

Randomised clinical trial: a leucine-metformin-sildenafil combination (NS-0200) vs placebo in patients with non-alcoholic fatty liver disease

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Summary

Background: Sirtuin 1 (Sirt1) is suppressed in non-alcoholic fatty liver disease (NAFLD), while its' stimulation or overexpression results in reduced disease severity in pre-clinical NAFLD models. Leucine allosterically activates Sirt1 and synergise with other Sirt/AMPK/NO pathway activators. We developed a triple combination of leucine, metformin and sildenafil (NS-0200), which was effective in a mouse model of non-alcoholic steatohepatitis (NASH).

Aim: To report the results from a Phase 2, randomised clinical trial of of NS-0200 in 91 subjects with NAFLD (liver fat $\geq 15\%$ by magnetic resonance imaging-proton-density fat fraction (MRI-PDFF)).

Methods: Subjects were randomised to placebo, low-dose (1.1 g leucine/0.5 g metformin/0.5 mg sildenafil) or high-dose NS-0200 (1.1 g leucine/0.5 g metformin/1.0 mg sildenafil) b.d. for 16 weeks; change in hepatic fat was assessed via MRI-PDFF, and lipid metabolism was assessed via changes in the lipidomic signature. Seventy subjects completed the trial and met a priori compliance criteria. Analyses were conducted on the full cohort and on those with alanine aminotransferase (ALT) values above median (50 U/L; n = 35).

Results: In the full cohort, active treatments did not separate from placebo. High dose NS-0200 reduced hepatic fat by 15.7% (relative change from baseline) in the high ALT group ($P < 0.005$) while low dose NS-0200 and placebo did not significantly change hepatic fat. Lipidomic analysis showed dose-responsive treatment effects in both overall and high ALT cohorts, with significant decreases in metabolically active lipids and up-regulation of fatty acid oxidation.

Conclusion: These data support further evaluation of high-dose NS-0200 for treating NASH, especially in those with elevated ALT (NCT 02546609).

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N. Chalasani and M. B. Zemel are the Co-corresponding authors for this article.

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1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disorders characterised by hepatic lipid accumulation and cellular degeneration, in the absence of significant alcohol consumption or secondary causes of hepatic steatosis.¹ NAFLD includes those with non-alcoholic steatohepatitis (NASH), who are most likely to develop progressive fibrosis, cirrhosis and hepatocellular carcinoma. The prevalence of NAFLD is 10%-25% in the general population, and increases to ~75% in patients with obesity and diabetes.¹⁻³ Currently, the most widely accepted view of the pathogenesis of NAFLD is based on a "two-hit," or a "multiple-hit" model, with insulin resistance leading to hepatic lipid accumulation, representing the first hit. Successive activation of multiple pathways resulting in oxidative and inflammatory stress are thought to represent additional hit(s) which mediate progression of disease and fibrosis;^{4,5} among individuals with NAFLD, 23.8% are reported to have \geq F2 fibrosis and 2.3%-9.7% exhibit \geq F3 fibrosis.⁶ However, despite the pivotal role of insulin resistance in the pathogenesis of NAFLD, improving insulin sensitivity with metformin has only a minimal independent effect on liver histology and therefore is not clinically recommended.^{1,7} There is some evidence that pioglitazone improves NASH;^{8,9} however, that primary outcome measure was not achieved in a large clinical trial.⁸ Furthermore, effects reported with pioglitazone do not appear to be a thiazolidinedione-class effect, as rosiglitazone has not been effective in multiple clinical trials.^{9,10} There are presently no FDA-approved therapeutic agents for treatment of NASH, although weight loss exceeding 5%, whether via hypocaloric diet, exercise or both improve hepatic steatosis, and more substantial weight loss (~10%) may improve necroinflammation (sustained inflammation leading to necrosis and fibrosis) as well.^{1,11}

The 5' adenosine monophosphate-activated protein kinase (AMPK)/Sirtuin 1 (Sirt1) pathway is a key regulator of mitochondrial biogenesis, energy and lipid metabolism, including hepatic lipid metabolism.¹²⁻¹⁷ Activation of this pathway via either enzyme prevents or reverses excess hepatic lipid accumulation and inflammation,¹⁸⁻²² thus representing possible therapeutic targets for NASH. We have demonstrated that L-leucine has a unique role as an activator of the Sirt1/AMPK pathway,^{14,23,24} serving as a partial mimetic of caloric restriction in pre-clinical models, and thereby modulates lipid and energy metabolism and increases insulin sensitivity.

Consequently, adding L-leucine to metformin results in a novel, synergistic interaction that enabled a 65%-80% metformin dose reduction with no loss of anti-diabetic efficacy in diabetic rodent models^{25,26} and a 40% metformin dose reduction in a recent clinical trial.²⁷ Moreover, this combination of leucine with low-dose metformin reversed hepatic steatosis in pre-clinical studies in murine models of type 2 diabetes and NASH despite the lack of observed effect of full-dose metformin in the absence of L-leucine,²⁸ and adding low-dose sildenafil synergistically enhances this effect by stimulating endothelial nitric oxide synthase (eNOS) activity leading to further stimulation of Sirt1, and thus further attenuating inflammation and fibrosis.^{15,29} Accordingly, this exploratory phase 2 study

was conducted to evaluate the efficacy of NS-0200, a leucine-metformin-sildenafil fixed-dose combination, in reducing hepatic steatosis and improving related metabolic parameters in a placebo-controlled multicentre 16-week clinical trial.

2 | METHODS

2.1 | Study design

This was a randomised, placebo-controlled, phase 2, double-blind study designed to evaluate the effects on hepatic steatosis of 2 fixed-dose combinations of leucine, metformin and sildenafil (NS-0200) administered twice daily for 16 weeks, compared to placebo (NCT 02546609). Active treatments consisted of capsules containing 1100 mg leucine, 500 mg metformin and either 0.5 or 1.0 mg sildenafil administered twice daily orally (Table 1).

Subjects were screened at clinical sites for basic eligibility criteria with a detailed medical history and physical examination and blood analysis 14 days prior to initiating the study. Those subjects who met eligibility criteria returned for a screening magnetic resonance imaging (MRI) exam to estimate hepatic fat fraction by proton-density fat fraction (PDFF) within 7 days prior to study initiation. Participants with hepatic fat fraction \geq 15% by MRI-PDFF were enrolled in the study. Participants who qualified by MRI-PDFF returned to the clinic on study day 1 for baseline laboratories and first dose of study medication, followed by visits on days 7, 28, 56, 84 and 112 (end of study). Subjects were also contacted by telephone on days 14, 42, 70 and 98 to enhance compliance. Study visits included routine blood tests, body weight and assessment of compliance via pill counts, and the final study visit included the endpoint MRI to assess PDFF change.

This study was approved by the Institutional Review Boards at each participating centre, and written informed consent was obtained from all participants prior to study enrolment and initiation of study-specific procedures.

2.2 | Eligibility

The key inclusion criteria were age 18-75 years at study entry, MRI-PDFF \geq 15%, ALT \geq 30 U/L for men and \geq 19 U/L for women, body mass index between 25 and 40 kg/m² and clinical and laboratory criteria consistent with NAFLD. Subjects with evidence of other chronic liver disease or with history of significant alcohol consumption, defined as >7 drinks/wk for both males and females for the prior 6 months were excluded from the trial.

2.3 | Randomisation and blinding

A computer-generated randomised block schedule was prepared by a third party, and treatment allocation was performed centrally for all sites, with randomization numbers assigned in chronological order. Study medication was provided to study centres in a blinded manner, and sponsor personnel, study staff, MRI analysis staff and subjects were blinded to treatment assignment.

TABLE 1 Treatment arms

Treatment arm	Subjects (N) randomised	Dosing regimen	Study Medication Dosed Twice/Day ^a		
			Leucine	Metformin	Sildenafil
A	24	Placebo	N/A	N/A	N/A
B	35	NS-0200	1100 mg	500 mg	0.5 mg
C	32	NS-0200	1100 mg	500 mg	1.0 mg

^aAdministration was twice daily (b.d.); therefore, the total daily dose of NS-0200 is twice that listed for each component.

2.4 | Primary and secondary outcomes

The primary outcome was 16-week change in hepatic fat fraction, estimated by MRI-PDFF. Secondary endpoints were 16-week changes in liver enzymes (ALT, AST, GGT); cytokeratin 18 (K18, M30 epitope); HbA1c, fasting glucose, insulin and insulin sensitivity; blood lipids (cholesterol, LDL, HDL and triglycerides); hsCRP; and serum metabolomic profile.

2.4.1 | MRI acquisition

An MRI of the liver was performed using a torso-array surface coil centred over the upper abdomen using one or both types of breath-hold, noncontrast, gradient-recalled-echo, axial 2D sequence: a multiple-echo (ME) 6-echo sequence, and a double-double-echo (DDE) sequence. MRI-PDFF results from these 2 types of sequence have been shown to be nearly identical.³⁰ Target MRI scanning parameters were selected to avoid or correct for possible confounding factors (eg, T1 bias, T2* decay, multi-frequency interference) that could introduce error in fat quantification.³¹⁻³⁶ Images in Digital Imaging and Communications in Medicine (DICOM) format were transferred from clinical trial sites to a central Radiology Coordinating Center (University of California at San Diego) for quality control (QC) inspection, and analysis.

2.4.2 | MRI QC and analysis

After intake QC inspection of images and DICOM-header parameter information, 3 circular, 1-cm radius regions of interest (ROIs) were placed in the right lobe of liver, preferentially on fifth-echo liver images for the ME sequence, and on the first-echo liver images of the first DDE sequence, avoiding major vessels and ducts, lesions, artefact, other organs and liver edges. Those ROIs were propagated to images for the other echoes. ROI signal intensities from those images were analysed with the MATLAB software analysis package (MathWorks, Natick, MA) using a custom fitting algorithm that assumed exponential T2* signal decay, and applied a multi-peak spectral model to account for fat-fat and fat-water multi-frequency interference effects, based on the work of Hamilton et al.³⁷ MRI-PDFF and effective transverse relaxation time (T2*) were calculated for each ROI location. Values of MRI-PDFF, T2* and r^2 (where r is the Pearson-r fitting coefficient for the custom fitting algorithm) were recorded for each ROI, and mean values of the 3 ROI-location PDFF values were recorded for each acquired sequence. To exclude

poorly fitted results, PDFF values for an ROI were excluded if the r^2 fitting parameter was $<.97$.³⁸

2.5 | Metabolomic assessment

Serum metabolomic profiles were semi-quantified by ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) as previously described.³⁹ Briefly, 2 separate UHPLC-Time of Flight-MS based platforms analysing methanol and chloroform/methanol serum extracts were combined with a UHPLC-single quadrupole-MS based platform used to analyse amino acids. A specific metabolite extraction procedure was performed for each platform. Identified ion features in the methanol extract platform included fatty acids, acyl carnitines, bile acids, monoacylglycerophospholipids, monoetherglycerophospholipids, free sphingoid bases, N-acyl ethanolamines and oxidised fatty acids. The chloroform/methanol extract platform provided coverage over glycerolipids, cholesterol esters, sphingolipids, diacylglycerophospholipids and acyl-ether-glycerophospholipids. Lipid nomenclature and classification follows the LIPID MAPS convention, www.lipidmaps.org. Metabolite extraction procedures, chromatographic separation conditions and mass spectrometric detection conditions are also detailed in.³⁹ Metabolomics data were pre-processed using the TargetLynx application manager for MassLynx 4.1 (Waters Corp., Milford, MA). Intra-batch (multiple internal standard response corrections) and inter-batch (variable specific interbatch single point external calibration using repeat extracts of a commercial serum sample) data normalisation is described in.⁴⁰

2.6 | Safety evaluation

Safety assessment included physical examination with vital signs, 12-lead electrocardiogram, clinical chemistry, haematology and urinalysis. Adverse events were categorised by severity, outcome and relationship to study drug.

2.7 | Statistics

The primary analysis population was per protocol (PP) for efficacy endpoints and intention to treat (ITT) for safety endpoints. The PP population consisted of all ITT subjects who completed all study visits, had a valid MRI measured at the endpoint and who adequately complied with the study protocol without major protocol deviations and received $\geq 80\%$ of provided study medication via pill counts. A

post-hoc subgroup analysis consisting of participants with ALT levels greater than median value (>50 U/L) was also conducted.

A mixed-model of analysis of covariance (ANCOVA) was used to analyse changes in MRI-PDFF from baseline to week 16, with baseline MRI-PDFF adjusted as a covariate. The least squares (LS) means, standard errors (SEs) and the corresponding 95% confidence intervals (CIs) for the changes from baseline to week 16 were derived from the model for each treatment. Each of the fixed-dose combinations of leucine, metformin and sildenafil treatment groups (low dose, treatment group B; and high dose, treatment group C) were compared to placebo group (treatment group A), and the LS means for the treatment difference (treatment B or C minus treatment A), the SEs, the associated 95% CIs and the *P*-values computed accordingly; unless otherwise noted, all changes in MRI-PDFF are reported as relative change, expressed as % change from baseline. Secondary efficacy endpoints were similarly analysed via mixed effects model, with baseline value used as covariate and with logarithmic transformation as necessary to ensure data normality.

Univariate analyses were performed on normalised metabolomic data. These analyses consisted in several binary comparisons over different defined subgroups of interest: paired comparisons between study endpoint and baseline and unpaired comparisons between treatment arms at baseline. For each metabolite, changes between subgroups were calculated as the base 2 logarithm of fold-change (mean of subgroup 1 divided by mean of subgroup 2). These values were accompanied by a significance level based on *P*-values from Student's *t*-test. Three levels of increasing significance were considered: $P < 0.05$, $P < 0.01$ and $P < 0.001$. All calculations were performed using statistical software package R v. 3.4.1 (R Core Team, 2017; <https://www.R-project.org/>). In order to visually compare results, base 2 logarithm fold-changes and significances were used to create detailed heatmaps, grouping metabolites by families.

Sample size requirements for the trial were initially estimated as 17/group based on 80% power to detect a mean treatment difference in MRI-PDFF, assuming a common standard deviation of 20%. This sample size was increased by approximately 35%, to 23/treatment group (69 total), to provide a larger sample size for assessment of secondary endpoints. This value was then adjusted for a predicted dropout rate of 24% to yield a final enrolment goal of 30/treatment arm (90 total).

3 | RESULTS

Two hundred and fifteen subjects were screened for participation. 124 of these did not meet enrolment criteria, primarily due to hepatic fat fraction $<15\%$. The remaining 91 subjects were randomised, 24 to placebo (treatment group A), 35 to the 1100 mg leucine/500 mg metformin/0.5 mg sildenafil group (treatment group B) and 32 to the 1100 mg leucine/500 mg metformin/1.0 mg sildenafil group (treatment group C). Of these, 90 participants received at least one dose of medication and constituted the ITT analysis group. Twenty of the enrolled (22.0%) participants discontinued study

treatment early; 2 from placebo (treatment group A, 8.3%), 11 (31.4%) from treatment group B and 7 (21.9%) from treatment group C. Of the 71/91 (78.0%) participants completing the trial, 1 was excluded from the PP analysis group due to non-adherence with a priori study medication compliance criteria. Thus, a total of 70 participants were analysed for this study for the PP group. Participants' baseline characteristics are shown in Table 2.

The relative change in MRI-PDFF between baseline and end of treatment was $-10 \pm 17.5\%$ in the placebo treated patients, whereas it was $3.08 \pm 25.5\%$ in low-dose NS-0200-treated patients ($P = 0.057$ compared to placebo) and $-4.01 \pm 24.6\%$ in high-dose NS-0200-treated patients ($P = 0.377$ compared to placebo) (Figure 1). There were no significant changes in ALT, AST, GGT or K18 in any of the treatment groups. Body weight did not significantly change in the placebo group (-0.4 ± 0.4 kg) or the low-dose NS-0200 group (-1.4 ± 0.6 kg, not significant vs placebo); there was a significant decrease in weight in the high-dose NS-0200 group (-2.4 ± 0.5 kg, $P = 0.025$).

In a subgroup analysis, we examined the effects of high and low-dose NS-0200 in participants with baseline ALT values above the median (> 50 U/L; $n = 35$). In addition to elevated ALT levels (by definition), this subgroup also had elevated K18, AST and GGT (Figure S1), as well as differential metabolomic profiling, as described below. There was 15.7% relative reduction in MRI-PDFF in the high-dose NS-0200 group in this high-ALT subgroup cohort ($P = 0.0032$), while there was no significant change in either the lower dose or placebo groups (Figure 2). In contrast, subjects with ALT below the median value of 50 U/L exhibited a decrease in MRI-PDFF only in the placebo group ($-20.5 \pm 5.7\%$ relative decrease, $P < 0.05$ vs active treatments), while those in the active treatment group exhibited no significant change from baseline (low dose, $9.5 \pm 9.1\%$; high dose, $4.3 \pm 6.1\%$). Subjects with high baseline ALT exhibited non-significant trends towards decreasing ALT and AST in the high-dose NS-0200 group and significant reductions in GGT in both NS-0200 treatment groups vs placebo (Figure S2).

Although the study population was nondiabetic, both low- and high-dose treatment arms achieved statistically significant reductions in HbA1c (placebo adjusted decreases of 0.21% [$P = 0.002$] and 0.17% [$P = 0.016$] for low- and high-dose, respectively), but there was no significant treatment effect on fasting glucose, insulin or HOMA-IR.

Metabolomic profiling demonstrated reductions in metabolically active lipids, including diglycerides (DG), triglycerides (TG), ceramides and multiple phospholipid and sphingomyelins and glycerophospholipid species in treatment vs placebo groups in the full *per protocol* cohort at study endpoint vs baseline (Figure 3), and pathway analysis of these data also demonstrate an increase in fat oxidation in the active treatment groups. However, a comparison between participants with baseline ALT > 50 U/L and those with < 50 U/L ALT demonstrated that only the high-ALT group exhibited treatment effects on metabolically active lipids, as the low-ALT group exhibited only minor increases in fatty acid species, but no treatment effect on DG, TG, ceramides or other lipid species of interest (Figure 4).

TABLE 2 Selected demographical and baseline characteristics by treatment^a

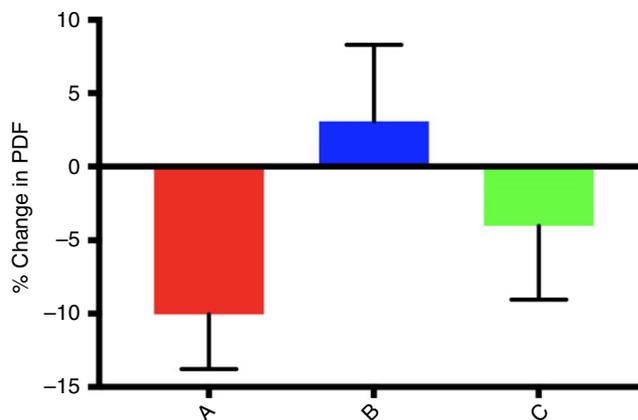
	Per protocol population (n = 70)		
	Placebo treatment A ^b (n = 22)	NS-0200 treatment B ^b (n = 24)	NS-0200 treatment C ^b (n = 24)
Sex, n (%)			
Male	11 (50.0)	8 (33.3)	12 (50.0)
Female	11 (50.0)	16 (66.7)	12 (50.0)
Age (y) ^c			
Mean (SD)	46.7 (10.59)	45.7 (11.77)	46.0 (13.55)
Race, n (%)			
American Indian or Alaska Native	0 (0.0)	0 (0.0)	1 (4.2)
Black or African American	2 (9.1)	1 (4.2)	0 (0.0)
White	19 (86.4)	22 (91.7)	22 (91.7)
White/Asian	1 (4.2)	1 (2.9)	0 (0.0)
Not Reported	0 (0.0)	1 (4.2)	1 (4.2)
Ethnicity, n (%)			
Hispanic or Latino	5 (22.7)	7 (29.2)	7 (29.2)
Not Hispanic or Latino	17 (77.3)	17 (70.8)	17 (70.8)
Weight (kg)			
Mean (SD)	98.16 (14.98)	94.21 (14.01)	96.70(17.84)
Height (cm)			
Mean (SD)	170.5 (9.8)	169.8 (9.4)	168.6 (10.9)
Body Mass Index (BMI) (kg/m ²)			
Mean (SD)	33.82 (4.60)	32.62 (3.39)	33.62 (3.84)
Fasting Plasma Glucose (mg/dL)			
Mean (SD)	109.1 (16.9)	103.0 (14.6)	112.1 (24.8)
HbA1c (%)			
Mean (SD)	5.62 (0.45)	5.62 (0.57)	5.74 (0.71)
PDFF (%)			
Mean (SD)	23.8 (4.7)	24.2 (7.0)	26.3 (6.0)
ALT (U/L)			
Mean (SD)	64.0 (27.6)	56.5 (35.5)	55.5 (28.5)
AST (U/L)			
Mean (SD)	37.4 (19.0)	33.1 (17.7)	34.1 (14.7)
GGT (U/L)			
Mean (SD)	49.5 (29.3)	55.0 (44.4)	58.3 (33.7)

SD, Standard deviation; PDFF, Proton-density fat fraction; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase.

^aNo statistically significant differences among treatment groups.

^bTreatment A, Placebo; Treatment B, 1100 mg leucine + 500 mg metformin + 0.5 mg sildenafil; Treatment C, 1100 mg leucine + 500 mg metformin + 1.0 mg sildenafil.

^cAge at consent.

**FIGURE 1** Effects of treatments on PDFF in the full *per protocol* cohort. A, Placebo; B, 1100 mg leucine/500 mg metformin/0.5 mg sildenafil b.d.; C, 1100 mg leucine/500 mg metformin/1.0 mg sildenafil b.d. PDFF changes are expressed as relative change from baseline ($[(16 \text{ wk HFF}-\text{Baseline HFF})/\text{Baseline HFF}] \times 100$)

Furthermore, the baseline lipidomic signature between the high- and low-ALT groups was different, with high-ALT subgroup exhibiting more metabolically active lipid species indicative of greater baseline disease.

Consistent with the improvements in lipidomic profile, there was a corresponding improvement in TG which was most pronounced among those with hypertriglyceridemia ($\geq 200 \text{ mg/dL}$) at baseline ($n = 22$; Figure S3).

Treatment-emergent adverse events (TEAEs) are summarised in Table 3. Overall, the frequency of TEAEs was similar between placebo (75% of subjects) and pooled treatment groups (77% of subjects). Similarly, comparable fractions of placebo (50%) and active treatment (54.5%) subjects reported TEAEs that were considered related to study treatment (Table 3). However, subjects in the active treatment groups did report a greater number of mild-to-moderate gastrointestinal events than the placebo group, including diarrhoea (40.9 vs 8.3%), nausea (16.7 vs 12.5%) and vomiting (4.2 vs 0.6%), consistent with the presence of metformin. There were no (0.0%) serious TEAEs that were related to study treatment in any of the treatment groups (placebo or NS-0200).

Subjects discontinued participation in the trial for the following reasons: noncompliance/protocol violation(s), $n = 5$; withdrawal of consent, $n = 3$; lost to follow-up, $n = 5$; and TEAEs, $n = 7$. TEAEs leading to discontinuation are summarised in Table 4.

4 | DISCUSSION

Data from this exploratory randomised, placebo-controlled, phase 2, double-blind clinical trial provide evidence supporting efficacy of NS-0200 in a subset of subjects with NAFLD. Although the primary endpoint of reduced MRI-PDFF was not achieved in the pre-defined *per*

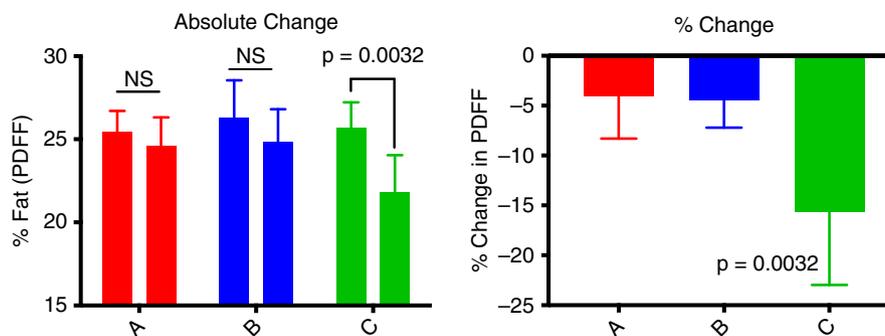


FIGURE 2 Effects of treatments on PDFF in subjects with elevated ALT ($n = 35$). A, Placebo ($n = 14$); B, 1100 mg leucine/500 mg metformin/0.5 mg sildenafil b.d. ($n = 11$); C, 1100 mg leucine/500 mg metformin/1.0 mg sildenafil b.d. ($n = 10$). Left panel shows absolute change and right panel shows relative % change ($[(16 \text{ wk HFF-Baseline HFF})/\text{Baseline HFF}] \times 100$)

protocol cohort, a post-hoc analysis indicates significant improvements in MRI-PDFF in a cohort exhibiting more advanced disease ($P < 0.0032$). The high-ALT cohort was defined as subjects with baseline ALT above the median value of 50 U/L; although the higher ALT levels in this cohort are not predictive of NASH *per se*, higher ALT levels are nonetheless associated with both NASH and fibrosis.^{41,42} At baseline, this group had a 2-fold elevation in ALT vs the low-ALT group, with corresponding elevations in other hepatic markers (AST, GGT and K18; Figure S1; $P < 0.001$). Further, this high-ALT group exhibited a baseline metabolomic signature characteristic of more pronounced disease³⁹ and exhibited decreases in multiple metabolically active lipid species with treatment, while the low-ALT group did not. These data are consistent with other reports demonstrating minimal efficacy of experimental NASH therapeutics in individuals with mild disease vs greater efficacy in NASH resolution in those with more advanced disease.⁴³

The *per protocol* placebo group exhibited an unexpectedly high placebo response which may have resulted from MRI-PDFF volatility in less diseased individuals. Previous studies have demonstrated similar volatility in hepatic fat, measured either via MRI-PDFF or magnetic resonance spectroscopy (MRS), and corresponding decreases in liver fat in study placebo groups the absence of biopsy-confirmed NASH. For example, Cui et al⁴⁴ reported a 13.9% relative reduction in MRI-PDFF in the placebo group of a 24-week study of sitagliptin vs placebo in subjects with NAFLD. Similarly, in a shorter-term (12-week) study of the effects of liraglutide and sitagliptin on hepatic fat as measured by MRS in subjects with NAFLD, the placebo group exhibited a 9.5% relative reduction from baseline in hepatic fat as measured by MRS,⁴⁵ consistent with the placebo group in the present study. However, placebo-treated subjects with more advanced disease, as evidenced by biopsy-confirmed NASH, exhibited only 4%–5% relative changes in MRI-PDFF,^{46,47} consistent with the observed change in high-ALT cohort placebo group in the present study.

Two doses of NS-0200 were evaluated, both containing 1.11 g leucine and 500 mg metformin, and differing from each other only in the quantity of sildenafil present (0.5 vs 1.0 mg). These sildenafil doses were extrapolated from rodent data,¹⁵ accounting for species differences in bioavailability. The lower dose produced little effect

on most variables, although it did result in improvements in the metabolomics profile, while the higher dose produced improvement in multiple variables. Thus, these data do not permit a conclusion with respect to optimal sildenafil dose, and higher doses may be warranted in follow-up studies. However, rodent studies (unpublished data) indicates that > 4 mg sildenafil (human equivalent dose) does not confer further benefit, and may in fact attenuate the efficacy of this combination. The other components of NS-0200 have been established at fixed levels, as follows. Pre-clinical data indicate that optimal activation of Sirt1 occurs with a plasma leucine concentration range of 0.38–0.50 mM,^{14,23–25} and dose-ranging studies with oral leucine administration in adults demonstrate a linear dose relationship, with doses of 1.0–1.5 g resulting in peak plasma leucine levels of 0.4–0.5 mM.⁴⁸ Further increases in leucine administration result in a slower increase, plateauing at ~ 0.8 mM,⁴⁸ but this higher level is associated with potential mTOR activation, while 0.5 mM leucine is not.²⁵ The metformin dose in NS-0200 is based on a previous assessment of leucine-metformin synergy in improving glycaemic control in patients with type 2 diabetes.²⁷

The combination of leucine, metformin and sildenafil in NS-0200 targets a synergistic interaction among elements of the Sirt1-AMPK-eNOS network.¹⁵ This network is a key regulator of integral factors required for progression or regression of NASH, namely hepatic lipid metabolism, oxidative stress and inflammation. Downregulation of this system promotes NASH progression, while activation improves hepatic steatosis, inflammation and fibrosis,^{19,49–51} and the combination of leucine, metformin and sildenafil correspondingly regressed hepatic steatosis, inflammation, ballooning and fibrosis in a mouse model of NASH.¹⁵ Synergy among the 3 components is evidenced by the observation that the individual components in this combination exert little or no independent effect in experimental systems or clinical studies. For example, the effects of metformin on liver fat content, liver enzymes and/or histology have been evaluated in several studies with little or no effect demonstrated.^{52–57} Further, a recent systematic review⁵⁷ of the effects of metformin on NASH outcomes in 11 papers with pre- and post-treatment biopsies found no histological improvement of NASH in nondiabetic adults (5 studies), in mixed populations of diabetic and nondiabetic adults (5 studies) or in adults with type 2 diabetes (1 study). Similarly, data from

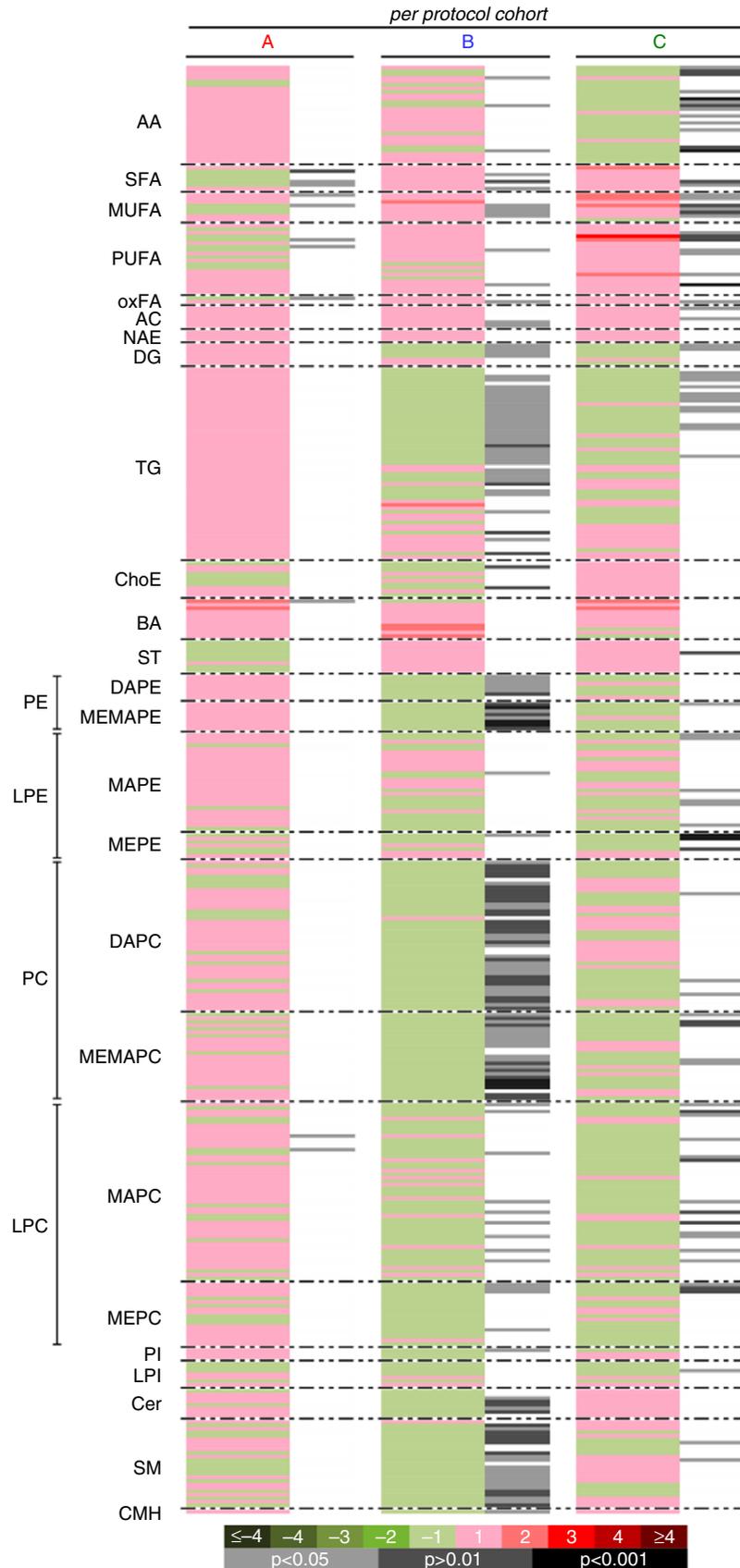


FIGURE 3 Effects of NS-0200 on serum metabolomic changes in the *per protocol* cohort (n = 70). A, Placebo; B, 1100 mg leucine/500 mg metformin/0.5 mg sildenafil b.i.d; C, 1100 mg leucine/500 mg metformin/1.0 mg sildenafil b.d. Comparisons are week 16 vs baseline. Green sections of the heat map denote metabolites that were reduced (negative log₂ fold-changes) and red sections denote metabolites that were increased (positive log₂ fold-changes); grey/black bars indicate significant changes with treatment (light grey, P < 0.05; dark grey, P < 0.01; black, P < 0.001)

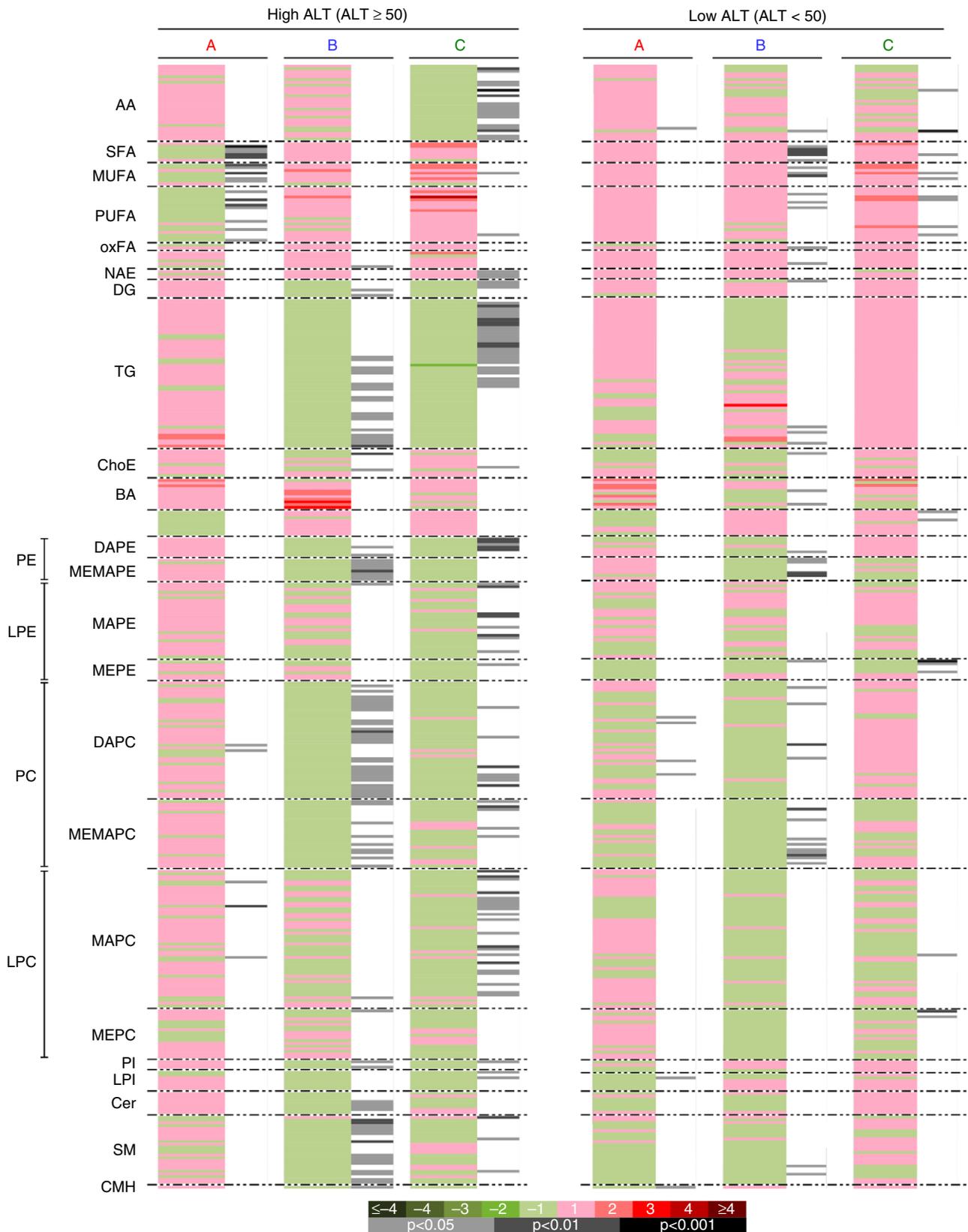


FIGURE 4 Effects of NS-0200 on serum metabolomic changes in subjects with ALT > 50 U/L (“High ALT,” n = 35, left panel) vs those with ALT < 50 U/L (“Low ALT,” n = 35, right panel). A, Placebo; B, 1100 mg leucine/500 mg metformin/0.5 mg sildenafil b.d.; C, 1100 mg leucine/500 mg metformin/1.0 mg sildenafil b.d. Comparisons in each panel are week 16 vs baseline. Green sections of the heat map denote metabolites that were reduced (negative log₂ fold-changes) and red sections denote metabolites that were increased (positive log₂ fold-changes); grey/black bars indicate significant changes with treatment (light grey, P < 0.05; dark grey, P < 0.01; black, P < 0.001)

TABLE 3 Summary of treatment-emergent adverse events, by treatment group

	Intent-to-treat population (n = 90)				Significance (P value)
	Placebo treatment A ^a (n = 24)	NS-0200 treatment B ^a (n = 34)	NS-0200 treatment C ^a (n = 32)	B and C Pooled (n = 66)	
All TEAEs ^b					0.741
Number of subjects, n (%)	18 (75.0)	25 (73.5)	26 (81.3)	51 (77.3)	
Number of events	49	91	90	181	
All Serious TEAEs					0.435
Number of subjects, n (%)	0 (0.0)	1 (2.9)	0 (0.0)	1 (1.5)	
Number of events	0	3	0	3	
All drug-related TEAEs					0.718
Number of subjects, n (%)	12 (50.0)	20 (58.8)	16 (50.0)	36 (54.5)	
Number of events	20	54	39	93	
All drug-related, serious TEAEs					1.000
Number of subjects, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Number of events	0	0	0	0	
All TEAEs leading to study discontinuation					0.053
Number of subjects, n (%)	0 (0.0)	5 (14.7)	1 (3.1)	6 (9.1)	
Number of events	0	5	1	6	

^aTreatment A, Placebo; Treatment B, 1100 mg leucine + 500 mg metformin + 0.5 mg sildenafil; Treatment C, 1100 mg leucine + 500 mg metformin + 1.0 mg sildenafil.

^bTreatment-emergent adverse event (TEAE) is defined as an adverse event occurring on or after the first dose of randomised study medication, or existing prior to the time of and worsening after the time of the first dose of randomised study medication.

TABLE 4 Treatment-emergent adverse events leading to discontinuation by system organ class, preferred term and treatment

System organ class preferred term n (%)	Intent-to-treat population (N = 90)				Significance (P value)
	Placebo treatment A n = 24	NS-0200 treatment B ^a n = 34	NS-0200 treatment C ^a n = 32	B and C pooled (n = 66)	
All TEAEs ^b leading to discontinuation	0 (0.0)	6 (17.6)	1 (3.1)	7 (10.6)	0.0526
Eye disorders	0 (0.0)	2 (5.9)	0 (0.0)	2 (3.0)	0.186
Vision blurred	0 (0.0)	2 (5.9)	0 (0.0)	2 (3.0)	0.186
Gastrointestinal disorders	0 (0.0)	4 (11.8)	1 (3.1)	5 (7.6)	0.118
Diarrhoea	0 (0.0)	1 (2.9)	0 (0.0)	1 (1.5)	0.435
Gastritis erosive	0 (0.0)	1 (2.9)	0 (0.0)	1 (1.5)	0.435
Gastrointestinal disorder	0 (0.0)	1 (2.9)	0 (0.0)	1 (1.5)	0.435
Nausea	0 (0.0)	1 (2.9)	1 (3.1)	1 (1.5)	0.689

^aTreatment A, Placebo; Treatment B, 1100 mg Leucine + 500 mg Metformin + 0.5 mg Sildenafil; Treatment C, 1100 mg Leucine + 500 mg Metformin + 1.0 mg Sildenafil.

^bTreatment-emergent adverse event (TEAE) is defined as an adverse event occurring on or after the first dose of randomised study medication, or existing prior to the time of and worsening after the time of the first dose of randomised study medication.

children to adolescents also indicate little to no independent effect of metformin; metformin exerted no effect on reducing ALT, hepatic steatosis, lobular inflammation, NAFLD activity score or resolution of NASH in the 96-week multicentre randomised trial (TONIC Trial) of 173 patients aged 8-17 years, although there was an improvement in ballooning degeneration score.⁵⁸

Similarly, although sildenafil has not been well-studied in NASH, extensive pre-clinical and clinical studies do not demonstrate effects

of sildenafil on hepatic function in the approved therapeutic dose range for labelled indications,⁵⁹ and pre-clinical toxicology studies demonstrate increases in absolute and relative liver weight in both rats and dogs. Furthermore, El-Mahmoudy et al⁶⁰ reported that administration of sildenafil failed to protect against high fat diet-induced impairment in hepatic biomarkers in hyperlipidemic rats. Thus, any therapeutic benefit seen when sildenafil is administered as a component of NS-0200 likely occurs as a result of synergy with leucine.

Although leucine has not been well-studied in NASH, pre-clinical data demonstrate little or no independent effect of leucine on either histology or relevant biomarkers in mouse models of NAFLD/NASH.^{15,29} Similarly, pre-clinical data demonstrate greater efficacy of

the leucine-metformin-sildenafil combination compared to combinations of any two of these agents (leucine-metformin, leucine-sildenafil, metformin-sildenafil) in regressing hepatic steatosis, inflammation and fibrosis in a mouse model of NASH.¹⁵ Thus, the efficacy of this

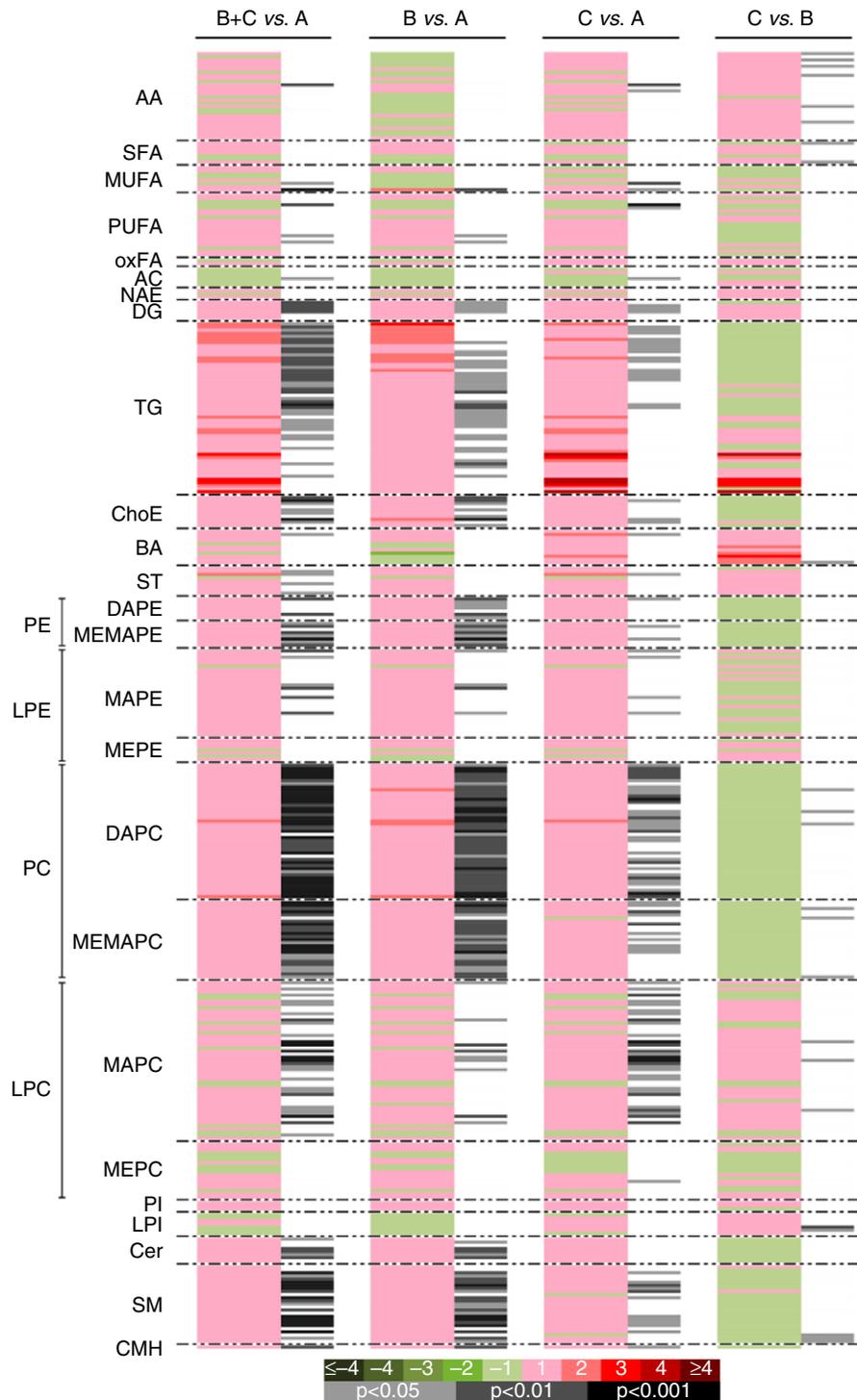


FIGURE 5 Metabolomic assessment of baseline differences among treatment arms. A, Placebo; B, 1100 mg leucine/500 mg metformin/0.5 mg sildenafil b.d.; C, 1100 mg leucine/500 mg metformin/1.0 mg sildenafil b.d. Comparisons shown are baseline of groups B+C vs A; B vs A; C vs A; and C vs B. Green sections of the heat map denote metabolites that were reduced (negative \log_2 fold-changes) and red sections denote metabolites that were increased (positive \log_2 fold-changes); grey/black bars indicate significant changes with treatment (light grey, $P < 0.05$; dark grey, $P < 0.01$; black, $P < 0.001$)

combination appears to be dependent upon synergistic interaction among leucine, metformin and sildenafil.

Overall, NS-0200 was well-tolerated in this trial, with no difference in TEAEs in treatment vs placebo groups, although subjects in the active treatment groups reported a greater number of mild-to-moderate gastrointestinal events than the placebo group, consistent with the metformin component of NS-0200.

This study has several limitations. Since the study was an exploratory proof-of-concept study of relatively short duration, liver biopsies could not be justified and therefore fibrosis and other histological outcomes cannot be assessed. Additionally, since subjects were selected on the basis of MRI-PDFF (a quantitative biomarker of hepatic steatosis) rather than biopsy-proven NASH, our study probably included individuals with mild NAFLD unlikely to respond to intervention. Additionally, the metabolomics data indicate heterogeneity in the extent of disease, with the placebo group presenting a metabolic signature demonstrating lower levels of relevant metabolically active lipids (DG, TG, cholesterol esters, glycerophospholipids, ceramides and sphingomyelins) than the 2 active treatment groups (Figure 5), suggesting less advanced disease and possibly contributing to greater volatility in MRI-PDFF in the placebo vs active treatment groups in the full cohort (*per protocol*) placebo group.

In conclusion, high-dose NS-0200 significantly reduced hepatic fat in NAFLD patients with elevated ALT, and correspondingly improved the NAFLD-associated metabolomic signature. NS-0200, either in high or low dose, did not improve hepatic fat in the ITT population, but the placebo-treated participants exhibited an unusually high response rate. These data support further development of NS-0200, especially high-dose NS-0200 in NAFLD patients with elevated ALT.

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SUPPORTING INFORMATION

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