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ABSTRACTS

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Hepatic proton density fat fraction correlates with histologic measures of steatosis and is responsive to change in those measures in a multi-center nonalcoholic steatohepatitis clinical trial

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Background: MRI-estimated proton density fat fraction (PDFF) is a noninvasive imaging biomarker of hepatic steatosis. Selonsertib (SEL, formerly GS-4997) is a selective inhibitor of apoptosis signal-regulating kinase 1 (ASK1) being studied for the treatment of nonalcoholic steatohepatitis (NASH). The objectives of this study were to examine correlations between hepatic PDFF with histologic measures of steatosis, and to assess associations between changes in these measures in a multi-center clinical trial of SEL in subjects with NASH.

Materials & Methods: PDFF was estimated by advanced MRI at baseline (BL) and W24 in a Phase 2, multi-center clinical trial of 72 patients with NASH (NAS ≥5 and F2-3 fibrosis) treated with SEL 6 mg or 18 mg orally QD alone or in combination with simtuzumab (SIM, 125 mg SQ weekly) or SIM alone for 24 weeks. For the purpose of this analysis, study groups were combined. Liver biopsies, including morphometric quantitation of fat content on picrosirius red-stained sections, were performed at BL and W24, and serum markers including cytokeratin 18 (CK-18) M30 and M65 subfractions, were measured. Associations between PDFF and other measures were determined using Kruskal-Wallis or Wilcoxon rank sum tests or Spearman correlations (rs).

Results: At BL and W24, PDFF was correlated with the NAS steatosis grade (BL: rs=0.57; W24: rs=0.59), and hepatic fat assessed by morphometry (BL: rs =0.61; W24: rs=0.87) (all p<0.05). Percent change in PDFF from BL to W24 was associated with NAS response; subjects with a ≥2-point reduction in NAS (n=12) had a 27% median relative reduction in PDFF (IQR: -4.2%, -39%), compared to a 4.6% median relative reduction (IQR: 17%, -22%) in non-responders (n=53; p=0.032). Relative changes in PDFF were also associated with percent changes in morphometric fat content, liver biochemistry, CK18 subfractions, glucose, and total cholesterol (Table 1).

Conclusions: MRI-estimated hepatic PDFF correlates with histologic measures of steatosis and is responsive to changes in these measures. These data help further validate PDFF as a biomarker of hepatic steatosis, and support the utility of PDFF for the noninvasive assessment of hepatic steatosis in clinical trials of patients with NASH.

Table 1: Associations between relative changes in PDFF and other markers from BL to W24

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Median % Change in Biomarker at W24 (n)</th>
<th>p-value</th>
<th>Correlation with % Change in PDFF at W24, rs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDFF &gt;30% reduction</td>
<td>PDFF ≤30% reduction</td>
<td></td>
</tr>
<tr>
<td>%Hepatic Fat</td>
<td>-35.6 (12)</td>
<td>-6.4 (52)</td>
<td>0.006</td>
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<td>ALT</td>
<td>-31.8 (12)</td>
<td>-2.9 (52)</td>
<td>0.004</td>
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<td>AST</td>
<td>-22.1 (10)</td>
<td>-5.1 (52)</td>
<td>0.027</td>
</tr>
<tr>
<td>GGT</td>
<td>-20.3 (12)</td>
<td>-0.5 (52)</td>
<td>0.018</td>
</tr>
<tr>
<td>CK18 M30</td>
<td>-28.8 (11)</td>
<td>-1.4 (52)</td>
<td>0.079</td>
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<tr>
<td>CK18 M65</td>
<td>-40.5 (11)</td>
<td>-21.0 (50)</td>
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<td>Glucose</td>
<td>-6.2 (11)</td>
<td>6.0 (52)</td>
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<tr>
<td>Cholesterol</td>
<td>-13.1 (12)</td>
<td>-2.2 (52)</td>
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</tbody>
</table>
A Meta-Analysis on Repeatability of Magnetic Resonance Elastography of Liver.

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Background: MR Elastography (MRE) non-invasively measures tissue shear wave stiffness, which is a potential non-invasive biomarker for quantification of liver fibrosis. In qualifying a biomarker for disease evaluation, it is essential to estimate the measurement error of the technique as well as to standardize and validate the acquisition and analysis techniques. With the expanding clinical and research applications of MRE, the literature on MRE repeatability has grown, but a key repeatability metric, namely the repeatability coefficient (RC), has not been reported in most published papers. Purpose: The purpose of this work was to conduct a meta-analysis to generate an estimate of the repeatability coefficient (RC) of MRE.

Materials & Methods: Our work is motivated by the activities of the Radiological Society of North America (RSNA) Quantitative Imaging Biomarkers Alliance (QIBA). A systematic search of databases was performed for publications on MRE during the ten-year period 2006-2016. This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. The identified studies were screened independently and verified reciprocally by all authors. Two reviewers independently determined the percent %RC and effective sample size from each article. A forest plot was constructed of the %RC estimates from the 12 studies. 95% percentile bootstrap CIs were constructed for the summary %RCs.

Results: Twelve studies comprising 274 patients met the eligibility criteria and were included for analysis. Flow diagram of included studies according to PRISMA guidelines was prepared for the inclusion and exclusion criteria. All studies included in the meta-analysis fulfilled 4 or more of the 7 categories of QUADAS-2 tool. The estimated summary RC was 22% with 95% CI of [16.1 – 28.2]. The three main sources for this heterogeneity were the trained vs untrained operator to draw contours to choose (Regions of Interest) ROIs, the time between two replicate scans, and finally the scanner field strength. The RC estimates tended to be higher for studies that did not use a well-trained operator, and with longer time intervals between scans.

Conclusion: The meta-analysis results provide the basis for the following draft longitudinal QIBA MRE claim: a measured change in hepatic stiffness of 22% or greater, at the same site, using the same equipment, and acquisition sequence indicates that a true change in stiffness has occurred with 95% confidence. Ongoing efforts to further standardize MRE examination protocols and drawing contours to obtain liver stiffness values may provide further increases in performance. Future investigations should also assess reproducibility of MRE measurements across different system and vendor platforms.

No conflict of interest
A Real-world, Observational Cohort of Patients with Nonalcoholic Fatty Liver Disease: The TARGET-NASH Study

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Background: Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in the industrialized world in both children and adults, and can lead to cirrhosis, hepatocellular carcinoma and death from liver disease. It is also associated with an increased risk of type II diabetes and cardiovascular events. At this time, treatment is limited to weight loss and exercise, which are difficult for patients to achieve and sustain. As such, pharmacologic therapies are greatly needed, and many are in various stages of development. Clinically meaningful benefit of a drug is defined by the FDA as improvement in how a patient “feels,” “functions” or “survives.” Because of the long natural course of NAFLD, these outcomes are reached over decades and are not practical endpoints for clinical trials. This has led to reliance on surrogate markers of benefit, primarily liver histology. Large, observational cohorts are needed to further validate histology and to validate noninvasive biomarkers so they can be accepted as surrogates of clinically meaningful benefits.

Materials & Methods: TARGET-NASH is a long-term observational study of pediatric and adult patients with NAFLD designed to address questions that go beyond registration trials. The four aims of the study are: 1) record the real-world natural history of NAFLD, 2) describe the real-world clinical practice of diagnosis and treatment of NAFLD, 3) once pharmacologic agents for the treatment of NAFLD are available, provide post marketing data on clinical effectiveness and safety, and 4) collect and maintain a bio specimen repository for translational studies and biomarker validation. The recruitment goal is 15,000 patients in the US, Europe, and Asia. Enrollment will take place at up to 100 adult and pediatric sites representing primary care, gastroenterology, hepatology, and endocrinology providers in academic and community settings. Enrolled patients have 3 years of retrospective clinical data collected, followed by prospective data capture of routine clinical care for 5 years, and bio specimens collected annually. Enrollment criteria is much less restrictive than those needed for treatment trials to allow assessment of real-world outcomes. Patient comorbidities, concomitant medications, interventions for NAFLD, and disease progression are being assessed. Adverse outcomes, including cardiovascular and neoplastic complications, and those related to medications are being monitored.

Results: To date, 26 sites in the United States have enrolled nearly 500 patients, and have begun collecting data and bio specimens. Interim analyses will be conducted after the first 1000 patients are enrolled.

Conclusions: TARGET-NASH is a large, diverse, real-world cohort of patients with NAFLD, who represent the full age range of the disease. Patients are being studied at both academic and community practices. Longitudinal collection of hard endpoints will be leveraged to develop and validate noninvasive biomarkers for the diagnosis and progression of NAFLD, and identify clinically meaningful end points for treatment trials of NASH.

No conflict of interest
Novel multiparametric magnetic resonance elastography (MRE) protocol accurately predicts NAS score for NASH diagnosis

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Background: The lack of a reliable, noninvasive method to diagnose nonalcoholic steatohepatitis (NASH) remains a major unmet need in nonalcoholic fatty liver disease (NAFLD). Magnetic resonance elastography (MRE) is the most accurate noninvasive biomarker of liver fibrosis. We aimed to determine the diagnostic performance of a multiparametric MRE protocol (the “Hepatogram”) for the detection of NAFLD activity score (NAS) components, using novel mechanical properties that detect early parenchymal viscoelastic changes in NASH.

Materials & Methods: The model was developed in animal studies and validated in humans. In both models, multifrequency 3D MRE was used to assess mechanical properties that correlate with hepatocyte ballooning and inflammation, while MRI proton density fat fraction (MRI PDFF) was used to quantify steatosis. Liver biopsies were obtained for histologic grading of steatosis, lobular inflammation, and ballooning, and NAS was calculated based on the NASH CRN criteria. Pairwise comparisons (nonparametric Dunn method for joint ranking) were performed for imaging parameters among different grades of inflammation, ballooning, and steatosis. The imaging parameters were included into predictive regression models which were tested with ROC analyses using NAS score as a continuous output. Ceiling operations were used to predict NAS score for NASH classifications.

Results: The preclinical model included 64 mice: 36 with NAFLD/NASH (fed a fast food diet and fructose water) and 28 controls. The clinical model included 51 human subjects: 38 obese (22 NAFLD and 16 NASH) and 13 healthy volunteers. From the complex shear modulus output generated by MRE at multiple mechanical frequencies, the parameters that best correlated with individual NAS components were selected. The damping ratio (loss modulus/storage modulus) correlated with lobular inflammation, while the complex shear modulus magnitude correlated with hepatocellular ballooning. The fat fraction obtained from MRI PDFF best correlated with steatosis (p<0.05 for all parameters). These 3 parameters were fit into a generalized linear model which successfully distinguished each NAS score with excellent accuracy (AUROC>0.89 for all)

Conclusion: This preliminary data shows that the Hepatogram can accurately predict NAS score and represents a promising alternative to liver biopsy for NASH diagnosis and monitoring.

No conflict of interest
05
The comparisons of non-invasive markers for assessing fibrosis in Asian patients with nonalcoholic fatty liver disease

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Background: The prevalence of nonalcoholic fatty liver disease (NAFLD) is growing worldwide. We investigated whether liver stiffness (LS) and controlled attenuation parameter (CAP), assessed using transient elastography (TE), could assess liver steatosis and fibrosis accurately.

Materials & Methods: In a total, 214 patients who underwent liver biopsy and concomitant TE were recruited from a tertiary hospital in Korea and finally analyzed between November 2011 and December 2014. We assessed liver fibrosis using APRI, NAFLD fibrosis score, and FIB-4.

Results: The study population included control group (n=103) and NAFLD group (n=111) according to the results of liver biopsy. Patients with NAFLD exhibited a mean age of 39.7 years and male predominance (n=85, 76.6%). The accuracy of CAP in detecting ≥S1, ≥S2, and ≥S3, assessed by the area under the receiver operating curve (AUROC), were 0.882, 0.906, and 0.870, respectively. The optimal cut-off values for steatosis were 248 dB/m for S1, 281 dB/m for S2, and 315 dB/m for S3. Also, the AUROC of LS in detecting ≥F2, ≥F3, and ≥F4 were 0.887, 0.958, and 0.986, respectively. The optimal cut-off values for fibrosis in patients with NAFLD were 7.65 dB/m for F2, 8.75 dB/m for F3, and 14.45 dB/m for F4. The sensitivity and specificity of the optimal cut-off for detecting ≥F3 and F4 were good (100 and 72% vs. 80.0 and 98.0%), as well as better than other noninvasive markers such as APRI, NAFLD fibrosis score and FIB-4. About 24 (21.6%) patients with NAFLD showed discordance between TE and histology. The predictive factors for discordance were age, body mass index (BMI), and the grade of steatosis.

Conclusion: TE showed the accurate detection of not only steatosis but also fibrosis in patients with NAFLD. In addition, TE showed better sensitivity and specificity for detecting advanced fibrosis and cirrhosis than other noninvasive markers.

No conflict of interest

06
Serum GP73 as a surrogate biomarker of significant liver fibrosis and cirrhosis in NAFLD

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Background: Currently the diagnosis of liver fibrosis in NAFLD requires a liver biopsy. There is an urgent need to develop better diagnostic biomarker for fibrosis.

Materials & Methods: The clinical data of 135 cases with NAFLD were collected retrospectively, the area under the receiver-operating characteristics curve (AUROC) of serum GP73 for the diagnosis of fibrosis and cirrhosis were assessed in all NAFLD patients who had satisfactory liver biopsy specimens. The in situ expression of GP73 was measured by immunohistochemistry.

Results: With the worsening of liver fibrotic stages, a step-wise increase of GP73 expression in the liver tissue was observed. In parallel, the
serum levels of GP73 increased gradually from normal (F0: 27.54 ± 2.82 ng/ml) to mild fibrotic changes (F1: 41.14 ± 2.62 ng/ml), significant fibrosis (F2: 59.51 ± 5.18 ng/ml), advanced fibrosis (F3: 82.38±12.01 ng/ml) and cirrhosis (F4: 139.07±14.93 ng/ml). Concordantly, the AUROCs of serum GP73 for the diagnosis were 0.898 for significant fibrosis and higher, 0.936 for advanced fibrosis and higher and 0.961 for early cirrhosis, respectively. Serum GP73 measurement exhibited a much better diagnosis performance, as compared to that of the aspartate aminotransferase–to–platelet ratio index (APRI), fibrosis index based on four factors (FIB-4) and NAFLD fibrosis score (NFS) with AUROCs ranged from 0.714 to 0.880, P<0.0001 for all comparisons.

Conclusions: Liver fibrosis is the most important histological lesion in non-alcoholic fatty liver disease (NAFLD). Serum GP73 is a valuable biomarker for the diagnosis of significant fibrosis, advanced fibrosis and cirrhosis in NAFLD.

No conflict of interest

Background: Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders in Western countries. Non-alcoholic steatohepatitis (NASH) is a more progressive phenotype, which has been associated with liver fibrosis and cirrhosis. Screening for NASH with advanced liver fibrosis in patients with metabolic risk factors has been recommended by the European Association for the Study of Liver (EASL) or by other scientific societies. Such tests may help identify high-risk patients while limiting the number of unnecessary liver biopsies. We investigated the ability of the type III collagen formation neo-epitope marker “Pro-C3”, known to be related to fibrogenesis, to differentiate NAFL from NASH and explored its association with the severity of hepatocellular ballooning, steatosis, lobular inflammation and fibrosis stage in patients screened for the CENTAUR study.

Materials & Methods: 532 liver biopsies from subjects screened for the CENTAUR study (NCT02217475), evaluating the efficacy and safety of cenicriviroc in adults with NASH and liver fibrosis (Stages 1-3, NASH CRN) were analyzed by a central pathologist. NASH was defined by the presence of each component of the NAFLD activity score (NAS). Demographics were collected and EDTA plasma was drawn at screening. Plasma Pro-C3 levels were determined using competitive ELISA. The ability of Pro-C3 to identify patients with fibrosis stage (F) ≥ 2 (moderate/advanced fibrosis) and ≥ 3 (advanced fibrosis) was determined by AUROC analysis. A previously defined cut-off (20.2 ng/mL) for high-risk patients based on hepatitis C cohorts was used to classify the patients into high or low Pro-C3.

Results: Plasma Pro-C3 significantly correlated to grade of hepatocellular ballooning (r=0.458;
p<0.0001), lobular inflammation (r=0.390; p<0.0001) and steatosis (r=0.304; p<0.0001), as well as fibrosis stage (r=0.443; p<0.0001). The AUC for detecting F≥2 was 0.71, p<0.001, and for detecting F≥3 AUC=0.73, p<0.001. Pro-C3 levels were 41% higher in F≥2 compared to F0-1 (p<0.001) and 39% in F≥3 compared to F0-2 (p<0.001). Pro-C3 was able to differentiate NASH from NAFL with an AUC of 0.73, p<0.001, with Pro-C3 levels 36% higher in NASH compared to NAFL (p<0.001). When classifying patients by high or low baseline Pro-C3 levels, AST, ALT, ALP, insulin and HOMA-IR were significantly higher in the high Pro-C3 group as compared to the low group (p<0.05-0.0001), whereas HDL and platelets were significantly lower (p<0.001-p<0.0001). Moreover, a higher grade of NAS was observed in the high versus low Pro-C3 group (p<0.0001).

Conclusions: Pro-C3 can detect moderate and advanced fibrosis in NAFLD patients, and can differentiate NASH from NAFL with fair accuracy. High baseline Pro-C3 correlates with markers of hepatocellular injury and insulin resistance. Pro-C3 is a novel non-invasive screening tool for selecting high-risk patients with NASH and fibrosis that are suitable for clinical trials. Current analyses of additional serological markers may further increase accuracy when combined with Pro-C3.

Financial relationship(s) with: Allergan

Next-Generation Sequencing (NGS) of two independent cohorts identifies eleven circulating miRNAs for diagnosis of NASH and fibrosis

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Background: Identification and qualification of novel circulating biomarkers to non-invasively diagnose patients with active NASH and fibrosis are needed. miRNAs exert biological activities and participate in the pathophysiology of NASH and liver fibrosis and circulating levels of miRNAs (e.g. miR-34a or miR-122) have been associated with NASH activity and fibrosis stage.

Materials & Methods: Serum samples from two independent patient cohorts and corresponding liver biopsies were studied (N=269 for GOLDEN-505 cohort; N=248 for OBESE cohort). Serum levels of 2,083 miRNAs were measured using HTG-EdgeSeq-NGS technology. The differential expressions in NASH patients at risk of fibrosis evolution (To-Be-Treated; TBT=NAS≥4, F≥2) vs Not-To-Be-Treated (NTBT) patients were assessed by bioinformatics approaches. miRNAs were then sorted by fold change and/or statistical significance (p<0.01) in the two cohorts. Correlation of relevant miRNAs with histological severity was assessed.

Results: When assessing differential expression of 2083 miRNA’s in TBT and NTBT, we identified more over-expressed than under-expressed miRNAs (36 vs 14 in GOLDEN and 17 vs 5 in OBESE). When selection of over-expressed miRNAs was based both on p-value (p<0.01) and fold change (>1.3), 21 (GOLDEN) and 14 (OBESE) miRNAs remained selected. After removing miRNAs with low expression levels in GOLDEN, cross-validation between the two cohorts gave the following list of commonly over-expressed miRNAs in TBT: Differential expressions in TBT vs NTBT of each miRNA were confirmed by RT-qPCR. As previously reported for miR-34a and miR-
122a, in both cohorts, serum levels of miR-A and miR-C increased with increasing NAS and NASH-CRN-fibrosis stage.

**Conclusion:** By analyzing >500 serum samples from two independent cohorts this NGS study has allowed a non-biased selection of circulating miRNAs associated with NASH and fibrosis. From a total number of 2,083 miRNAs, a panel of 11 miRNAs has been identified and validated that hold promise for non-invasive of NASH patients at risk of fibrosis evolution.

**Financial relationship(s) with:** Genfit

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**09**

**A new non-invasive diagnostic score to monitor change in disease activity and predict fibrosis evolution in patients with NASH.**

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**Background:** There are urgent needs for new non-invasive methods for diagnosis of NASH and to monitor disease evolution. We describe the diagnostic performances of a new algorithm including four circulating markers and assess its value to monitor disease evolution.

**Materials & Methods:** miRNA’s were measured in serum samples collected at inclusion of NASH patients (NAS≥3; N=238) in GOLDEN-505. A logistic regression approach was used to generate the best algorithm for identification of patients to be treated (TBT=NAS≥4; F≥2, N=104). After ROC analysis, the correlations of non-invasive score (NIS) with histological scores (NAS; Activity Index-AI= Inflammation + ballooning; steatosis, fibrosis score) at inclusion were examined. Using the second liver biopsy and corresponding samples collected one year later, we examined whether changes vs baseline (ΔNIS) are correlated to changes of histological scores. Results are expressed as mean±SEM. Statistical analyses were performed using Kruskal-Wallice test and Dunn’s test.

**Results:** Compared to the previous algorithm obtained with miRNA’s measured in plasma, we identified a simplified algorithm with 4 independent variables: miR34a + YKL-40 + HBA1C + A2M and comparable diagnostic performances: AUROC=0.82, Sensitivity=73%, Specificity=78%, Positive Predictive Value=72%, Negative Predictive Value=79%. NIS increased with NAS (0.35±0.04,NAS=3 vs 0.58±0.04, NAS=7, p<0.01), AI (0.34±0.02, AI=2 vs 0.74±0.01, AI=5, p<0.01) and fibrosis score (0.24±0.02, F=0 vs 0.66±0.03, F=3, p<0.001). but not with steatosis (0.39±0.04 for S=1 vs 0.47±0.2 for S=3, NS). After 1 year, ΔNIS correlated with histological evolutions In NAS≥4; F≥2 at inclusion, patients who improved fibrosis after 1 year had significantly lower NIS at baseline than patients who worsened fibrosis.

**Conclusion:** This study reports a new algorithm for identification of to-be-treated NASH patients. It could identify patient at risk of fibrosis evolution and could be used to monitor disease evolution in NASH patients with or without treatment. Cross validation in other longitudinal cohorts is warranted.

**Financial relationship(s) with:** Genfit
A precision medicine approach to comprehensive NAFLD diagnosis via metabolomics-based liquid biopsy

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Background: Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease with a prevalence between 10-40% in Western countries. Nonalcoholic steatohepatitis (NASH) is the most severe form of NAFLD and is a progressive disease that may lead to further liver injury, advanced fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Patients with NASH and fibrosis are identified for therapeutic intervention. However, increasing evidence points to different subtypes of NAFLD which progress at different rates and may respond differently to treatment. Considering the prevalence and potential severity of disease, there currently exists an urgent need for a simple, inexpensive, non-invasive point-of-care diagnostic tool. Such a tool must aid in identifying different pathological mechanisms leading to NASH, while identifying the associated biomarkers within NASH subtypes in order to more fully support development of effective drug treatments and more precise disease diagnosis.

Aims: To develop a metabolomics-based liquid biopsy model to: (1) differentiate NAFLD from normal liver controls, (2) assess severity of steatosis, (3) distinguish between simple steatosis vs. NASH, (4) assessment of fibrosis, and (5) identify and isolate metabolic signatures of relevant NAFLD subtypes.

Materials & Methods: Models were developed in an initial cohort (N=652) and validated in subsequent independent cohorts (N=118) from Europe and USA or by leave-one-out cross validation (LOOCV). In a subset of the test cohort (n=114), concomitant hepatic fat content using MR-fat fractions was available to compare the model to hepatic triglyceride content. Samples were collected under fasting conditions at the point of invasive liver biopsy. Only those samples without discrepancies in histological diagnosis of NAFLD between pathologists of each participating hospital and the core lab analysis were considered in the evaluation (NASH CRN criteria). Metabolomics was performed as previously described (J Prot Res 2012,11:2521).

Results: NAFLD Diagnosis: A BMI-dependent model discriminated between controls and NAFLD, (controls=90; NAFLD=377; AUC=0.90±0.02). Applied to the validation cohort, the performance of the model was AUC=0.87±0.04.

Steatosis Severity: There was a strong concordance with hepatic triglyceride content (r=0.81, p<0.0001). NASH diagnosis: Simple steatosis (n=246) and NASH (n=131) could be distinguished by a BMI-dependent model (AUC=0.95±0.01). The AUC...
was 0.92±0.03 in the validation cohort.

Fibrosis Assessment: Performed in 185 patients (F0=71; F1=69; F2=11; F3=29; F4=5), 4 algorithms were calculated discriminating between F0 vs. F1-4 (AUC=0.92±0.02; LOOCV: AUC=0.85), F0-1 vs. F2-4 (AUC=0.89±0.02; LOOCV: AUC=0.86), F1 vs. F2-4 (AUC=0.86±0.03; LOOCV: AUC=0.81), and F1-2 vs. F3-4 (AUC=0.89±0.03; LOOCV: AUC=0.86).

NAFLD Subtypes Discovery: Cluster analysis and validation process revealed 2 main subtypes, effectively classifying 50% and 40% of the patients (>70% reproducibility). Remaining patients (10%) showed <70% reproducibility. Biomarkers significantly differentiating between NASH and simple steatosis per subtype were listed based on ≥70% reproducibility.

Conclusions: Metabolomics-based liquid biopsy testing can be routinely applied as a diagnostic tool in NAFLD patient management, patient triage, and also provide clinical guidance in predictive and personalised medicine.

Financial relationship(s) with: OWL Metabolomics

Histological, biochemical and molecular disease progression in a diet-induced obese mouse model of non-alcoholic steatohepatitis

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Background: The Gubra diet-induced obese (DIO) mouse model of non-alcoholic steatohepatitis (DIO-NASH) displays key hallmarks of metabolically driven steatohepatitis and fibrosis. Here we aimed to investigate the temporal disease progression of the Gubra DIO-NASH mouse model by assessing metabolic parameters, liver histopathology and molecular markers.

Materials & Methods: Male C57Bl/6J mice (5 weeks of age) were offered ad libitum access to AMLN diet (40% trans-fat, 22% fructose and 2% cholesterol). Animals were terminated at 0-50 weeks on diet for histopathological evaluation of NAFLD Activity Score (NAS, steatosis, inflammation, ballooning degeneration) and fibrosis stage. In addition, immunohistochemically detection of macrophages and collagen was applied and quantified and supported by biochemical analysis of liver lipids and collagen (hydroxyproline) content. Furthermore, plasma analysis of liver enzymes (alanine/aspartate aminotransferases; ALT/AST) and lipids (total cholesterol; TC, triglycerides; TG) were included. Finally, RNA sequencing was used to analyze activity in key liver metabolic and NASH pathways.

Results: From week 10 and throughout the remainder of the study, DIO-NASH mice exhibited hepatomegaly, steatohepatitis, elevated liver cholesterol content and increased plasma levels of TC and ALT/AST. In conjunction, DIO-NASH mice developed progressive NAS, predominantly by increased steatosis and inflammation scores. Importantly, development of fibrosis was observed from around week 20 and onwards. Hence, mean fibrosis stage increased from F1 to F3-4 at study end. Collagen deposition was confirmed by increased liver hydroxyproline content and increased Col1a1 immunoreactivity. RNA sequencing revealed dysregulation of genes known to be involved in hepatic lipid handling, inflammation and macrophage recruitment as well as collagen deposition, thus supporting the histopathological findings. Furthermore, the expression of key genes in fibrosis development allowed us to fingerprint the temporal dynamics underlying fibrosis development in the DIO-NASH model.
Conclusions: We demonstrate that the Gubra DIO-NASH mouse model exhibits key clinical biomarkers of NASH and liver fibrosis which aggravates progressively with the level of adiposity and hepatomegaly. The highly detailed histopathological phenotyping provides an improved understanding of the onset and progression of diet-induced metabolically-driven NASH at both the whole animal, histological and molecular level. The Gubra DIO-NASH mouse model is therefore highly applicable for gaining further insight into the pathogenesis and novel targets in the treatment of NASH.

Financial relationship(s) with: Gubra

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Validation of the SAF score of non alcoholic fatty liver disease in an independent cohort of morbidly obese patients.

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Background: NAFLD (non alcoholic fatty liver disease) is actually the most frequent cause of liver disease worldwide. The spectrum of lesions regroups, at one end, non-alcoholic fatty liver (NAFL), with benign prognosis and, at the other end, non-alcoholic steatohepatitis (NASH), with a potential of progression to cirrhosis. Histological scores of activity and fibrosis have been established and adopted in order to select patients with an increased risk of evolution towards cirrhosis, and to facilitate their follow up. In 2005, the NASH-CRN (Clinical Research Network) established the NAFLD activity score (NAS) presumed to mirror disease activity. Although very useful in the research field, this score has weakness points in clinical practice, since steatosis, which is part of the activity grading may lead to an over-estimation of NASH. Furthermore, the degree of fibrosis is not taken into account. Recently, a scoring system (SAF score) was proposed, consisting in a semi-quantitative estimation of steatosis (S), activity (A) and fibrosis (F), where an algorithm (FLIP for Fatty Liver Inhibition of Progression) is used to determine activity. This score has the advantage of combining three histopathological features of NAFLD in the same score, thus allowing the selection of patients with significant liver disease and following their progression in time. On the other hand, it has been proven to be highly reproducible among pathologists. The aim of this study is to re-examine the validity of this scoring system in a population of morbidly obese patients and to compare the results with the NAS value and the biological parameters.

Materials and Methods: One hundred and sixty-three morbidly obese patients candidate for bariatric surgery were selected on the basis of the absence of significant alcohol consumption and the absence of underlying known liver disease. Liver biopsy was performed during surgery and a blood sample was collected in order to determine the levels of liver enzymes. For each biopsy, SAF score and NAS were determined.

Results: Among the 163 patients, 102 (62.6%) had features of NAFLD, of which 81 (49.7% of the total patients) met the criteria for NASH according to the FLIP algorithm. The remaining 21 patients (12.9%) were classified as NAFL. Concerning fibrosis, 61 of the 102 patients (59.8%) were classified as F1, 31 (30.4%) were F2 and 7 (6.9%) were F3. Ninety patients (88%) met the criteria for significant liver disease (A ≥ 2 and or F ≥ 2). The levels of AST and ALT increased progressively with the grade of activity. NAS correlated absolutely with the diagnosis of NASH in the extreme values (≤ 2 or ≥5). However, 82.2% of the patients classified as borderline NAS were diagnosed as having NASH and 17.8% as NAFL according to the
Conclusion: We confirm that SAF score and FLIP algorithm allow a simple and reproducible histopathological classification of NAFLD patients and are more discriminating than NAS in distinguishing patients with severe liver lesions.

No conflict of interest

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Quantitative imaging biomarker of disease progression in hepatocellular carcinoma and NASH

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Background: NASH is a growing contributor to chronic liver disease and already represents the second highest cause of hepatocellular carcinoma in the United States. Current diagnosis and severity assessment typically relies on histopathological analysis from biopsy samples. With novel NASH therapies under development there is a need for reliable non-invasive assessment of disease progression. The aim of the study is to demonstrate the feasibility of using routine CT imaging protocols combined with big data processing for extracting radiological signatures for assessing disease progression.

Materials and Methods: A cohort of 200 patients presenting with focal tumoral liver lesions for liver surgery or transplantation at the Hôpital La Pitié Salpêtrière in Paris was administered a non-contrast CT exam as well as a standard triphasic (arterial, venous portal and delayed phases) contrast CT scan. The patients also had tissue samples surgically excised and graded by histopathology according to the METAVIR score (F0-F4) for fibrosis. The liver was automatically segmented from the CT scans. An agnostic textural feature signature comprising a 4,096 dimensional vector was automatically extracted from a moving window scanning the entire liver image. A similarity metric based on the earth mover’s distance (EMD) was used to measure the similarity between signatures. Robustness of the signatures was tested against variations in reconstruction algorithms, equipment models and slice thickness as well as variations in intensity and contrast injection. Temporal stability was also assessed using test-retest procedures at different time points. The ability of the textural signatures to discriminate between tissues was evaluated against the variability within a tissue type. Finally, the ability to assess progression of disease was assessed longitudinally.

Results: The signatures were generally insensitive to variations in equipment configuration, reconstruction or slice thickness of acquisition as well as variations in intensity or contrast. Local or temporal variations from matched tissue samples were also small as compared to variations across tissue types. Finally, longitudinal assessment of fibrosis progression could be assessed by calibrating the signature similarity measure to known tissue samples.

Conclusions: Quantitative imaging biomarkers extracted from textural analysis of CT images using Big Data analytics could be used as surrogate markers of disease progression in NASH patients and to assess treatment response.

Financial relationship(s) with: Median Technologies
Omics Characterization of Non-alcoholic fatty liver disease (NAFLD)

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Background: Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in the United States with an estimated prevalence of 10-46% of the population and is projected to be the leading cause of liver transplantation by 2020. The application of advanced mass spectrometry based omics platforms can provide an unbiased assessment of the metabolome and proteome in patient samples to help characterize complex systemic disease conditions. The current study utilizes these platforms to study NAFLD including comparative liver tissue from repository sources and blood plasma from ongoing NAFLD treatment trials utilizing betaine (trimethylglycine), a nutritional supplement which has been shown to reduce hepatic lipid accumulation and reverse insulin resistance in animal models. The overall aim is to apply omics technologies to characterize NAFLD in both the liver and plasma for understanding the underlying disease mechanisms and origin of potential biomarkers.

Material and Methods: 50 liver tissues obtained from the Liver Tissue Cell Distribution System (LTCDS) at University of Minnesota repository, age and sex matched, were utilized for tissue comparisons of NAFLD versus other liver diseases and normal liver tissue. The betaine treatment trial analysis includes 21 patients with a clinical diagnosis of NAFLD and ALT ≥ 50 IU/mL, divided into type 2 diabetes mellitus (8) or non-diabetic (13) cohorts. Plasma samples analyzed included baseline (prior to treatment), W4 (betaine treatment), W8 (betaine treatment), W12 (end of betaine treatment), W16 (4 weeks off betaine treatment), and W24 (12 weeks off betaine treatment, back to baseline). Instrument analysis included label free high mass accuracy liquid chromatography-tandem mass spectrometry (LC-MS/MS), Velos Orbitrap, platform pipelines for both proteomic and lipidomic approaches, with variations in the LC component and detector mode (positive/negative) for the lipidome. Data analysis was performed using the AMT tag quantitative method and statistics were based upon ANOVA comparisons with the reported p value thresholds.

Results: Initial proteome analysis comparing matched 10 NAFLD liver tissue specimens with 20 normal controls, 10 chronic hepatitis C infected, and 10 alcoholic cirrhosis (AC) using a single analysis label free quantification LC-MS/MS platform identifying 38K peptides for quantification resulting in 2.5K proteins for comparison. >750 proteins were identified as significantly altered in abundance (pval <0.01) in NAFLD compared to normal control. Pathway enrichment comparison using WikiPathways identified 75 pathway as significantly altered with many unique to NAFLD, compared to AC or HepC, including sphingolipid metabolism and SUMOylation pathways. We are currently performing identification of the altered lipid profiles in plasma of the betaine treatment study (baseline and longitudinal comparison of 21 patients versus 15 matched normal controls) and anticipate presenting the correlation of these results to both the tissue pathway networks as well as to functional ALT results.

Conclusions: Identification of specific protein and pathway alterations in NAFLD liver tissue provides a baseline to help correlate additional omics data, i.e. the lipidome, in orthogonal treatment studies to help determine specific relevant metabolic pathways indicative of NAFLD.

No conflict of interest
A Physiological Biomarker, Hepatic Reserve, Predicts the Risk of Varices in NASH Patients and in Chronic HCV Patients

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Background: The emerging epidemic of NASH will require new methods to predict the risk of serious complications, such as esophageal varices, especially the clinically relevant medium-to-large varices. Static biomarkers cannot reflect the dynamic pathophysiological changes during disease progression. The goal of this pilot study was to determine if a liver physiology-based assessment of Hepatic Reserve (HR), the HepQuant®-SHUNT test, could predict the risk of varices in NASH patients and in chronic HCV patients.

Material & Methods: The HepQuant®-SHUNT test utilizes serum sampling at 5, 20, 45, 60, and 90 minutes after simultaneous administration of IV cholic acid-24-13C and oral cholic acid-2,2,4,4-d4 to determine the labeled cholates' pharmacokinetic clearances, which are used to calculate HR. The scale of HR was set using healthy controls (HR 100%) and extremely advanced liver disease patients (HR 0%). HCV patients (N=217) enrolled in the HALT-C trial were tested and had protocol endoscopies. NASH patients (N=31) were tested and endoscopic findings were captured from patient histories. HCV patients had a range of METAVIR fibrosis stages (F1 – F4, all compensated) and NASH patients had a range of Brunt-Kleiner fibrosis stages, F1 – F4, half of cirrhotics were decompensated. The ability of HR to predict the risk for any size varices and for medium-to-large varices was evaluated by logistic regression analysis and ROC analysis.

Results: HR was highly significant in univariate logistic regression models for predicting the risk of any size varices (p<0.0001) and of medium-to-large varices (p<0.0001) in HCV patients, and of any size varices (p=0.0037) and of medium-to-large varices (p=0.002) in NASH patients. The 50% probability of any size varices was reached at HR<50.6% in HCV patients, and at HR<50.7% in NASH patients. The 50% probability of medium-to-large varices was reached when HR further declined to <33.6% in HCV patients, and <39.1% in NASH patients. By ROC analysis, HR could identify NASH patients at higher risk of any size varices (c-statistic 0.87) and the optimum cutoff was an HR<59.6% at a Youden Index of 0.68. Further decline in HR could identify NASH patients at higher risk of medium-to-large varices (c-statistic 0.93) and the optimum cutoff was an HR<50.3% at a Youden Index of 0.80. Similarly, HR could identify HCV patients at higher risk of any size varices (c-statistic 0.71) and the optimum cutoff was an HR<57.9% at a Youden Index of 0.42. HR could identify HCV patients at risk of medium-to-large varices (c-statistic 0.82) and the optimum cutoff was an HR<56.8% at a Youden Index of 0.53.

Both logistic regression and ROC analyses found that the relation of HR to varices risk was very similar in both NASH and HCV patients.

Conclusions: This pilot data suggests that a physiological biomarker, Hepatic Reserve, could be utilized to assess the risk for any size varices and the risk for medium-to-large varices in NASH patients. The relationship of Hepatic Reserve to risk for varices was very similar in NASH patients and in chronic HCV patients, suggesting that the same pathophysiological mechanisms underlie both types of liver disease.

Financial relationship(s) with: HepQuant, LLC
Circulating microRNAs as noninvasive markers for the assessment of liver histology in patients with nonalcoholic steatohepatitis

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Background: MicroRNAs (miRs) are small, non-coding RNA molecules involved in post-transcriptional regulation of gene expression. Since miRs are secreted into the circulation, are stable, and are easily measured, we aimed to determine their utility as noninvasive markers of liver histology in subjects with nonalcoholic steatohepatitis (NASH).

Materials & Methods: Differential miR analysis was performed on plasma samples from 72 subjects with suspected NASH (sNASH) who failed screening for a phase 2 trial of GS-4997, an inhibitor of apoptosis signal-regulating kinase 1 (ASK1; mean age 52 years, 36% male, mean BMI 36 kg/m2), and 50 age/gender-matched controls (mean BMI 26 kg/m2). miRs were isolated using a total RNA isolation kit (Norgen Corp, Canada); complementary DNA was generated (QuantmiR kit); and 380 miRs were measured by qPCR (miRnome Profiler; System Biosciences, Mountain View). Differential miR expression was evaluated using the -△△Ct method with either U1 spliceosomal RNA or spike-in C. elegans control. Statistical analyses were performed using the Significance Analysis of Microarray (SAM) method. miRs with a minimum 2-fold difference in expression and P≤0.01 between groups were selected, and correlations with fibrosis stage and NAFLD Activity Score (NAS) were determined. Using a random decision forest method, the diagnostic performance of a miR panel for distinguishing sNASH subjects from controls was evaluated.

Results: Samples from 56 sNASH and 39 controls passed quality-control standards. Among 30 sNASH subjects with available biopsies, 57% had NAS ≥5 and 73% had stage 2-3 fibrosis. 96 miRs were differentially expressed between sNASH subjects and controls; 25 miRs were significantly up-regulated and 10 were down-regulated using the pre-defined selection criteria (highest fold-change, 4.99). Greater than 60% of these miRs have been associated with NASH pathogenesis and several novel miRs were also identified. One of the most up-regulated miRs distinguished sNASH subjects from controls with an area under the ROC curve (AUROC) of 0.891 and was strongly correlated with fibrosis stage (Spearman ρ=0.66, P=7.9×10⁻¹⁰) and NAS Score (ρ=0.67, P=3.5×10⁻¹⁰). A panel of 5 of the most highly up-regulated and 5 most highly-down regulated miRs had an AUROC of 0.925 for the differentiation of sNASH subjects from controls (sensitivity 80%, specificity 100%).

Conclusions: In this exploratory study, a panel of miRs were differentially expressed between sNASH patients and healthy controls and correlated with liver histology. Although validation of these findings is necessary, miRs have potential utility for the noninvasive diagnosis of NASH.

Financial relationship(s) with: Gilead Sciences
Treatment with selonsertib, an inhibitor of apoptosis signal-regulating kinase 1 (ASK1), reduces markers of hepatocellular apoptosis and necrosis in patients with nonalcoholic steatohepatitis (NASH)

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Background: ASK1 is a serine/threonine kinase that promotes hepatocyte apoptosis, necrosis, inflammation, and fibrosis in the setting of oxidative stress. We evaluated the therapeutic effect of selonsertib (SEL, formerly GS-4997), an inhibitor of ASK1, in a multi-center clinical trial of subjects with NASH using biomarkers of apoptotic and necrotic cell death and inflammation.

Materials & Methods: We included 72 subjects with NASH (NAS ≥ 5) and F2-3 fibrosis treated with SEL 6 mg or 18 mg orally QD alone or in combination with simtuzumab (SIM, 125 mg SQ weekly) or SIM alone for 24 weeks. Liver biopsies were performed at baseline (BL) and W24, and serum markers including cytokeratin 18 (CK-18) M30 and M65 subfractions and highsensitivity C-reactive protein (hsCRP), were measured. As no differences were observed between combination and monotherapy, data are presented by SEL treatment group only.

Results: At W24, subjects treated with SEL had dose-dependent decreases in CK18 M30 and M65 (Table). Subjects with a ≥1-stage reduction in fibrosis (fibrosis responders) or ≥1-point improvement in lobular inflammation (LI) score (LI responders) had greater reductions in CK18 compared to fibrosis or LI non-responders (Table). Consistent with reductions in CK18, improvements in ALT and GGT were also seen in SEL-treated subjects with the greatest reductions observed in LI responders. Trends toward reductions in hsCRP were also seen in SEL-treated subjects with greater reductions occurring in LI responders.

Conclusion: Consistent with its proposed mechanism of action, these data suggest that SEL decreases markers of hepatocyte apoptosis and inflammation in subjects with NASH and moderate to severe liver fibrosis.

Financial relationship(s) with: Gilead Sciences

Table 1: Relative Change (%) of Biomarkers by Selonsertib Treatment and Histologic Responses at W24

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Fibrosis Responder</th>
<th>LI Responder</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK18 (M30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEL 18mg vs SIM</td>
<td>-31.1% (±61.6, 26.3)</td>
<td>-31.5% (±62.2, 0.9)</td>
<td>0.0072</td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-6.1% (±68.4, 43.6)</td>
<td>-22.4% (±26.2, 54.8)</td>
<td></td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-41.0% (±62.2, 3.3)</td>
<td>-2.9% (±40.5, 53.4)</td>
<td></td>
</tr>
<tr>
<td>CK18 (M65)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEL 18mg vs SIM</td>
<td>-44.4% (±66.3, 33.3)</td>
<td>-48.7% (±46.9, 26.2)</td>
<td>0.011</td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-34.7% (±62.1, 37.1)</td>
<td>-31.1% (±62.1, 37.1)</td>
<td></td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-3.8% (±31.6, 13.6)</td>
<td>-22.1% (±31.6, 13.6)</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEL 18mg vs SIM</td>
<td>-10.3% (±23.1, 30.0)</td>
<td>-37.5% (±41.4, 18.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-15.5% (±33.1, 33.1)</td>
<td>-25.0% (±39.3, 8.5)</td>
<td></td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-4.8 (±25.6, 1.1)</td>
<td>-6.3% (±29.4, 14.1)</td>
<td></td>
</tr>
<tr>
<td>GGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEL 18mg vs SIM</td>
<td>-14.79% (±24.2, 13.7)</td>
<td>-21.3% (±29.6, 13.8)</td>
<td>0.13</td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-3.5% (±28.3, 23.9)</td>
<td>-12.2% (±30.6, 10.0)</td>
<td></td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-3.2% (±16.9, 2.0)</td>
<td>-3.2% (±21.3, 25.9)</td>
<td></td>
</tr>
<tr>
<td>hsCRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEL 18mg vs SIM</td>
<td>-12.2% (±40.5, 34.0)</td>
<td>-20.9% (±42.8, 6.6)</td>
<td>0.66</td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-4.2% (±34.7, 29.9)</td>
<td>-14.1% (±45.3, 29.9)</td>
<td></td>
</tr>
<tr>
<td>SEL 6mg vs SIM</td>
<td>-7.7% (±45.2, 8.5)</td>
<td>-5.7% (±37.4, 25.6)</td>
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</tr>
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</table>

* Median % change from baseline (Q1, Q3) at W24 and n with available samples.
Limitations of HOMA-IR in Assessing Insulin Resistance in NAFLD

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Background: Non-alcoholic fatty liver disease (NAFLD) is often accompanied by insulin resistance (IR). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) is an approximation of IR (predominantly hepatic) and is used in clinical trials. Values > 2.0-2.5 are considered indicative of IR and possibly of NAFLD. However, HOMA-IR is based on two highly variable parameters (fasting insulin and glucose) and its reliability is unclear.

Aim: To assess the intra-individual variability of HOMA-IR and thus, its reliability as a metric, in a well-characterized cohort of NAFLD subjects with frequent visits in a 6-week period.

Materials & Methods: This is a retrospective analysis from an ongoing clinical trial of vitamin E in patients with biopsy-proven NAFLD. Subjects were admitted for an inpatient stay at the NIH Clinical Center (CC) 1-2 weeks prior to starting vitamin E treatment. Patients were then seen for weekly outpatient visits immediately before and at weeks 1, 2 and 3 after starting treatment. On week 4, they were admitted again for inpatient evaluation. Fasting blood was collected on each of the 6 visits (2 inpatient and 4 outpatient) and insulin and glucose levels measured at the CC Department of Laboratory Medicine using standard assays. HOMA-IR was calculated as insulin[μU/ml]/glucose[mg/dL] /405. Liver fat content was measured by 1H-magnetic resonance spectroscopy at baseline and week 4.

Results: Data was available from 19 subjects. At baseline, liver fat content was 15.6±10.3%, glucose 108±16 mg/dl, insulin 20.2±10.7 μU/ml and HOMA-IR 5.6±3.7. HOMA-IR varied widely between visits; the range of values (maximum-minimum) per patient during a 6-week period was 4.63±2.79 and the within-subject coefficient of variation (wsCV) was 29.4% (range 11.3%-49.7%). The wsCV for glucose was 8.9±6.8% and for insulin 27.0±10.8%, suggesting variability in insulin levels as the main driver of HOMA-IR variability. The fluctuations in HOMA-IR values did not correlate with changes in body weight or liver fat content during the study period and did not show a significant trend over time, suggesting the changes are not due to the vitamin E treatment. The variability between the 2 inpatient measurements (where prior-day meal, fasting, bed rest and time of draw were uniform) was not different from the variability of outpatient visits (45.4±34.7% vs. 45.3±21.5%) suggesting this was not due to test timing, duration of fasting or physical activity. In 4 of the 19 patients, there were HOMA-IR values both above and below the 2.5 cut-off during the 6-week period.

Conclusions: HOMA-IR has a very high inherent intra-individual variability in subjects with NAFLD, which cannot be overcome by standardizing experimental procedures. Despite its ease of use and relative low cost, HOMA-IR is a noisy measure and its utility as a biomarker in NAFLD studies is limited.

No conflict of interest
Identification of divergent gene expression profiles in diet-induced obese (DIO) mice and DIO mice showing hallmarks of non-alcoholic steatohepatitis (NASH) for NASH biomarker development

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1Gubra Aps, Hørsholm, Denmark

Background: With the increased prevalence of non-alcoholic fatty liver diseases (NAFLD) there is a growing need for diagnostic procedures distinguishing patients with liver steatosis from non-alcoholic steatohepatitis (NASH). In order to identify potential biomarkers for NASH, we characterized the gene regulatory events associated with NASH development in a DIO-NASH model showing histological hallmarks of NASH as compared to standard DIO mice.

Materials & Methods: Male C57BL/6J mice (5 wks old) were offered ad libitum access to a diet high in trans-fat (40%), fructose (22%) and cholesterol (2%) (AMLN diet), or a diet high in fat (60%) (60% DIO diet), for 30 wks. Comparator group of animals were fed standard chow. After diet-induction, animals were terminated and a liver sample was obtained for blinded histopathological assessment of NASH disease progression using a clinically derived NAFLD Activity Score (NAS), including Fibrosis Stage. Frozen liver samples were used for mRNA purification followed by RNAseq and subsequent bioinformatics analysis. Differential expression between groups was assessed using the DESe2 package for R.

Results: The AMLN NASH diet induced a distinct metabolic and hepatic phenotype with pronounced steatosis, inflammation and ballooning degeneration, and with 9 of 10 animals showing fibrosis stage of 1 or higher. The 60% DIO diet induced prominent steatosis with a more microvesicular profile, as compared to DIO-NASH mice. No development of inflammation, ballooning and fibrosis was detected in DIO mice. The gene regulatory analyses identified marked changes in prominent lipid metabolism pathways as compared to chow, including a dramatical reduction in cholesterol synthesis for DIO-NASH as compared to chow and DIO. Moreover, the inflammatory expression profile of DIO-NASH shifted from a predominately CD163 genotype to a CD68 genotype, indicating a decreased M1/M2 macrophage ratio in comparison to DIO and chow. c-c chemokine receptor 2 was significantly elevated in DIO-NASH as compared to chow and DIO with a further, widespread regulation of cytokines and Toll-like receptors (TLRs), including TLR4. Finally, the expression of a wide panel of fibrillary collagens was dramatically increased for DIO-NASH compared to DIO and chow, in alignment with the fibrotic state of the NASH livers.

Conclusions: We have successfully applied RNAseq for characterization of global regulatory events in DIO-NASH compared to standard DIO and chow mice. DIO-NASH mice displayed a genetic profile significantly different from both chow and DIO with elevated expression of inflammatory markers such as cytokines and TLRs in combination with elevated expression of fibrillary collagens. These findings may be employed for identification of potential biomarkers related to NASH development.

Financial relationship(s) with: Gubra
**FibroScan-based score to identify patients with non-alcoholic steatohepatitis: development in a multi-centric British cohort and validated in French and American cohorts**

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**Background:** Reliable non-invasive biomarkers are needed for the diagnosis and monitoring of patients with non-alcoholic steatohepatitis (NASH). Echosens has developed a score based on a single Fibroscan (liver stiffness measurement (LSM) and controlled attenuation parameter (CAP)) to diagnose NASH. The aim of this study is to present this score developed in a British multi-centric cohort and to show its performance in two external validation cohorts.

**Materials & Methods:** Derivation cohort: Patients with suspected NASH prospectively underwent FibroScan and liver biopsy (LB) at seven British centres. LB were read in a blinded manner by two expert pathologists. NASH was diagnosed using the FLIP algorithm. To develop the NASH score, the cohort was split randomly into training (80%) and validation (20%) sets. Only patient with body mass index (BMI)<40 kg/m2 were considered. LSM and CAP were combined using statistical modeling and repeated split sampling leading to the selection of the optimum model. Score performance was assessed using area under receiver operating curve (AUROC), together with its 95% confidence interval, and was internally validated using bootstrap method.

American validation cohort: Patients referred for a routine colon cancer screening in a single American center were enrolled. They were screened for evidence of NAFLD using FibroScan®, LiverMultiScan® (magnetic resonance imaging proton density fat fraction (PDFF), liver inflammation and fibrosis (LIF) score) and magnetic resonance elastography (MRE). Patients with PDFF≥5% or LIF≥2 or LSM ≥7kPa on FibroScan or ≥3kPa on MRE were recommended a LB, which were all read by a single expert pathologist.

**Results:** Derivation cohort: 144 patients with a median BMI of 32.9 [IQR=6.9] kg/m2 and age of 54 [21] years. 58% were male and 58% had NASH. French cohort: 43 patients with a BMI of 30.0 [8.0] kg/m2 and age of 53 [22] years. 67% were male and 58% had NASH. American cohort: 443 patients were screened. 154 (35%) patients underwent a LB. They had a BMI of 32.6 [6.1] kg/m2 and age of 57 [10] years. 64% were male and 16% of had NASH. In the training set (N=116), performance of the NASH score in terms of AUROC was 0.84 [0.77-0.91]. In the interval validation set (N=28), AUROC=0.88 [0.76-0.78]. Bootstrap validation led to an AUROC=0.85 [0.79-0.91]. AUROC in the French external validation was 0.89 [0.75-
and 0.85 [0.77-0.93] in the American external validation cohort.

In each cohort, optimal cutoff was appraised maximizing the Youden index and leading to the following sensitivities (Se) and specificities (Sp) in the: training set: Se=0.82/Sp=0.73; validation set: Se=0.88/Sp=0.75; bootstrap validation Se=0.80/Sp=0.81; French cohort: Se=0.97/Sp=0.71; American cohort: Se=0.88/Sp=0.72.

**Conclusion:** A novel score based a single FibroScan examination (LSM & CAP) has shown a good diagnostic performance for NASH in the derivation cohort. When applied to the external validation cohorts this score shows excellent sensitivity and acceptable specificity.

Financial relationship(s) with: Echosens

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The LiMAX test - iv 
13C-methacetin breath testing – non-invasively diagnoses cirrhosis

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**Background:** The LiMAX test, a 13C-methacetin breath test that uses intravenous administration of the substrate has demonstrated to be a valuable and reliable tool for accurately measuring the functional capacity of the liver (Stockmann 2009). The LiMAX test has shown to correlate well with clinical scoring systems, while avoiding the pitfalls (Malinowski 2014). Using the data from various studies we have analyzed the ability of the LiMAX test to non-

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**Materials & Methods:** The LiMAX test is a breath test that measures CYP1A2 activity after intravenous dosing of 13C-methacetin. The LiMAX test is manufactured and marketed by Humedics GmbH, Berlin, Germany. 727 patients who underwent surgical resection or transplantation were included in 3 different studies. Exclusion criteria were acute on chronic liver disease and acute manifestations of complications of liver disease. Histological analysis (according to Ishak) was performed by two pathologists on the liver specimen available after liver resection or liver explantation.

**Results:** A LiMAX value of 240 ug/kg/h was selected as cut-off value for cirrhosis. Based on this cut off value the sensitivity and specificity was calculated provided that sufficient patients were available.

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<td>Sample size</td>
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<td>403</td>
<td>74</td>
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<td>Study type</td>
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<tr>
<td>BMI &gt;30</td>
<td>40 (16.3%)</td>
<td>92 (22.8%)</td>
<td>15 (20.3%)</td>
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<tr>
<td>Cirrhosis</td>
<td>77 (31.3%)</td>
<td>403 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

ALL PATIENTS

| Sensitivity | 87.0% (67/77) | 91.3% (368/403) | - (0,0%) |
| Specificity | 94.1% (159/169) | - (0,0%) | 94.6% (70/74) |

PATIENTS WITH BMI >30

| Sensitivity | 92.9% (13/14) | 94.6% (87/92) | - (0,0%) |
| Specificity | 80.8% (21/26) | - (0,0%) | 86.7% (13/15) |

**Conclusion:** The LiMAX test reliably diagnoses cirrhosis in patients.

Financial relationship(s) with: Humedics GmbH
Serum assessed “True collagen type III formation” (Pro-C3) levels as a marker of non-alcoholic steatohepatitis (NASH) in a prospective cohort

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Background: Majority of patients with non-alcoholic fatty liver disease (NAFLD) have simple steatosis, while 10-30% have non-alcoholic steatohepatitis (NASH) which has been associated with progressive liver fibrosis and cirrhosis. Although screening high-risk populations for NASH has recently been recommended by the European Association for the Study of Liver (EASL), lack of accurate non-invasive tests for NASH is hindering an effective evaluation of people at risk.

Aim: To investigate the ability of the type III collagen formation neoepitope marker “Pro-C3”, known to be related to severity of disease, its progression and response to treatments in chronic liver disease in detecting and grading NASH in patients with risk factors for NAFLD.

Materials & Methods: We enrolled 103 well characterised patients with clinically established NAFLD prospectively. We estimated serum concentrations of Pro-C3 by using competitive ELISA. We correlated Pro-C3 levels with clinical, demographic, imaging parameters and liver histology.

Results: There was a significant correlation with serum Pro-C3 levels and NAFLD activity score (NAS). Pro-C3 levels were 50-150% higher with hepatocyte ballooning score 1 or 2-3 compared to 0 (P<0.001) and 58-130% higher with a lobular inflammation score of 1 or 2-3 compared to 0 (P<0.01-0.001; Figure 2), and 78-115% higher with liver fibrosis scores 2 or 3-4 compared to 0 (P<0.01-0.001). Furthermore, it was shown that Pro-C3 was correlated to percentage of fibrosis and fat (P=0.01-0.0007).

Conclusions: In conclusion, elevated serum Pro-C3 was significantly associated key components of NASH correlating well with NAS and degree of liver fibrosis. Pro-C3 was a potential marker of NASH for monitoring disease progression.

Financial relationship(s) with: Nordic Bioscience

13C-Methacetin Breath Test accurately assesses clinically significant portal hypertension in patients with NASH cirrhosis

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Background: Determining prognosis for patients with compensated NASH cirrhosis using noninvasive tools is a significant unmet need. Sub-staging of compensated cirrhosis is based on the presence (or absence) of clinically significant portal hypertension (CSPH), as defined by a hepatic venous pressure gradient ≥ 10 mmHg, which is the main predictor of decompensation. However, measurement of
HVPG is invasive and not used routinely. The 13C-methacetin breath test (MBT) is a non-invasive, non-operator dependent, real-time molecular correlation spectroscopy assay that measures the abundance of 13CO2 produced by hepatic cytochrome p450 metabolism of ingested non-radioactive 13C isotope-labeled methacetin in expired breath, using the Exalenz BreathID© MCS System. MBT has been shown to assess the degree of liver function in patients with cirrhosis and has been shown to correlate with HVPG in mostly viral cirrhosis.

Aim: To investigate the correlation of MBT with HVPG in patients with biopsy-proven compensated NASH cirrhosis.

Materials & Methods: Baseline data was collected from NASH patients screened for the Galectin Therapeutics Phase II clinical trial (NCT02462967) evaluating GR-MD-02 who underwent MBT and HVPG measurement within 19 (±14) days from each other with an average HVPG of 11.3 (±4.6) mmHg. All patients had cirrhosis and had never had ascites, variceal hemorrhage or encephalopathy. Demographic information, MBT, HVPG results, liver stiffness and lab tests, were collected and analyzed by logistic regression modeling.

Results: Analysis was conducted on 155 patients (53 males; 34%) with 91 (59%) having CSPH. Average age was 58.3 (±8.7) years, with average BMI 34.9 (±6.6) kg/m2. MBT-derived model detected CSPH with an AUROC of 0.83 (95%CI: 0.77-0.90). Selecting two cutoff points in the model with 85% sensitivity and 85% specificity, CSPH could be ruled in or ruled out in 73.5% of these patients with 89% PPV and 80% NPV, resulting in an AUROC of 0.89 (95%CI: 0.82-0.95). Liver Stiffness as measured by Transient Elastography was available for 120 of the patients and resulted in an AUROC of 0.71 (95%CI: 0.61-0.80). Platelet count and APRI showed an AUROC of 0.76 (95%CI: 0.68-0.83) and 0.71 (95%CI: 0.63-0.80), respectively.

Conclusion: MBT non-invasively detects CSPH with high sensitivity and specificity, and may serve as a useful tool in the stratification of patients with compensated NASH cirrhosis at point-of-care.

Financial relationship(s) with: Exalenz Bioscience, Galectin Therapeutics

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A combination of serum Pro-C3 and various clinical parameters is superior at identifying advanced fibrosis in patients with non-alcoholic fatty liver disease compared to established serological fibrosis tests

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Background: The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing in conjunction with that of obesity. However, only a small proportion of NAFLD patients have a fibrogenic phenotype and are at risk of developing cirrhosis and liver related adverse outcomes. Non-invasive tests for the early detection of significant liver fibrosis are needed to overcome the inherent problems with diagnosing via liver biopsies. Identifying patients at high-risk would enable timely interventions. We compared the diagnostic power of a novel biomarker score to several blood based fibrosis tests; APRI, FIB-4 and NAFLD fibrosis score.

Materials & Methods: 150 patients with extensive clinical phenotyping including liver biopsy were included in this cross-sectional study. Serum concentrations of Pro-C3 were measured by the use of a competitive ELISA.
An expert pathologist carried out liver histology; the biopsies were scored according to the NASH CRN score. Ninety patients had a fibrosis score of F=0-1, 52 had F=2-3 and 8 patients were found to be cirrhotic (F=4). Thirty-seven percent of patients were diagnosed with NASH. Advanced fibrosis was defined as fibrosis F≥3. APRI, FIB-4 and the NAFLD fibrosis score was calculated using published algorithms. Clinical and laboratory variables were subjected to multivariate modelling to predict the presence of advanced fibrosis. An algorithm was developed from the multivariate modelling; the components included in the model were platelet count, presence of diabetes, log Pro-C3 level, and waist/hip ratio. The Youden Index determined by ROC analysis was used to calculate the cut-off points of the developed algorithm to rule-in or rule-out advanced fibrosis.

Results: The ability of non-invasive tests to identify patients with F≥3 (advanced fibrosis) was assessed by determining the AUROC for the various non-invasive fibrosis tests. APRI gave an AUROC of 0.73 (NPV= 86%, PPV= 55%), the AUROC for FIB-4 was 0.80 (NPV= 46%, PPV=91%) and for the NAFLD fibrosis score the AUROC was 0.82 (NPV= 46%, PPV=93%). The Pro-C3 FIB score was found to be significantly superior to all other tests with an AUROC = 0.91 (P=0.03) (NPV= 97%, PPV= 56%). Using cut-off values previously published, the NAFLD Fibrosis score and the FIB-4 score correctly classified 54% and 55% respectfully. Whereas APRI correctly classified 48%. The Pro-C3 FIB score, however, was able to correctly classified 82% of patients using a cut-off value of 1.6738.

Conclusion: The Pro-C3 FIB Score was shown to be significantly better at identifying patients with advanced fibrosis (F≥3) from a cohort of NASH/NAFLD patients when compared to other previously validated non-invasive fibrosis tests. By applying this model, a liver biopsy could have been avoided in 82% of patients.

Financial relationship(s) with: Nordic Bioscience

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Palmitate breath test: A dynamic marker of fatty acid oxidation in non-alcoholic fatty liver disease

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Background: Non-alcoholic fatty liver disease (NAFLD) reflects impaired energy balance. The fate of an oral caloric load and especially that of fatty acids (FA) may be different in NAFLD, particularly its rate of oxidation (FAO), one of the paths for elimination. We aimed to measure total body FAO in response to an oral FA load in NAFLD and controls using a breath test.

Materials & Methods: Fasting subjects with NAFLD and healthy lean controls were given an oral load of 10mg/kg of 13C-palmitic acid (PA) in a standardized liquid meal (Ensure®) over 5 minutes. 13CO2/12CO2 ratio in expired air was measured continuously at rest over 6 hours by the BreathID molecular correlation spectrometer. The percent dose recovered (PDR), reflecting the appearance of ingested 13C in expired air at any given time point, was calculated. Cumulative percent dose recovered (CPDR), the total amount of ingested 13C label that was recovered during the 6 hours, reflects rate of PA oxidation and was the study primary end point.

A small subset of NAFLD subjects underwent 12 weeks of lifestyle modification including dietary intervention and moderate-intensity physical activity. The breath test was performed at baseline and after 12 weeks of lifestyle intervention.

Results: The study population included 28 subjects with NAFLD (57% male, median age 53 [27-73], BMI 32.6 [21.3-41.8], ALT 34.5 [20-244], liver fat content by 1H-MRS 16.6% [7.3-28.7]) and 11 healthy controls (45% male, age 33 [21-51],
BMI 22.3 [20.4-24.7]). NAFLD subjects oxidized a lower fraction of the oral PA (by CPDR) over 6-hours compared to controls (8.38 vs. 13.09%, p=0.0001). Absorption rates, assessed by time to peak PDR, did not differ between groups (4.91 [NAFLD] vs. 4.67 hours, p=0.51). Of the 4 subjects who were assessed again after lifestyle intervention, two had a marked decrease of >40% in ALT, and concomitantly showed an increase in CPDR by 54 and 68% from baseline levels. In contrast, in the 2 other subjects who had no impact of intervention on their ALT, CPDR was unchanged.

**Conclusion:** Patients with NAFLD oxidize a lower proportion of an oral FA load suggesting a greater proportion is retained. Improvement in NAFLD after lifestyle intervention seems to be associated with improved handling of oral FA. Whether this is unique to NAFLD or is a generalized part of the metabolic syndrome remains to be seen. The palmitate breath test can potentially be a dynamic marker of FAO rates in subjects with NAFLD.

No conflict of interest

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**On the importance of assessing the entire liver in MRI-PDFF studies of liver fat**

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**Background:** Magnetic Resonance Imaging using Proton Density Fat Fraction MRI-PDFF has been used in multiple intervention studies of drugs affecting liver lipids. The evaluation of liver fat can be done in several ways from MRI-PDFF data. A single Region-Of-Interest (ROI), Multiple ROI’s or assessment of the entire liver, i.e. outlining the liver in all slices excluding bile ducts and liver veins. The later one also allows liver volume to be quantified. The purpose of this study was to investigate the importance of the various techniques on ability to detect significant changes in liver fat%.

**Materials & Methods:** 10 morbidly obese subjects undergoing low calorie diet (LCD) were included in the study. MRI-PDFF was performed at baseline, day 0 and at day 3,7,14 and 28 following initiation of the VLCD diet in all subjects. Fat fraction maps was generated and the fat fraction (%) was measured using 1,2,3 and 4 ROI’s as well as the entire liver excluding bile ducts and liver veins. The change in liver fat was measured either by taking the mean of the different ROI’s or the mean of the entire liver. In addition, the liver volume was quantified. The added mean coefficient of variance of the different number of ROI’s used using the whole liver assessment as the ground truth was calculated using every timepoint in all subjects.

**Results:** The added coefficient of variance was 20.6%, 13.7%, 13.2% and 12.1% respectively when using the mean from 1,2,3 or 4 ROI’s in all 10 subjects. Examples from two patients are shown in figure 1. Furthermore, the assessment of liver volume showed a disconnect between reduction in liver volume and liver fat where the reduction in liver volume plateaued after 14 days while liver fat continued to decrease. This indicated that other factors such as glycogen levels and water bound to glycogen affect the liver volume.

**Conclusion:** The current study demonstrates the potential advantage of assessing the entire liver and liver volume when quantifying intervention induced changes in liver fat.

**Figure 1:** Shows examples from two patients undergoing LCD with repeated MRI-PDFF measurements at day 0,3,7,14 and 28. The whole liver data is shown as the solid line.

Financial relationship(s) with: Antaros Medical
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