yes; Villanueva et al; Tiss et al
1352.6	Fibrinopeptide A	SGEGDFLAEGGGVR	yes; Villanueva et al; Tiss et al
2084.1	Fibrinogen alpha	GGSTSYGTGSETESPRNPSSAG	Koomen et al;
2212.3	HMW kininogen	KHNLGHGHKHERDQGHGHQ	Villanueva et al; Tiss et al
2556.8	Fibrinogen alpha	SSSYSKQFTSSTSYNRGDSTFES	Villanueva et al; Tiss et al
2935.7	Fibrinogen alpha	SSSYSKQFTSSTSYNRGDSTFESKSY	Villanueva et al; Tiss et al
5812.6	Fibrinogen alpha	SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRP	yes
1364.9			no
2606.6			no
2906.0*			no
5070.7			no
8779.6			no

bold=discriminatory peak = used in model BTC vs healthy

Conclusions

- Applied a semi-automated MALDI-TOF MS serum peptidome profiling strategy to a set of BTC case control samples

- Identified discriminatory peaks for BTC vs. healthy. Less robust discrimination of BTC vs. benign group (inflammatory response?)

- Tested a model on an independent validation set that accurately classified BTC cases from healthy control

- Identified peaks used in the model; mostly fragments of abundant serum proteins, suggesting tumour-specific exopeptidase activities

- Need to further define and test models for discrimination of BTC vs. benign group

- Further work: specific assays to develop a clinical test (e.g. using SRM)

Acknowledgements

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