Serum metabolic profiling reveals novel biomarkers associated with NAFLD progression

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NAFLD and Metabolomics

The identification of novel serum biomarkers differentiating between normal liver, steatosis and NASH is of fundamental importance for effective NAFLD diagnosis and treatment assessment. Liver biopsy is currently the standard for NASH diagnosis, but is an expensive, invasive and subjective procedure, associated with potential complications and prone to sampling error.

The emergent field of metabolomics has the potential to reveal such biomarkers. Recent technological breakthroughs have provided researchers with the capacity to measure hundreds or even thousands of small-molecule metabolites in as little as a few minutes per sample, paving the way for hypothesis generation studies ideally suited to complex disease scenarios such as NAFLD. The approach is particularly applicable to liver injury assessment, where the most commonly available sample for laboratory tests is serum or urine – ideal for metabolomics analysis.

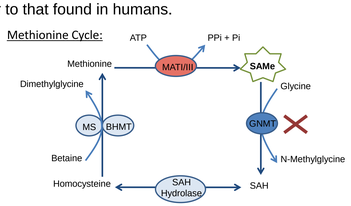
The results illustrate the potential of metabolite profiling to provide biomarkers for staging, prognosis and therapy selection in NAFLD management.

GNMT-KO mice, a NAFLD animal model

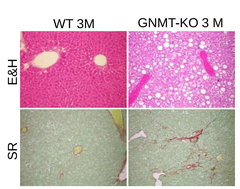
The suitability of the metabolomics approach for the study of NAFLD has been evaluated in our laboratory using the GNMT knockout mouse model.

This animal model is based on perturbations of the methionine cycle, long recognised as being of key importance in liver disease. The deletion of glycine-N-methyltransferase (GNMT) results in an increase of methionine and SAMe. S-adenosylhomocysteine (SAMe) is the principal biological methyl donor and participates in multiple cellular reactions. The GNMT-KO mice develop liver disease with a pathology very similar to that found in humans.

A. Methionine Cycle:

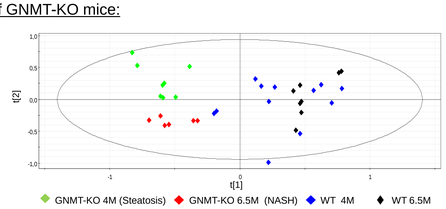


B.



A) SAMe metabolism. B) Deletion of GNMT leads to steatosis and fibrosis. The Hematoxylin/eosin staining reveals at 3 months of age macro and microvesicular steatosis as could be seen through the hepatic lobule in GNMT-KO mice compared to wild type animals. Collagen deposits (sirius red staining) indicate moderate liver fibrosis.

Metabolic profile of GNMT-KO mice:



PCA scores plot of the UPLC®-MS serum metabolic profiling data obtained from WT and GNMT-KO mice. Clear differentiation between the WT (C57BL6j) and GNMT-KO mice is observed in the first principal component (t[1]); Additional separation, between the KO mice at 4 (steatosis) and 6.5 (NASH) months is also apparent in the second principal component (t[2]).

Metabolomics study in morbidly obese human NAFLD patients

Sample collection:

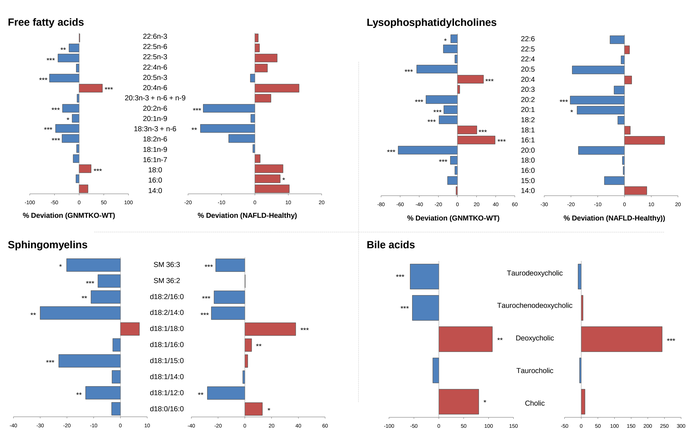
Sera was collected from 43 middle-aged, non-diabetic, female patients, (See Table), each with a diagnosis (steatosis grade 1-3, NASH grade 1) established histologically in liver biopsy samples, in the absence of other (viral-, alcohol-, or drug-induced) causes of NAFLD.

Group	N	BMI (kg/m²)	AST (IU)	ALT (IU)	Glucose (mM)	Cholesterol (mM)	Triglycerides (mM)
S0	9	47.0 ± 1.9	23.3 ± 2.3	25.1 ± 3.2	5.0 ± 0.2	4.8 ± 0.2	1.2 ± 0.2
S1	8	45.4 ± 1.7	21.8 ± 3.5	24.8 ± 3.1	5.0 ± 0.2	6.2 ± 0.6	1.9 ± 0.4
S2	7	43.5 ± 2.0	24.9 ± 2.3	34.9 ± 3.2	5.2 ± 0.2	5.6 ± 0.7	1.6 ± 0.2
S3	9	45.5 ± 2.7	27.8 ± 2.5	40.8 ± 7.2	5.3 ± 0.2	4.8 ± 0.4	1.4 ± 0.2
NASH	9	43.2 ± 1.5	32.8 ± 3.2	44.6 ± 5.6	5.5 ± 0.4	5.1 ± 0.3	1.4 ± 0.2

Common markers in mice and humans

Having established that the animal model metabolic profiles were correlated with NAFLD progression, the proceeding analysis was focused towards the identification of biomarkers with similar trends in the human NAFLD samples.

Significant overlap was found between the two data sets, with common perturbed groups of compounds, differentiating between the healthy and NAFLD samples. All these compound classes are involved in key hepatic metabolic pathways.



Mean percent changes of groups of compounds in human NAFLD (S0 vs S1, S2, S3, S3+NASH - right) and GNMT mice (GNMT-WT vs GNMT-KO - left) sera. Positive and negative percentages indicate higher levels of metabolites in NAFLD (GNMT-KO) and healthy (GNMT-WT) sera, respectively. Unpaired Student's *t*-test *p*-values are indicated where appropriate: *P < 0.15, **P < 0.1, ***P < 0.05.

Metabolite biomarkers discriminating between human steatosis and NASH

The human samples were further subjected to univariate statistical analyses, focusing on metabolite biomarkers differenciating between the S3 (severe steatosis) and S3+NASH sample groups.

Metabolite	% Change (NASH – S3)	*p*-value (NASH – S3)
PC (14:0/20:4)	73.6	0.033
PC (18:1/18:2)	14.4	0.086
PC (18:1/0:0)†	60.8	0.028
PC (P-18:1/20:4)	24.7	0.068
PC (P-24:0/0:0)	-38.9	0.049
PC (P-22:0/0:0)	-48.0	0.097
PC (O-20:0/0:0)	-47.5	0.051
Arachidonic acid†	-32.5	0.083
Glutamic acid†	-32.1	0.061

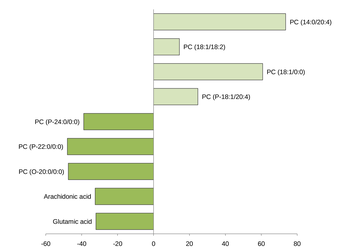


Table 3: Biomarker metabolites found in human sera. Mean percentage changes are provided, comparing the S3 + NASH and S3 sample groups. Positive and negative percentages indicate higher levels of metabolites in S3 + NASH and S3 sera, respectively. Statistical *p*-value calculated using the unpaired Student's *t*-test. †Metabolite identifications performed by comparison of mass spectra and chromatographic retention times with those obtained using commercially available standards. All other identifications were performed by accurate mass database searching with fragment ion analysis. Lipid nomenclature follows the LIPID MAPS convention (www.lipidmaps.org).

- *sn*-2 *arachidonoyl phospholipids* [PC(14:0/20:4) and PC(P-18:1/20:4)] play a key role in arachidonic acid storage / mobilization.

- Two *lyso plasmalogen species* [PC(P-24:0/0:0) and PC(P-22:0/0:0)] were significantly decreased in the S3+NASH patients, as compared to the S3 group. Previous evidence has shown that plasmalogens have anti-oxidant properties.

- *Arachidonic acid* has been observed in higher quantities in the NAFLD samples. In the S3+NASH group is decreased with respect to S3 patients. This finding may reflect the increased utilization of arachidonic acid by the NASH patients in eicosanoid synthesis and / or its reduced mobilization from phospholipids, as suggested by the increased *sn*-2 arachidonoyl species.

- *Glutamic acid* was also found to be reduced in the NASH patients, as has been previously found in other non-alcoholic liver diseases. A similar decrease of glutamic acid has also been reported in high-fat diet rat livers.

CONCLUSIONS

-To the best of our knowledge, this is the first serum global metabolite profiling study correlating with biopsy proven NAFLD histology in a BMI matched, non-diabetic subject population.

-The results from parallel metabolic profiling experiments in human NAFLD and in the GNMT-KO NAFLD mouse model, show evidence for strong metabolic correlation with progression of the disease. Common, putative biomarker metabolites were observed in the GNMT-WT/KO animal model and human NAFLD patients, supporting the viability of the markers/metabolites used to differentiate healthy and NAFLD individuals.

-UPLC/MS metabolic profiling was found to be a suitable platform for the study of NAFLD. The serum metabolic profiles obtained enable differentiation between healthy and NAFLD patients. Furthermore this study has identified a series of putative biomarkers discriminating between steatosis and NASH.