Imperial College London

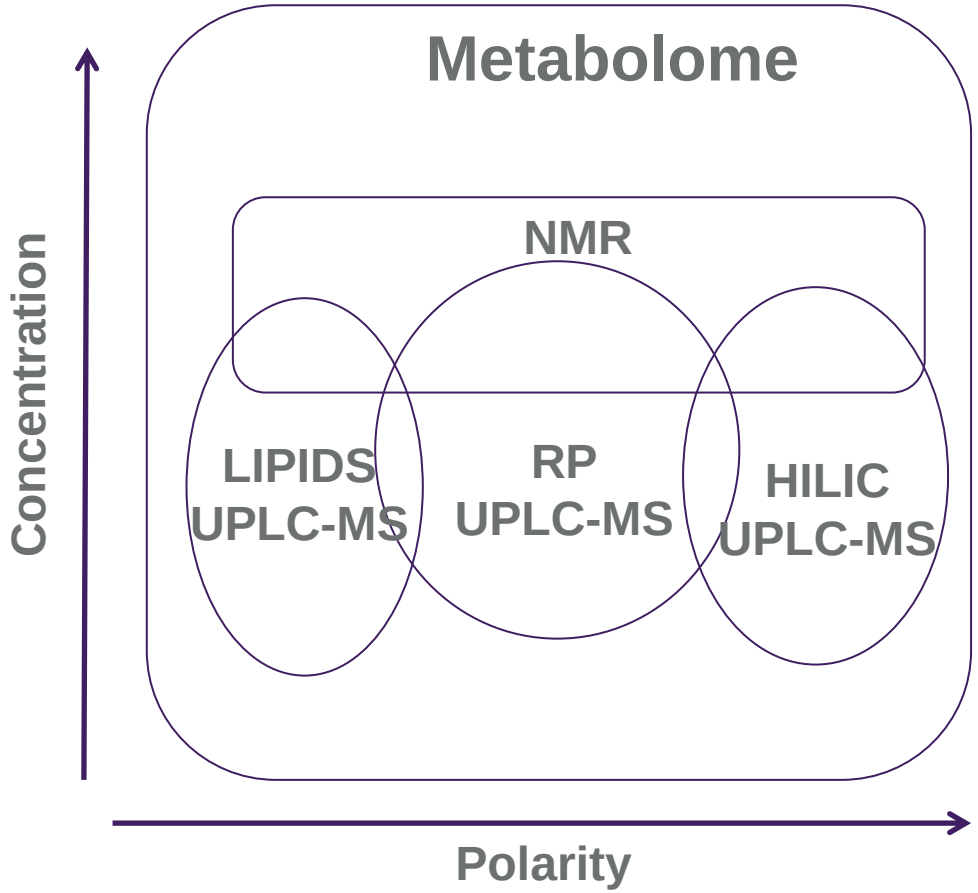
From Metabolomic profiling...
to semi-targeted Metabolomics...
to targeted assays...
...to working examples...

Dr Alexandros Pechlivanis

Imperial College London

Untargeted Metabolomics

- Top down approach
- Qualitative/unbiased screening
- Discovery phase



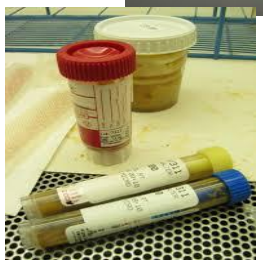
Samples

Urine

Serum/plasma

Faeces

Other biological specimens



Imperial College London

Semi-targeted Metabolomics vs Targeted Assays

A class of compounds is already known as relevant to the biological question

- Chromatographic condition are “tailored” to that specific class. e.g. BAs, lipids, FAs etc.
- Saves time (and money) for the analysis.
- Results can be interpreted more readily
- TOF or triple quad instruments

Few compounds of interest, or compounds identified using untargeted assays

- Chromatography shorter
- Targeting and quantifying those compounds
- Triple quad/ trap instruments

Imperial College London

Stratified Medicine in Primary Biliary Cholangitis (PBC):

Understanding Disease Mechanisms and Targeting Therapies (UK-PBC)

Imperial College London

Few facts about the disease

- *Infects mainly women (9:1) at the age 40-60 (35/100.000)*
- *Individuals who express the characteristic PBC autoantibody, anti-mitochondrial antibody (AMA) but who do not have liver injury (normal liver biochemistry);*
- *The daughters of mothers with PBC who run a 30-fold increased risk of PBC;*
- *Patients who have undergone liver transplantation for PBC and have a 1 in 3 chances of developing recurrent PBC within 5 years of their graft.*
- *UDCA as a damage control therapy, mitigating the toxic effects of hydrophilic BAs*

Currently no approaches proven effective at preventing PBC in such "at risk" individuals

Imperial College London

Breaking news

UK-PBC HOME ABOUT NEWS EVENTS PROJECTS RESOURCES INDUSTRY PATIENTS PROJECT TEAM CONTACT US

FDA Support for Obeticholic Acid (OCA)

POSTED ON APRIL 8, 2016 BY UKPBC

UK-PBC team helps new PBC drug to the point of licensing. UK-PBC investigators have been heavily involved in the FDA approval process for Obeticholic Acid, a novel drug for use in PBC patients unresponsive to UDCA.

On Thursday 7th April an Independent Advisory Committee voted 17 to 0 to support FDA approval of OCA which will now be the first new drug approved for use in PBC in 20 years. Data from the UK-PBC played a key role in convincing the committee that there is significant unmet need in PBC and thus a need for new drugs.

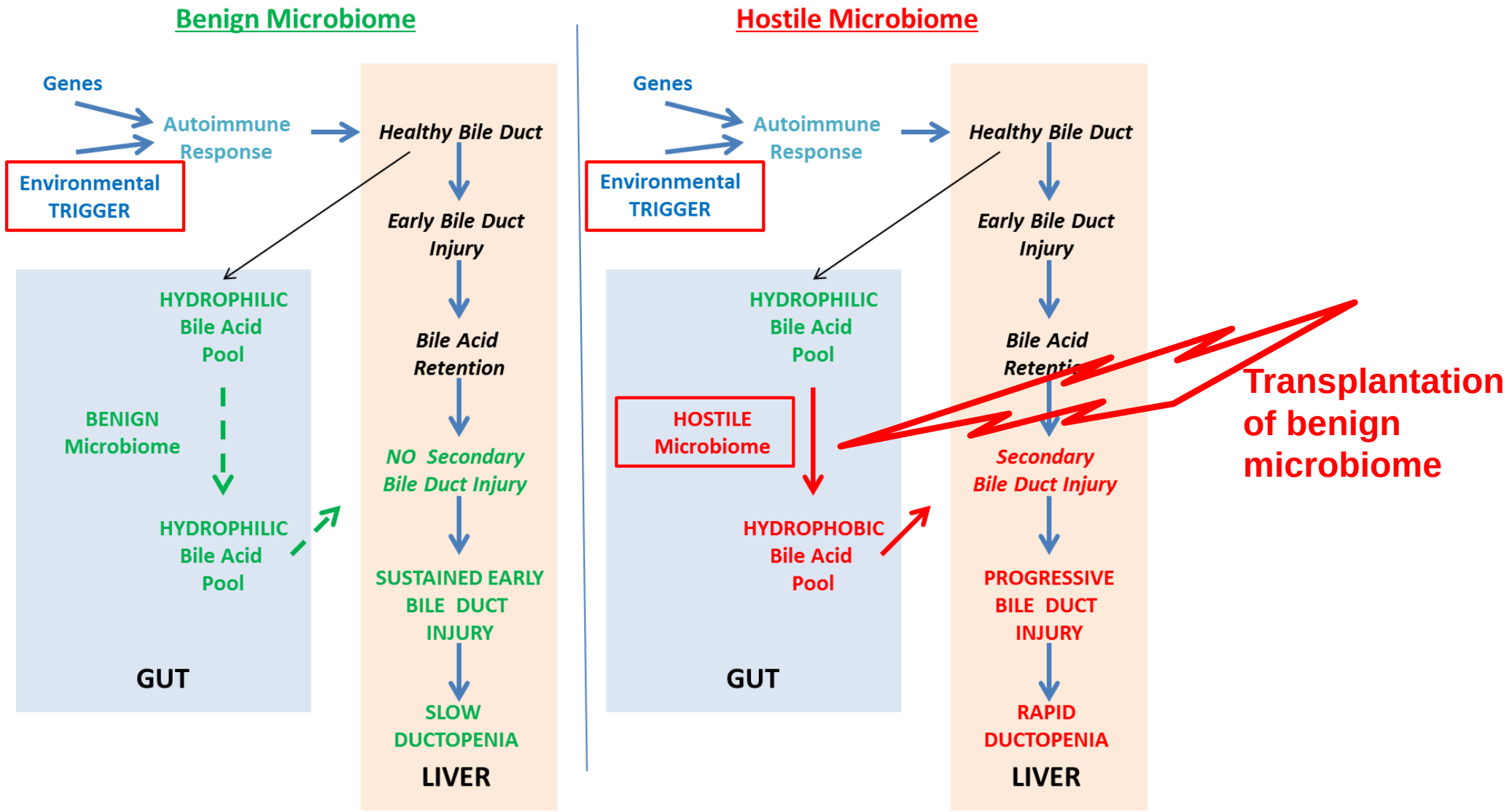
NEWS BY DATE

April 2016 January 2016
December 2015 October 2015
September 2015 August 2015
May 2015

the first new drug approved for use in PBC in 20 years!!!

Imperial College London

Hypothesis



Imperial College London

1) Profiling the PBC Metabolome

1) Untargeted screening
   i. Using Nuclear Magnetic Resonance (NMR) Spectroscopy
   ii. Using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)
   iii. Using MS imaging to map the distribution of BAs and lipids in liver tissue
2) Targeted screening of bile acids, eicosanoids and lipids using UPLC-MS

TARGET

Identification of biomarkers that are highly discriminatory between patient subgroups (responders and non-responders to UCDA)

Imperial College London

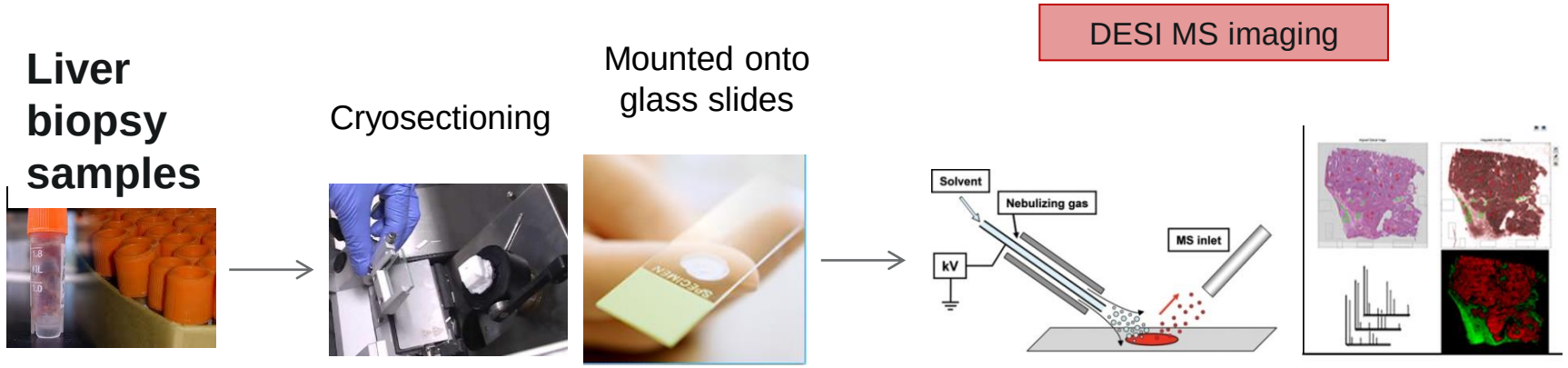
2) Profiling the gut microbiota using 16S rRNA genes and the 454 platform

1) Create inventories of the bacterial diversity using 454 sequencing
2) Taxonomic and diversity data
3) Determine total numbers of Bacteria and Archaea using qPCR for each kingdom's 16S rRNA genes
4) Identify bile salt hydrolase (BSH) and 7β hydroxysteroid dehydrogenases (HSDH) diversity using 454 sequencing

Imperial College London

3) Profiling topographical distribution of biomolecules using Mass Spectrometry Imaging

1) Assess the distribution of bile acids (in particular UDCA) across liver biopsies from responders and non-responders
2) Obtain a global spectral profile of the topographical differences in tissue biochemistry using DESI Imaging Mass Spectrometry



Imperial College London

Development of an efficient extraction method of Bile Acids from faecal material

The problem and how we tackle it

The problem:

1. BAs are a very chemically diverse group of compounds. (from very hydrophilic to very lipophilic)
2. Faecal samples are very inhomogeneous.
3. Efficiency of currently used extraction protocols is poor.
4. There are currently in the literature many different approaches.

Our approach:

1. Use of fresh or lyophilized material?
2. Testing different solvent mixtures according to the literature/ lab practise;
3. Solid phase extraction (time consuming/ efficiency);
4. Single stage extraction (incomplete extraction of the different BAs);
5. Sequential extraction (more time consuming but more complete extraction)

The criteria:

- Reproducibility, robustness, ease of use;
- The quantitative measurement of 56 BAs with use of UPLC-MS;
- Extraction efficiency varies 50-110%

Imperial College London

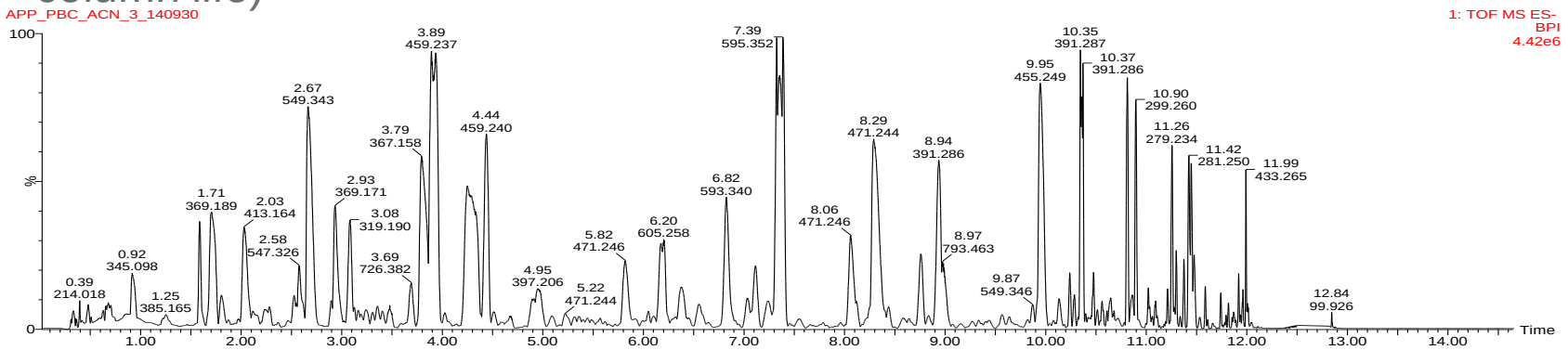
Development of an efficient extraction method of Bile Acids from faecal material

Samples lyophilisation

1. Solid phase extraction
2. Single stage extraction
3. Sequential extraction

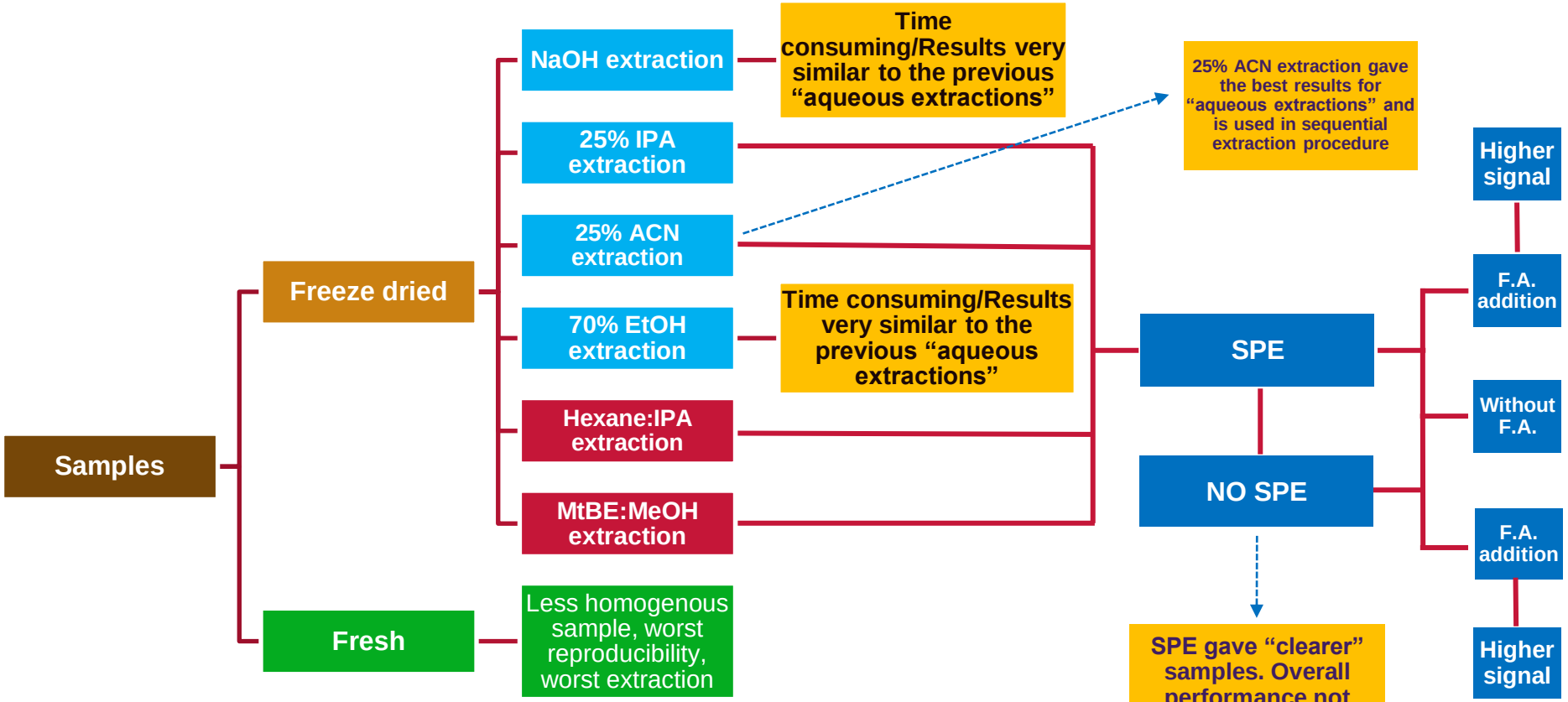
Crucial steps in extraction procedure:

- Complete drying of the samples (stability/ homogeneity)
- The choice of the extraction solvent mixture(s)
- The cleanliness of the final extract (MS ion suppression/ chromatographic column life)



Imperial College London

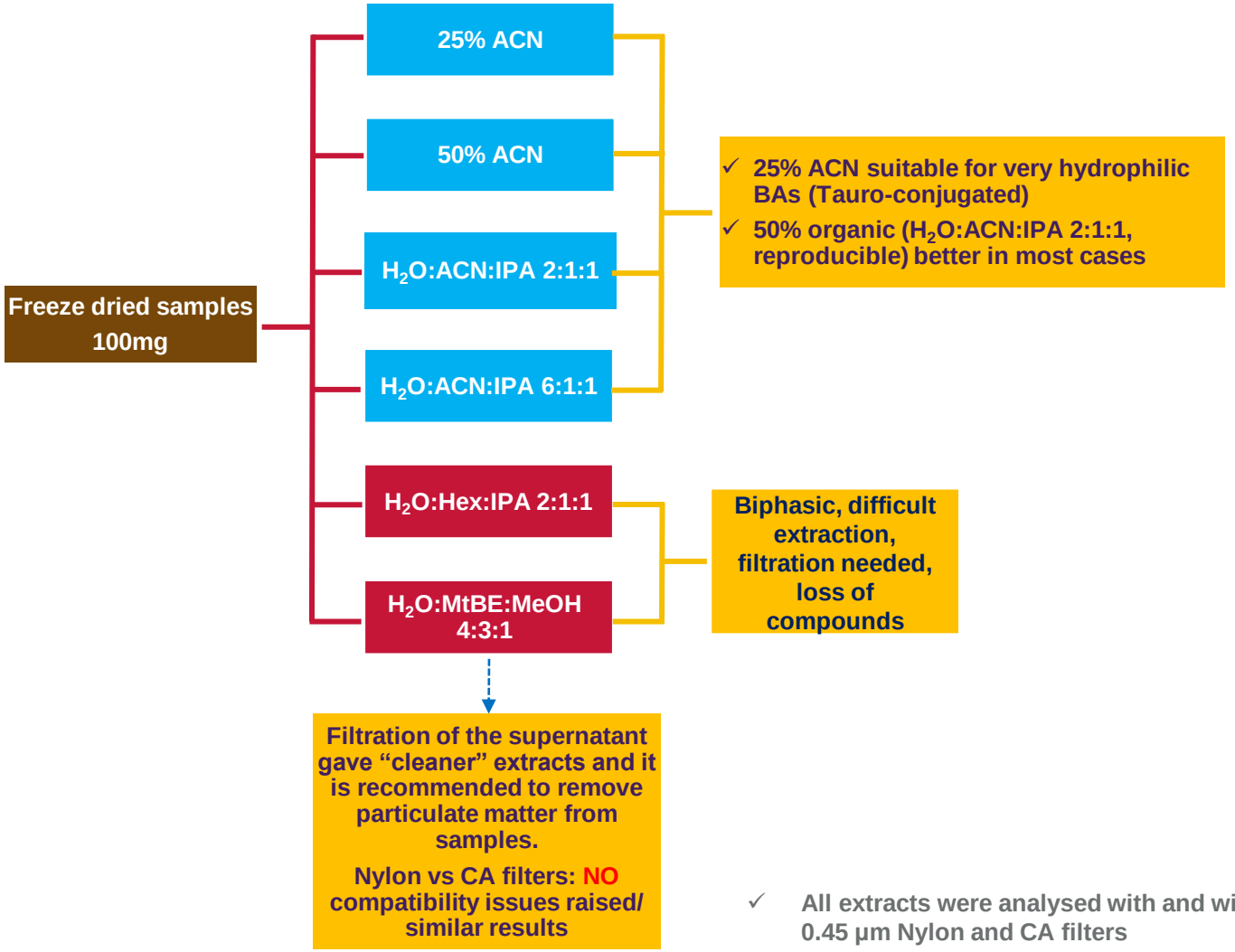
SPE for the extraction of BAs from fecal material



- Extractions were done using 0,5 or 1 ml of solvent per 100 mg of dry sample
- Percentage of H_2O in fresh samples ~80%
- SPE optimisation of loading/washing/elution steps was performed on Waters Oasis HLB plates

Imperial College London

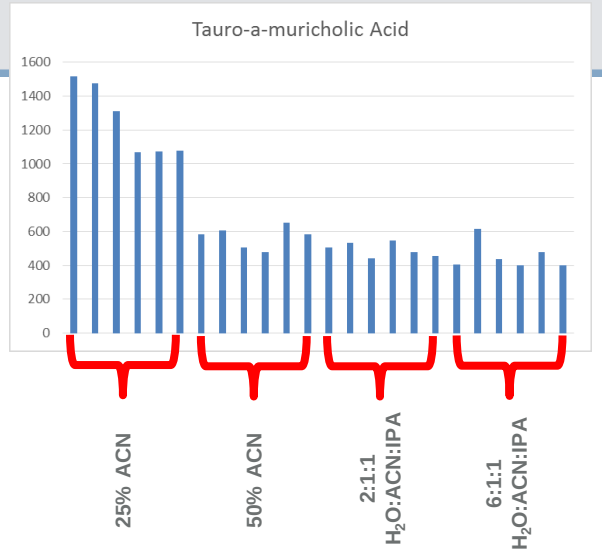
Single stage extraction of BAs

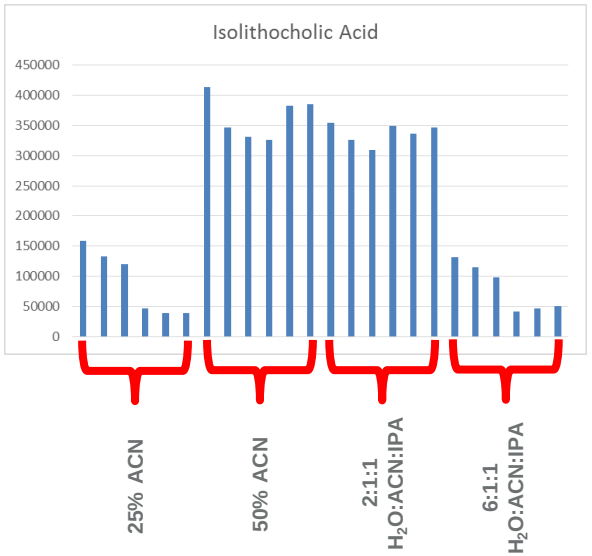


✓ All extracts were analysed with and without filtration with 0.45 µm Nylon and CA filters

Imperial College London

Single stage extraction of BAs





RT	Method	BA name
3.08	25% ACN	Tauro-a-muricholic Acid
3.08	25% ACN	Tauro-b-muricholic Acid
3.08	25% ACN	Tauro omega-Muricholic Acid
3.83	50% ACN	3,7,12 Dehydrocholic Acid
4.21	50% ACN	Taurohyocholic Acid
4.39	50% ACN	3a-OH-7,12-Diketocholanic Acid
4.55	50% ACN	Tauro-ursodeoxycholic Acid
4.87	H2O:ACN:IPA 2:1:1	Taurohyodeoxycholic Acid
5.07	H2O:ACN:IPA 2:1:1	Taurocholic Acid
5.19	50% ACN	Glycoursodeoxycholic Acid
6.22	50% ACN	12 Dehydrocholic Acid
6.66	50% ACN	a-Muricholic
6.74	50% ACN	b Muricholic Acid
6.85	H2O:ACN:IPA 2:1:1	Taurochenodeoxycholic Acid
6.85	H2O:ACN:IPA 2:1:1	Taurochenodeoxycholic Acid
7.29	H2O:ACN:IPA 2:1:1	3 Dehydrocholic Acid
7.29	50% ACN	Taurodeoxycholic Acid
7.34	H2O:ACN:IPA 2:1:1	Murocholic Acid
7.61	50% ACN	Glycochenodeoxycholic Acid
7.76	50% ACN	5a-Cholanic Acid-3,6-dione
7.86	H2O:ACN:IPA 2:1:1	Hyocholic acid
7.92	50% ACN/ H2O:ACN:IPA 2:1:1	3,7-Diketocholanic Acid
7.92	50% ACN	3,6-Diketocholanic Acid
7.92	H2O:ACN:IPA 2:1:1	3,12-Diketocholanic Acid
8.08	H2O:ACN:IPA 2:1:1	Glycodeoxycholic Acid
8.16	50% ACN/ H2O:ACN:IPA 2:1:1	5a-Cholanic Acid-3a-ol-6-one
8.16	50% ACN	Ursodeoxycholic acid
8.18	H2O:ACN:IPA 2:1:1	9(11), (5b)-Cholenic Acid-3a-ol-12-one
8.41	50% ACN/ H2O:ACN:IPA 2:1:1	Cholic acid
8.61	H2O:ACN:IPA 2:1:1	3a-Hydroxy-7 Ketolithocholic Acid
8.69	H2O:ACN:IPA 2:1:1	Hyodeoxycholic acid
8.94	H2O:ACN:IPA 2:1:1	3a-Hydroxy-12 Ketolithocholic Acid
8.96	50% ACN/ H2O:ACN:IPA 2:1:1	5b-Cholanic Acid-3b, 12a-diol
8.96	50% ACN	Tauro-ursocholanic Acid
9.57	50% ACN/ H2O:ACN:IPA 2:1:1	23-nor-5b-Cholanic Acid-3a, 12a-diol
9.83	50% ACN/ H2O:ACN:IPA 2:1:1	Glycolithocholic Acid
9.85	H2O:ACN:IPA 2:1:1	5b-Cholenic Acid-7a-ol-3-one
10.16	H2O:ACN:IPA 2:1:1	5-Cholenic Acid-3b-ol
10.26	50% ACN/ H2O:ACN:IPA 2:1:1	Chenodeoxycholic Acid
10.34	50% ACN/ H2O:ACN:IPA 2:1:1	Allolithocholic Acid
10.37	50% ACN	Deoxycholic Acid
10.48	50% ACN	Isolithocholic Acid
10.68	50% ACN/ H2O:ACN:IPA 2:1:1	3-Ketocholanic Acid
10.68	50% ACN	Lithocholenic Acid
10.82	50% ACN/ H2O:ACN:IPA 2:1:1	Lithocholic acid
10.93	H2O:ACN:IPA 2:1:1	Taurolithocholic Acid

Imperial College London

Sequential extraction of BAs (aqueous/organic)

Faeces samples

100 mg Feces
+
1 ml of organic solvent mixture

Vortex
1min

Centrifugation
20 min- 4ºC- 16,000 g

Supernatant
dried in fume hood

ORGANIC EXTRACT

Solid Precipitate

Solid Precipitate
+
1 ml of acueous solvent mixture

Vortex
1min

Centrifugation
20 min- 4ºC- 16,000 g

Supernatant
dried in speed-vacuum Concentrator

"AQUEOUS" EXTRACT

Mass Spectrometry: UPLC-MS

Waters XEVO G2 QTOF

UPLC mode

RP Chromatography
Lipid Profiling

BAs profiling

BAs profiling

MS mode

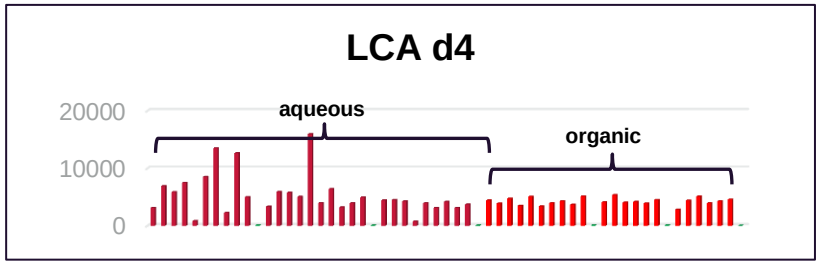
ESI+

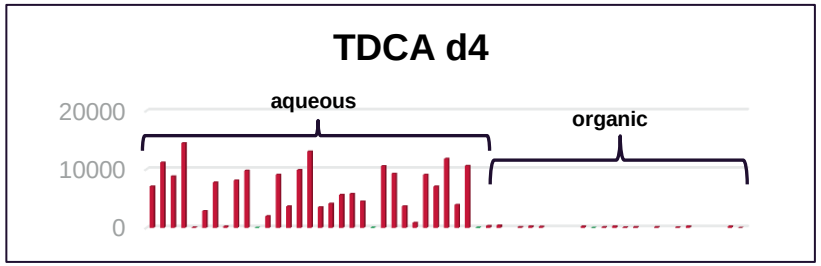
ESI-

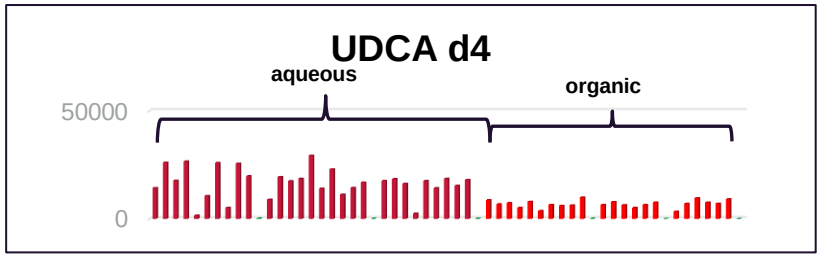
ESI-

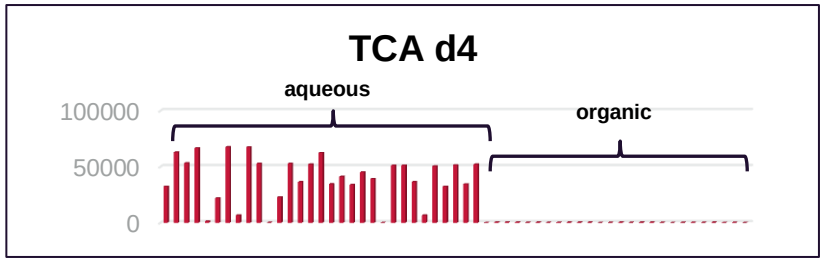
Imperial College London

Sequential extraction of BAs (aqueous/organic)







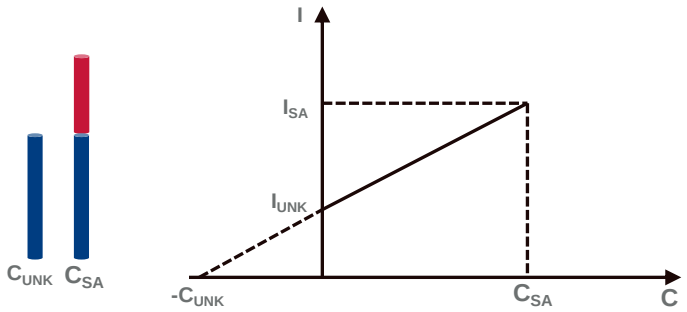


★ Aqueous solvent mix consist of 50% organic solvent (ACN)

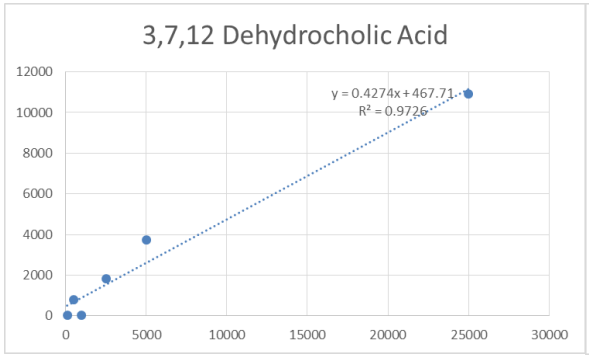
		Acqueous				Organic			
	Retention time	Average intensity	St.dev	RSD%	% of total signal	average	St.dev	RSD%	% of total signal
LCA-d4	*10.77*	4121.047	2436.668	59.12741	56.36	3189.929	344.178	10.78952	43.64
UDCA-d4	*7.96*	14333.67	5792.54	40.41212	75.22	4722.497	1010.89	21.40584	24.78
TDCA-d4	*6.8*	5212.718	3286.081	63.03969	99.13	45.63335	28.38799	62.20885	0.87
TCA-d4	*4.67*	38207.5	14835.3	38.82825	99.71	110.469	42.61309	38.57468	0.29

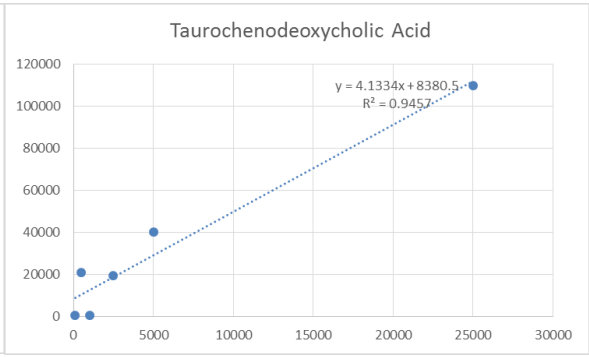
Imperial College London

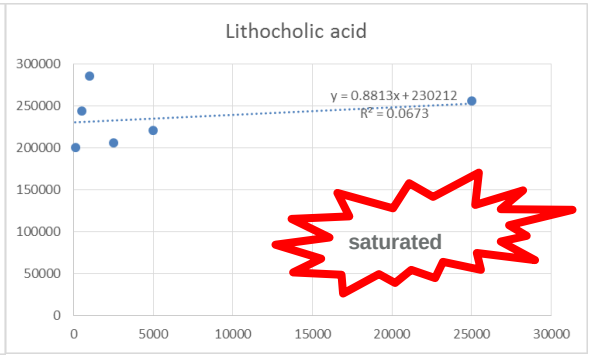
Quantitation of BAs via the SA-IS method.



- More labour intensive and less precise than the calibration curve method.
- It provides better accuracy on complicated samples.
- Compensates for the matrix effect on accuracy and precision
- The use of IS compensates for incomplete and variable extraction







✓ **Quantifications presented were performed using Quanlynx software**

Imperial College London

Take home message

- Samples have to be thoroughly dried (stability/extraction efficiency)
- The best overall extraction solvent H_2O:ACN:IPA, 2:1:1.
- Filtration of the extract with the use of spin-filter is recommended.
- The use of H_2O:ACN:IPA, 2:1:1 as an extraction solvent obviates the need of drying down/reconstitution.
- Use of 1% F.A in the extraction solvent can increase yield.
- Use of SA-IS method to build the calibration curves (laborious…but worth the pain!)
- Due to the big dynamic range of concentrations, might need to rerun some of the samples diluted (make sure enough volume)
- Attention has to paid when preparing LC solvents (RT highly dependent on pH)
- Always run the samples using MS^E function to get information about conjugation

Targeted UPLC-MS bile acid assay

analytical chemistry

Article

pubs.acs.org/ac

Bile Acid Profiling and Quantification in Biofluids Using Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

Magali H. Sarafian,[†,#] Matthew R. Lewis,[†,‡,#] Alexandros Pechlivanis,[†] Simon Ralphs,[§] Mark J. W. McPhail,[§] Vishal C. Patel,[⊥] Marc-Emmanuel Dumas,[†] Elaine Holmes,[†] and Jeremy K. Nicholson*[,†]

[†]Imperial College of London, Division of Computational Systems Medicine, Department of Surgery and Cancer, Sir Alexander Building, Exhibition Road, South Kensington, London SW7 2AZ, United Kingdom

[‡]Imperial College of London, MRC-NHR National Phenome Centre, Department of Surgery and Cancer, IRDB building, Du Cane Road, London W12 0NN, United Kingdom

[§]Imperial College of London, Department of Hepatology, St. Mary's Hospital, Paddington, London, United Kingdom

[⊥]King's College London, Institute of Liver Sciences, Hospital NHS Foundation Trust, Division of Transplantation Immunology and Mucosal Biology, MRC Centre for Transplantation, London, United Kingdom

Imperial College London

Faecal microbiota transplantation (FMT) is really working…

Hypothesis:

- Faecal microbiota transplantation (FMT) as a cure for *Clostridium difficile* infection (CDI)
- CDI is characterised by perturbed bile acid metabolism, and FMT may exert its efficacy through re-establishment of gut microbiota that restore this process to normal.

Methods:

- Stool samples from healthy volunteer donors participating in an FMT programme
- serial stool samples from a patient successfully treated with FMT for refractory CDI, pre- and post-transplantation.
- Samples were assayed for structure of the gut microbiota using 16S rRNA gene sequencing, and for BA profiling via UPLC-MS.
- Presence of bile salt hydrolases (responsible for deconjugation of glycine- and taurine-conjugated primary bile acids within the gut) was assessed via PCR of bacterial DNA extracted from stool.

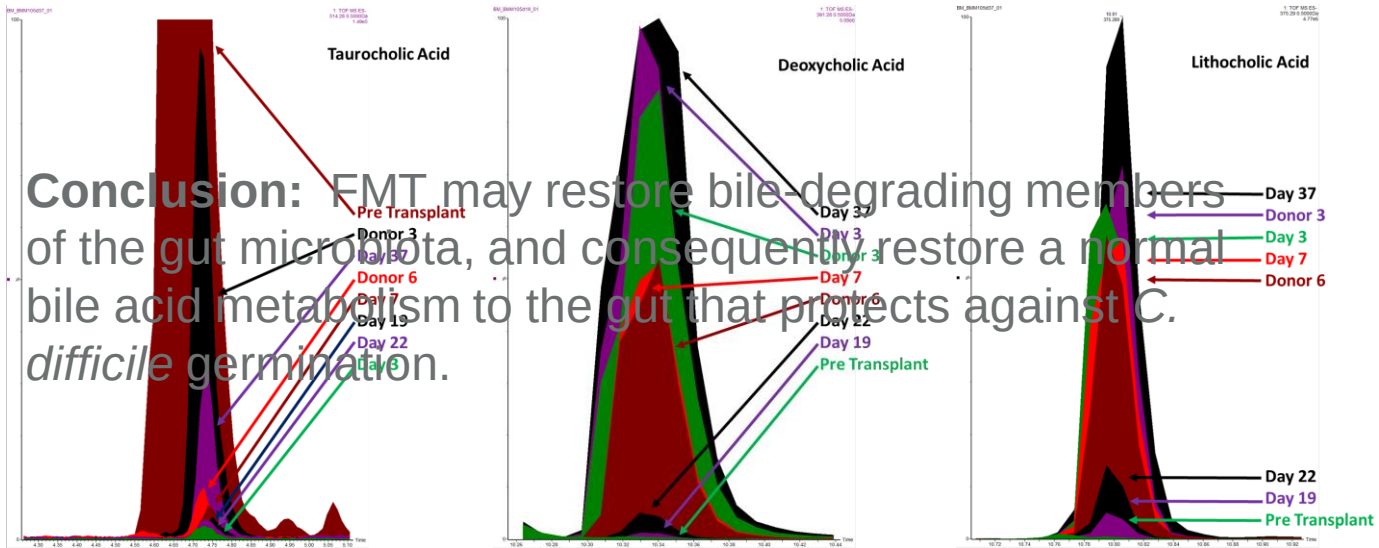
.

Imperial College London

Faecal microbiota transplantation (FMT) is really working…

Results:

- Modest improvement in diarrhoea after a first FMT, but an immediate, complete and sustained resolution of symptoms after a second FMT from a different donor (performed two weeks after the first).
- 16S rRNA gene sequencing demonstrated a pattern of faecal bacterial communities that closely resembled that of the healthy donors by one week after the second FMT.
- Faecal LC-MS analysis revealed the patient's gut bile acid profile pre-FMT to be enriched sixfold in TCA, Post-FMT the patient's gut bile acid profile resembled that of healthy donors, with loss of TCA and enrichment of secondary bile acids
- PCR of bacterial DNA displayed no detectable BSH genes in the recipient either pre-FMT or by day 7, but BSH presence was confirmed in both donors, as well as in the recipient by one week following the second FMT.



Conclusion: FMT may restore bile-degrading members of the gut microbiota, and consequently restore a normal bile acid metabolism to the gut that protects against *C. difficile* germination.

Imperial College London

Let's fly to a different place....

Imperial College London

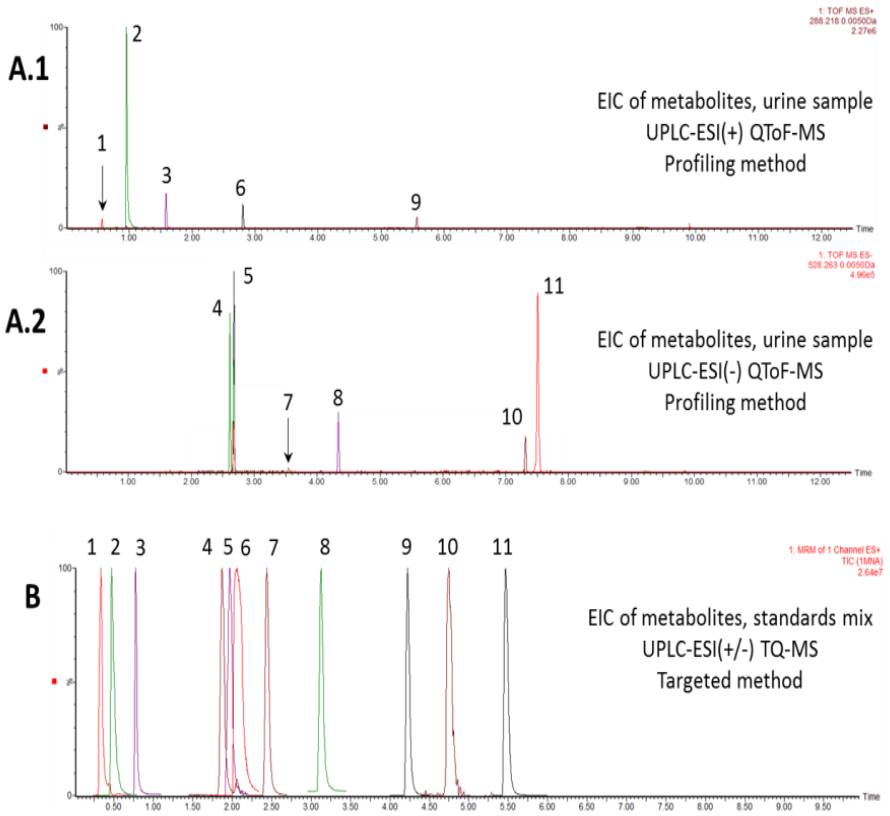
The PROLIFICA project

Discovery and validation of urinary biomarkers for the diagnosis of HCC in West Africans

- Hepatocellular carcinoma (HCC), the most common primary liver cancer, carries a poor prognosis. The mortality-to-incidence ratio of HCC approaches **unity** in most developing countries as a result of very late diagnosis.
- We have used a two-stage metabonomic strategy for the discovery and validation of a biomarker panel for the early diagnosis of HCC.
- In an initial phase, untargeted profiling of two sets of urine samples by UPLC-ESI- QTof MS was performed at Imperial College London for the detection and identification of disease biomarkers. Urine of patients from Nigeria and Gambia with HCC, cirrhosis, non-cirrhotic liver disease and healthy controls were compared.
- In the second step, the identified metabolites were used to develop a targeted MS method using the triple quadrupole (TQ) mass spectrometer donated by Waters in 2012 to the PROLIFICA project (UPLC-TQ MS system based in the MRC-The Gambia unit).

Imperial College London

Methods



UPLC method	Profiling method	Targeted method
Run time	25 min per sample (1 chromatographic run of 12.5 min per polarity)	12.5 min per sample (1 single run, both polarities)
Column type	HSS T3, 2.1 X 150 mm	HSS T3, 1 X 100 mm
Column temperature	45 °C	40°C
Flow rate	0.6 ml/min	0.21 ml/min

Reduction in run time and in flow rate allows decreasing significantly the consumption of solvent, which is critical in the developing world.

Imperial College London

The metabolites panel

	ID	RT (min)	m/z	Ion	Formula
1	1-Methylnicotinamide	0.57	137.071	$[M+H]^+$	$C_7H_9N_2O$
2	Acetylcarnitine	0.97	204.124	$[M+H]^+$	$C_9H_{17}NO_4$
3	Propionylcarnitine	1.58	218.139	$[M+H]^+$	$C_{10}H_{19}O_4$
4	Hydroxyphenyllactic acid	2.65	181.05	$[M-H]^-$	$C_9H_{10}O_4$
5	Homovanillic acid	2.78	261.006	$[M-H]^-$	$C_9H_{10}O_7S$
6	Kynurenic acid	2.85	190.051	$[M+H]^+$	$C_{10}H_7NO_3$
7	4-Hydroxybenzaldehyde	3.59	121.028	$[M-H]^-$	$C_7H_6O_2$
8	Indole lactic acid	4.38	204.065	$[M-H]^-$	$C_{11}H_{11}NO_3$
9	L-Octanoylcarnitine	5.59	288.217	$[M+H]^+$	$C_{15}H_{29}NO_4$
10	Glycocholic acid	7.38	464.301	$[M-H]^-$	$C_{26}H_{43}NO_6$
11	Glycochenodeoxycholic acid-3-sulfate	7.85	528.263	$[M-H]^-$	$C_{26}H_{43}NO_8S$

Imperial College London

The LCMS lab in The MRC Gambia

Imperial College London

Future Directions

- Further statistical analysis ongoing
- Added investigations on the performance of the panel of identified biomarkers as an early diagnostic tool for liver cancer
  - ✓ Association of urinary metabolites with HCC stage
  - ✓ Testing diagnostic accuracy of panel - AUROC curves
- Analysis of new urine samples by the developed targeted method
  - ✓ Larger cohort of samples
  - ✓ Use of 8 internal standards for absolute quantification

The ultimate goal of this study is the development of a urinary-based test (dipstick type) for use at the village level in Africa for screening of at-risk populations.

Imperial College London

Acknowledgements

Imperial College

- Dr Konstantina Spagou
- Dr Maria Gomez Romero
- Dr Magalie Sarafian
- Dr Matthew Lewis
- Dr Panos Vorkas
- Dr Benjamin Mullish
- Anna Mroz
- Prof Elaine Holmes
- Prof Simon Taylor-Robinson
- Prof Mark Thursz
- Prof Zoltan Takats

PROLIFICA Project

- Dr Ramou Njie
- Dr Haddy KS Fye
- Omar Jawara
- The PROLIFICA Team
- Waters


Спасибо
Gracias
شكر
Obrigado
Спасибо
Dank U
Grazie
Ευχαριστώ
Danke
Ευχαριστώ
Merci
Diolch
Ngiyabonga
Thank You
Dank U
Obrigado
Thank You
Ngiyabonga
Tack
Merci
Ευχαριστώ
Ευχαριστώ
Dziękuję
Danke
Grazie
תודה
Dank U
Diolch
Terima Kasih
Grazie
Merci
Dziękuję
Diolch
Gracias
Merci
תודה
Tack
Tack