METABOLIC PROFILING AS A NON-INVASIVE TOOL FOR NON-ALCOHOLIC STEATOHEPATITIS DIAGNOSIS

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Introduction

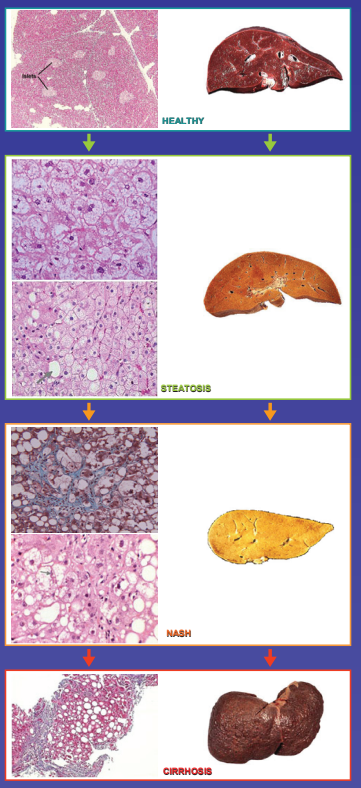
Non alchoholic fatty liver disease (NAFLD) encompasses a wide range of conditions characterised by the build-up of fat in the liver cells of people who do not drink alcohol excessively.

At one end of the scale is the relatively harmless simple fatty liver, or steatosis, that does not cause significant liver damage. If left unattended this condition may progress to more advanced conditions, some of which may be life threatening. Non alchoholic steatohepatitis (NASH) is a significant development in NAFLD, corresponding to an aggressive condition characterised by swelling and tenderness in the liver. With intense, on-going inflammation a build up of scar tissue (fibrosis) may form, eventually leading to cirrhosis where irregular bumps, known as nodules, replace the smooth liver tissue and the liver becomes harder. The effect of this, together with continued scarring from fibrosis, means that the liver will run out of healthy cells to support normal functions. This can lead to complete liver failure.

There is currently no specific laboratory test for NASH, making it extremely difficult to diagnose since even people who go on to develop fibrosis and cirrhosis may undergo liver damage for many years before symptoms become apparent. Although scanning the liver with imaging equipment such as magnetic resonance imaging (MRI) may reveal significant deposits of fat (steatosis), a potentially painful and hazardous biopsy is the only widely accepted test for distinguishing NASH from other forms of disease. This process involves passing a fine hollow needle through the skin and into the liver, withdrawing a small tissue of sample that is submitted for histological examination. Apart from the obvious discomfort induced by this invasive procedure, assessment is often subjective and prone to sampling error.

Most people with a fatty liver are overweight or obese. As more and more people lead inactive lives and carry extra weight around with them, so the number of cases of fatty liver, in particular NASH, is rising. Cleary there is a need for a non-invasive alternative to existing diagnosis methods, reducing patient discomfort and hospital-stay costs whilst providing a more robust, standardised assessment. The current work hopes to take a significant step to addressing this need, investigating the use of metabolic profiling of patient serum samples as a non-invasive alternative for NASH diagnosis.

NAFLD Evolution



Histological liver section Representative liver photo

UPLC-MS^E Metabolomics

Metabolomics involves the extraction and measurement of 100s-1000s of small molecules from tissues or biofluids to produce metabolic profiles. Comparison of profiles from different phenotypes can be used to identify specific metabolic changes leading to the understanding of disease progression. Mass spectrometry (MS) based metabolomics offers selective, sensitive and quantitative analyses with the potential to identify metabolites. A particular advantage of the technique for diagnostics applications is the ability, in conjunction with the high peak capacity afforded by sub-2 µm particle chromatography (UPLC), to selectively analyse individual metabolites involved in a given biochemical pathway. This allows disease specific components identified in initial fingerprinting experiments to be validated in different samples sets whilst avoiding possible confounding effects resulting from, for example, varying patient dietary habits or sample collection procedures.

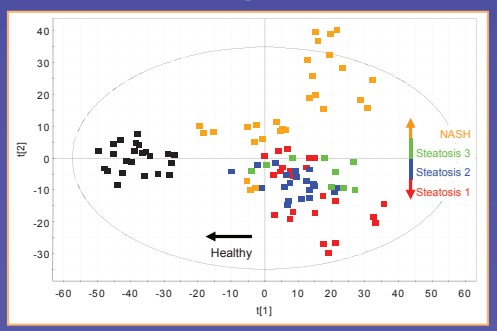
In the current work the UPLC-MS^E technique is used to compare metabolic profiles in the serum of a set of patients grouped according to the histological examination of a liver biopsy (European Standard: steatosis grade 1,2, 3 or NASH).

PLASMA	MALE	FEMALE	WEIGHT	HEIGHT	BMI	TECHNIQUE
Healthy	5	5	71 ± 6	169 ± 4	25 ± 2	Analysis
Steatosis Grade 1	1	7	90 ± 3	160 ± 3	36 ± 1	Biopsy
Steatosis Grade 2	2	5	99 ± 9	162 ± 5	38 ± 3	Biopsy
Steatosis Grade 3	1	2	116 ± 15	164 ± 4	42 ± 3	Biopsy
NASH	1	10	93 ± 5	155 ± 2	39 ± 2	Biopsy

Proteins were precipitated from the serum samples by liquid-liquid extraction (LLE) techniques and transferred to vials for UPLC-MS^E analysis.

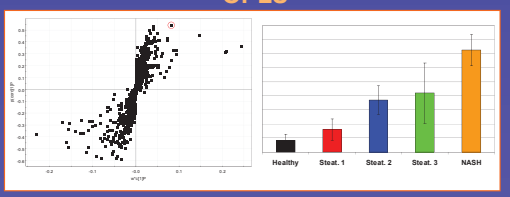
Chromatography was performed on a reverse phase packed column eluted with a 10 minute linear gradient using a Waters Acquity UPLC system. The eluent was introduced by electrospray ionisation into a Waters Q-TOF Premier mass spectrometer operating in positive and negative modes, in separate experiments. The spectrometer was operated with an alternating collision energy, allowing both precursor and product ions to be generated in the same analytical run. In many cases these ions can be linked using either retention times, mass defects or a combination of both to provide similar information to that obtained by conventional MS/MS analysis without having to re-analyse the samples. Triplicate sample injections were performed, with appropriate standard compounds used to examine the retention time stability, mass accuracy and sensitivity of the system throughout the course of the run.

PLS-DA



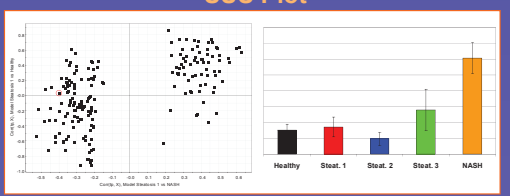
A PLS-DA scores plot distinguishing NAFLD (+ve t[1]) and healthy (-ve t[1]) metabolic profiles; NAFLD evolution is apparently explained by the second component (NASH samples have a positive t[2]).

OPLS



OPLS model of steatosis 1 ($Y = 0$) vs NASH samples ($Y = 1$), where X (RT – m/z) variation is separated into two parts linearly related, and orthogonal to Y. Markers with predictive component scores such as that which is highlighted correlate with NAFLD progression through steatosis to NASH.

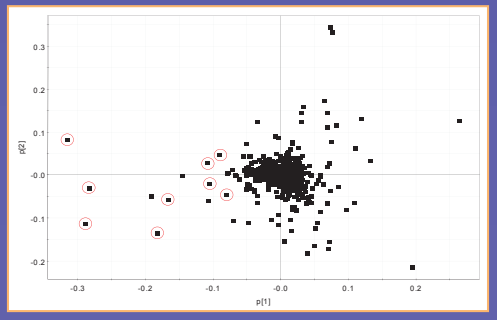
SUS Plot

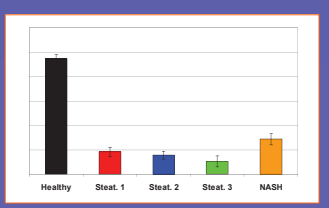


The shared and unique structure (SUS) plot enables one to compare several OPLS models. The utility of this approach is shown here where the highlighted marker has little correlation between healthy and steatosis 1 samples, but is significant in the steatosis 1 vs NASH model. This marker may be more indicative of the significantly more severe liver damage – inflammation / swelling – found in NASH patients as opposed to the more continuous progression through the different stages of steatosis.

Loadings PLS-DA

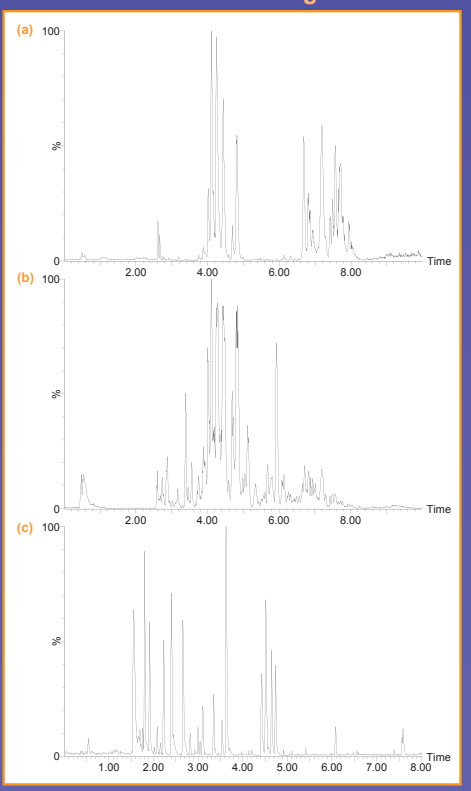
Accompanying PLS-DA loadings plot detailing the metabolites that most contribute to the separation observed above. Highlighted markers, identified by analysis of high energy MS^E spectra, belong to a sub-class of phospholipid (patent pending).





Analysis of similar markers in obese patients with little or no steatosis (data not shown) indicate that the trend observed in the highlighted markers is mainly related to obesity factors.

UPLC-MS Chromatograms



Complementary extraction methods and ionisation techniques are required for optimum metabolome coverage: (a) One-step LLE ESI +ve, (b) Single-step LLE ESI –ve, (c) Sequential two-step LLE with derivatisation ESI +ve.

Conclusions:

- ✓ UPLC-MS metabolic profiling can distinguish between patients at different stages of the NAFLD process, demonstrating the potential of the technique as a non-invasive alternative to biopsy diagnosis.
- ✓ Data mining techniques have allowed the identification of three different classes of biomarker correlating with obesity, progressive NAFLD, and NASH.
- ✓ Biomarker validation experiments for larger numbers of patients originating from different parts of Europe are currently in progress.

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