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1 **RIFAXIMIN IN NON-ALCOHOLIC STEATOHEPATITIS: AN OPEN-LABEL PILOT**  
2 **STUDY**

3 **Running title: Rifaximin in Non-Alcoholic Steatohepatitis**

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30

31 List of abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic  
32 steatohepatitis; LPS, lipopolysaccharide; ALT, alanine aminotransferase; HGP, hepatic  
33 glucose production; <sup>1</sup>H NMR, proton nuclear magnetic resonance; IHCL, intrahepatocellular  
34 lipids; PCA, principal components analysis; OPLS-DA, orthogonal partial least squares  
35 discriminant analysis; HOMA-IR, homeostasis model assessment-estimated insulin  
36 resistance index; HDL, high density lipoprotein.

37

38

39 **ABSTRACT**

40 **Aim:** Gut microbial dysbiosis is implicated in the pathogenesis of non-alcoholic  
41 steatohepatitis (NASH). We investigated downstream effects of gut microbiota modulation  
42 on markers of hepatic inflammation, steatosis, and hepatic and peripheral insulin sensitivity  
43 in patients with NASH using Rifaximin therapy.

44 **Methods:** Patients with biopsy-proven NASH and elevated aminotransferase values were  
45 included in this open-label pilot study, all receiving 6 weeks Rifaximin 400mg twice daily,  
46 followed by a 6 week observation period. The primary endpoint was change in ALT after 6  
47 weeks of Rifaximin. Secondary endpoints were change in hepatic lipid content and insulin  
48 sensitivity measured with a hyperinsulinaemic euglycaemic clamp.

49 **Results:** Fifteen patients, 13 male, 2 female, with median (range) age 46(32-63) years were  
50 included. Seven had diabetes on oral hypoglycaemic medications and 8 had no diabetes.  
51 After 6 weeks of therapy, no differences were seen in ALT (55 [33-191] versus 63 [41-  
52 218]IU/L,  $p=0.41$ ), peripheral glucose uptake (28.9 [19.4-48.3] to 25.5 [17.7-47.9]  
53  $\mu\text{mol/kg/min}$ ,  $p=0.30$ ), hepatic insulin sensitivity (35.2 [15.3-51.7]% versus 30.0 [10.8-  
54 50.5]%,  $p=0.47$ ), or hepatic lipid content (21.6[2.2-46.2]% before and 24.8[1.7-59.3]% after  
55 Rifaximin,  $p=0.59$ ) before and after Rifaximin treatment. After 12 weeks from baseline,  
56 serum ALT increased to 83(30-217)IU/L,  $p=0.02$ . There was a significant increase in HOMA-IR  
57 ( $p=0.05$ ). The urinary metabolic profile indicated a significant reduction in urinary hippurate  
58 with treatment, which reverted to baseline after cessation of Rifaximin, although there was  
59 no consistent difference in relative abundance of faecal microbiota with treatment.

60 **Conclusion:** These data do not indicate a beneficial effect of Rifaximin in patients with  
61 NASH.

62 **Abstract 248 words (max 250)**

63

64 Key words:

65 Antibiotic; Hippurate; Insulin resistance; Microbiota; NAFLD; Non-alcoholic steatohepatitis

66

67

## 68 INTRODUCTION

69 Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction and  
70 an increasing cause of liver-related morbidity and mortality globally(1, 2). NAFLD, and its  
71 inflammatory and potentially progressive subtype, non-alcoholic steatohepatitis (NASH),  
72 represents a complex disease trait, with genetic and environmental influences on incidence  
73 and disease progression(2, 3). While lifestyle measures in order to achieve sustained weight  
74 loss, including dietary changes and regular exercise are the mainstay of current  
75 management(4), many patients do not respond to such measures and specific therapies are  
76 lacking(5, 6).

77

78 The gut microbiota is increasingly recognised as a key metabolic influence in the body and a  
79 potentially modifiable environmental target in disorders of energy metabolism and fat  
80 storage(7). Mechanisms include increase of calorific yield of meals by co-digestion,  
81 production of short chain fatty acids and bacterial endotoxin (7, 8)(9).

82

83 Microbial interventions, such as transfer of caecal contents from conventionally-raised mice  
84 to germ free mice have been shown to alter the host phenotype(7), while a study in patients  
85 with the metabolic syndrome demonstrated improved insulin sensitivity in patients  
86 receiving a faecal allogenic enteric infusion from a lean donor than from an autologous  
87 infusion(10). Besides direct microbial transfer, other methods for alteration of the gut  
88 microbiota include use of prebiotics, probiotics and antibiotics(11). Antibiotic therapy in  
89 obese mice reduced LPS and improved the metabolic phenotype(12), while Rifaximin was

90 found to reduce endotoxaemia in patients with decompensated cirrhosis, associated with  
91 improvements in hepatic synthetic function, but not aminotransferase values(13).

92

93 Rifaximin is a minimally-absorbed, broad spectrum antibiotic, which has been found to have  
94 clinical utility in a number of gastrointestinal settings with few side effects(14-16). With  
95 standard oral dosing, intraluminal drug levels exceed the minimum inhibitory  
96 concentrations for most bacterial species by up to 250-fold, while systemic absorption is  
97 <0.4% of the dose(17).

98

99 We hypothesised that modulation of the gut microbiota, using Rifaximin, in humans with  
100 NASH would lead to improvement in hepatic inflammation, hepatic lipid content and insulin  
101 sensitivity. Thus, we conducted a pilot prospective clinical trial to evaluate the efficacy and  
102 safety of such an approach. We examined the faecal microbiota, urinary metabolome and  
103 inflammatory cytokine profile as secondary analyses to assess whether any changes  
104 observed were linked to detectable differences in bacterial populations, to microbial co-  
105 metabolism and whether this could be mediated by inflammatory signalling.

106

## 107 **METHODS**

108 Ethical approval (REC 10/H0711/58) was obtained and the study was registered on the  
109 European Clinical Trials Database (EudraCT 2010-021515-17). Patients were recruited from  
110 Hepatology clinics at a single UK centre (Imperial College Healthcare NHS Trust) between  
111 May 2011 and June 2012. Informed consent was obtained from all patients included in the

112 study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of  
113 Helsinki as reflected in *a priori* approval by the institution's human research and ethics  
114 committee (West London REC 2). Male and female patients were eligible for inclusion if  
115 aged between 18 and 70 years with non-alcoholic steatohepatitis histologically-proven, as  
116 evidenced by the presence of all of: steatosis, hepatocyte ballooning and lobular  
117 inflammation, and scored according to Kleiner(18) by a single experienced histopathologist  
118 (RDG) within the previous year, with or without mild to moderate fibrosis (stage 0-3/4) and  
119 with persistently elevated alanine aminotransferase (ALT) values on at least two occasions  
120 in the three months prior to recruitment. Patients were excluded if there was histological  
121 evidence of cirrhosis; hepatic decompensation; regular alcohol consumption exceeding 14  
122 units/week (16g ethanol/day) for a woman or 21 units/week (24g ethanol/day) for a man;  
123 evidence of viral, autoimmune or other metabolic liver disease on a chronic liver disease  
124 screen; a history of malignancy or systemic inflammatory conditions; myocardial infarction  
125 or cerebrovascular events in the preceding 6 months; a history of bariatric surgery, blind  
126 loop or short bowel; use of any treatment known or suspected to change bowel flora within  
127 3 months of enrolment; initiation or major dose change of metformin, thiazolidiones,  
128 biguanides, statins, fibrates, anti-obesity medications or insulin within 3 months of  
129 enrolment.

130

### 131 **Study design**

132 This was an open-label study of Rifaximin (Normix, Alfa Wasserman S.p.A, Bologna, Italy)  
133 400mg twice daily for six weeks followed by a further six weeks observation period during  
134 which patients received standard care. Compliance with treatment was checked by



135 collection of empty blister packs. Subjects were asked to provide a structured dietary and  
136 lifestyle history as previously described(19). The primary endpoint was change in ALT after 6  
137 weeks' Rifaximin therapy. Secondary endpoints were change in hepatic and whole-body  
138 insulin sensitivity assessed by the two-stage hyperinsulinaemic euglycaemic clamp and  
139 change in hepatic triglyceride content assessed by proton nuclear magnetic resonance  
140 spectroscopy at 6 weeks from baseline. Serum ALT, biochemistry and anthropometrics were  
141 also measured at 12 weeks to look for longer-term effects. Stool microbiota, urinary  
142 metabolic profile and serum cytokine profile were measured before and after intervention.

### 143 **Laboratory measurement**

144 Routine biochemistry was undertaken by the hospital biochemistry laboratory on the  
145 Aeroset (ALT, AST, HDL, triglyceride) or Architect (insulin) clinical chemistry analyser  
146 platforms (Abbott Diagnostics, Illinois, USA). Insulin concentrations were determined using a  
147 one-step chemiluminescent immunoassay. Cytokine analysis was performed by Aushon  
148 Multiplex Immunoassay Analysis (Aushon Biosystems, Billerica, USA).

### 149 **Hyperinsulinaemic euglycaemic clamp**

150 The two-step hyperinsulinaemic euglycaemic clamp combined with a [6,6-<sup>2</sup>H<sub>2</sub>]glucose  
151 infusion to measure insulin sensitivity was performed as previously described and detailed  
152 in the supplementary information (20). Patients consumed nothing but water orally after  
153 eating a low-fat pre-prepared meal (identical before and after intervention) 10 hours prior  
154 to the clamp study.

155 Plasma glucose concentration and enrichment time-courses were smoothed using optimal  
156 segments analysis(21) and non-steady-state equations(22)

157

**158 Proton nuclear magnetic resonance spectroscopy**

159 Patients fasted for at least 10 hours prior to scanning. Rapid T<sub>1</sub>-weighted magnetic  
160 resonance images were acquired using a 1.5T Phillips Achieva™ scanner (Philips Medical  
161 Systems, Best, Netherlands), as previously described(23). Proton nuclear magnetic  
162 resonance (<sup>1</sup>H NMR) spectra were acquired at 1.5T, using a surface coil. Transverse images  
163 of the liver were used to ensure accurate positioning of the (20x20x20 mm) voxel in the  
164 liver, avoiding blood vessels, the gall bladder and fatty tissue. Spectra were obtained from  
165 the right lobe of the liver using a PRESS sequence (TR 1500ms, TE 135 ms) without water  
166 saturation and with 128 signal averages. Intrahepatocellular lipids (IHCL) were measured  
167 relative to liver water content, as previously described(24).

**168 Faecal microbiota**

169 Faeces were collected in a sterile container at each assessment visit and frozen at -70°C  
170 within 10 minutes. DNA was extracted using a Qiagen DNA stool extraction kit (Qiagen,  
171 Manchester, UK), with an additional bead beating step added before the ASL buffer was  
172 added to the stool sample. The extracted DNA was quantified using a Qubit platform and all  
173 DNA samples were normalised to 10 ng/μL. The 16S rRNA gene was amplified using primers  
174 for the V1 to V3 regions and sequenced using paired end 250bp chemistry on an Illumina  
175 MiSeq platform (Illumina Inc, San Diego, California). The data were analysed using  
176 bioinformatics statistical packages (Mothur, STAMP) and R (R Foundation, Vienna, Austria)  
177 to determine whether any statistically significant changes in the profiles of the faecal  
178 microbiota had occurred(25-27).

179

**180 Urinary metabolomics**

181 Urine was collected, processed and buffered as detailed in the supplementary information.

182 All NMR spectra were referenced, phased and baselined corrected as detailed in the

183 supplementary information. Data were initially modelled using unsupervised principle

184 components analysis (PCA) and subsequently combined with clinical data and modelled

185 using orthogonal partial least squares discriminant analysis (OPLS-DA). For univariate

186 analyses Topspin (Bruker, Billerica, USA) was used to integrate under spectral resonances

187 for metabolites of interest and the quantitative data was analysed in the statistics package

188 SPSS (IBM, Armonk, USA).

**189 Statistical analysis**

190 Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, USA). Based on

191 historical data from 20 patients with paired ALT data in response to lifestyle and standard of

192 care intervention from the NAFLD clinic at our institution, a sample size of 16 would provide

193 80% power and  $\alpha$  of 0.05 to detect a change in ALT of 25IU/L with an expected standard

194 deviation of the difference of 33IU/L. Data were non-parametrically distributed, so are

195 displayed as median (range). Significance of differences in endpoints before and after

196 intervention was tested by the Wilcoxon Signed Rank test.

197

**198 RESULTS**

199 Of the 77 patients with biopsy-proven NAFLD evaluated in clinic over the recruitment  
200 period, 41 met inclusion criteria and were invited to take part in the study. On receipt of the  
201 patient information, 18 patients declined to participate and 23 were screened. Of these, a  
202 further two declined to participate further at the screening visit and three were excluded by  
203 the screening questionnaire. Of the 15 patients who initiated the study protocol, one  
204 participant was unable to tolerate MR scanning owing to claustrophobia and another  
205 participant declined the hyperinsulinaemic euglycaemic clamp having already started the  
206 study. Baseline patient characteristics are displayed in Table 1. 100% compliance with  
207 Rifaximin therapy was reported by all participants. One subject noted loose stools for 36  
208 hours during therapy, which resolved spontaneously and therapy was not discontinued. No  
209 other adverse events were recorded. Recruitment was halted after enrolment of 15 subjects  
210 because of difficulty in recruitment to the full study protocol over the defined study time  
211 period.

### 212 **Hepatocellular inflammation**

213 Alanine aminotransferase (ALT) values, the primary endpoint in this study, were 55IU/L (33-  
214 191) before Rifaximin, 63IU/L (41-218) after 6 weeks' Rifaximin ( $p=0.41$  compared to  
215 baseline) and 83IU/L (30-217) after a further 6 weeks follow-up ( $p=0.017$  compared to  
216 baseline), Figure 1A. Anthropometrics, HOMA-IR and lipid profile before and after Rifaximin  
217 are shown in Table 2. There was a significant increase in HDL and HOMA-IR at 12 weeks.

### 218 **Hepatic Lipid content**

219 Hepatic lipid content (IHCL) was 21.6% (2.2-46.2) before and 24.8% (1.7-59.3) after  
220 Rifaximin,  $p=0.59$ . Figure 1B.

**221 Insulin sensitivity**

222 Hepatic insulin sensitivity as assessed by suppression of hepatic glucose production was  
223 35.2% (15.3-51.7) before Rifaximin and 30.0% (10.8-50.5) after Rifaximin,  $p=0.47$ , Figure 1C.  
224 Peripheral insulin sensitivity as assessed by glucose Rd was 28.9  $\mu\text{mol/kg/min}$  (19.4-48.3)  
225 before Rifaximin and 25.5  $\mu\text{mol/kg/min}$  (17.7-47.9) after Rifaximin,  $p=0.30$ , Figure 1D.

**226 Cytokine analysis**

227 There were no differences in serum cytokine values, including  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$ , before and  
228 after treatment with Rifaximin, and over the observation periods (Supplementary Table 1).

229

**230 Urinary metabonomics**

231 Urinary metabolites were identified as seen in the representative urinary metabolic profile,  
232 Figure 2A. Principal components analysis of urinary metabolic profiles demonstrated close  
233 clustering of quality control samples and case samples clustered by patient, Figure 2B.  
234 Supervised partial least squares discriminant analysis (OPLS-DA) failed to produce robust,  
235 predictive models based upon the effect of treatment upon urinary metabolic profiles.  
236 Examination of the loadings plots for the OPLS-DA models identified three metabolites,  
237 alanine, creatinine and hippurate, with modest correlation with treatment status, Figure 2C.  
238 These metabolites were examined further in univariate analyses, Figure 2D. A significant  
239 decrease in hippurate levels was observed following treatment with rifaximin ( $p=0.048$ ). A  
240 significant increase in hippurate levels was seen from immediately after treatment to 6  
241 weeks after its discontinuation ( $p=0.035$ ); no difference was seen between 6 weeks post-

242 treatment and pre-treatment hippurate levels ( $p=0.721$ ). There were no statistically  
243 significant changes in alanine or creatinine levels with treatment.

244

#### 245 **Stool Microbiota**

246 No consistent differences were observed in the relative abundance of gut microbiota at the  
247 phylum level in faeces with Rifaximin treatment (Figure 3). However significant differences  
248 in the microbiota were seen at the genus level in individual patients with Rifaximin  
249 treatment (Supplementary Figure 1, published online), although these differences were not  
250 common to all subjects.

251

#### 252 **DISCUSSION**

253 In this study, we performed an open-label clinical trial of Rifaximin in patients with NASH to  
254 test effect and safety. Although there was no evidence of change in markers of hepatic  
255 inflammation, hepatic lipid content or insulin sensitivity after 6 weeks of therapy, serum ALT  
256 values increased significantly from baseline to 12 weeks, in association with increased  
257 insulin resistance as assessed by the HOMA-IR score. An increase in serum HDL values was  
258 also observed. Univariate analysis of urinary hippurate levels suggests that treatment  
259 transiently suppressed the production of this metabolite. However, robust changes were  
260 not demonstrated in the faecal microbiota, or a panel of pro- and anti-inflammatory serum  
261 cytokines. No adverse events were recorded. These results contrast with another recent  
262 open label study of Rifaximin in NAFLD/NASH which reported an improvement in liver  
263 biochemistry, body mass index and IL-10 after 28 days of Rifaximin 1200mg per day in 27

264 patients with NASH, although insulin sensitivity, liver fat and gut microbiota were not  
265 assessed specifically in that study(28).  
266  
267 Ours was a prospective clinical study in which subjects were intensively investigated to look  
268 for signals of biological effect of Rifaximin on NASH in human subjects that might form the  
269 basis of larger studies of longer duration. The sample size is relatively small, but the study  
270 was powered to detect a difference in ALT of 25IU/L with treatment, which was not seen.  
271 This study included more patients than studies using the hyperinsulinaemic euglycaemic  
272 clamp to assess the effects of antibiotic administration and faecal transfer on insulin  
273 sensitivity(10, 29), so might be expected to show a difference in insulin sensitivity if  
274 Rifaximin were to cause an effect of similar magnitude to those interventions. The study was  
275 of similar size to studies assessing the microbial and metabolic effects of Rifaximin in  
276 cirrhosis(30, 31) and the effect of Rifaximin on liver biochemistry in patients with PSC(32).  
277 Nevertheless, the study was not powered to detect differences in subgroups, such as those  
278 with and without type 2 diabetes mellitus. The six-week course of therapy may be  
279 considered short, but metabolic effects of antibiotics are seen at 1 week(29) and changes in  
280 hepatocellular inflammation are detectable rapidly in serum. The dose of Rifaximin used in  
281 this study is lower than in other recent clinical trials which have used 550mg twice daily, a  
282 dose licenced for use in the secondary prophylaxis of hepatic encephalopathy(14). This  
283 difference reflects the Rifaximin preparations and dosing information available, and the  
284 clinical usage for gastrointestinal infections at the time of study initiation. Assessment of  
285 changes to the intestinal microbiota using sequencing of faecal bacterial DNA is limited as  
286 the faecal microbiota may not reflect the metabolically active microbiota at the small bowel

287 mucosa, which are implicated in the effects of Rifaximin and more readily sampled in animal  
288 studies(33).

289

290 Although the primary and secondary outcome measures were not altered by Rifaximin in  
291 this study, some additional markers changed post-treatment. These differences were not  
292 specified in the *a priori* analysis so should be interpreted tentatively at this stage. However,  
293 this, and other studies, suggest that some broad spectrum oral antibiotics, including  
294 Rifaximin, may be associated with adverse metabolic and hepatic responses. For example,  
295 oral administration of a short course of vancomycin reduced peripheral insulin sensitivity in  
296 patients with the metabolic syndrome, in association with reduced gut microbial  
297 diversity(29). In another study of patients with cirrhosis before and after Rifaximin  
298 administration, there was a reduction in the ratio of secondary to primary bile acids(31),  
299 suggesting a possible mechanism for any Rifaximin-induced insulin resistance. As in the  
300 present study, previous work using a systems biology approach to evaluate metabolic and  
301 microbial effects of Rifaximin in patients with cirrhosis and minimal hepatic encephalopathy  
302 demonstrated no significant difference in the overall microbiome composition of stool(30).  
303 So, in contrast with *in vitro* studies, which demonstrate activity against a broad-spectrum of  
304 bacteria(34), the effects of Rifaximin *in vivo* may be on bacterial function and virulence,  
305 rather than simply a reduction in numbers(35, 36). The observation in the present study that  
306 urinary hippurate levels decreased with Rifaximin therapy is relevant as urinary hippurate is  
307 influenced by the intestinal microbiota (as well as age, sex and dietary intake, which were  
308 controlled for in the present study)(37). Hippurate is a glycine conjugate of benzoic acid and  
309 a normal constituent of the human urinary metabolite profile. Germ-free mice have  
310 significantly lower levels of urinary hippurate than conventionally raised mice(38) and



311 administration of vancomycin to mice leads to changes in the faecal microbiome and  
312 associated suppression of urinary hippurate levels(39). Metabolism of high-molecular  
313 weight polyphenolic compounds by colonic microbiota leads to production of benzoic acid  
314 which may be excreted as hippurate(37). Differential capacities of microbiota species to  
315 metabolise polyphenolic compounds(40) means that antibiotic-mediated changes in  
316 bacterial numbers or population composition may alter the bioavailability of upstream  
317 metabolites of benzoic acid and this lead to changes in urinary hippurate levels. Benzoic  
318 acid is converted to hippurate predominantly in hepatic mitochondria and impaired hepatic  
319 function is associated with a decreased capacity to produce hippurate from orally or  
320 intravenously administered precursors(41, 42). Thus there is some evidence that the  
321 transient depression in urinary hippurate levels with Rifaximin in this study is mediated by  
322 suppression of such activity by colonic microbiota.

323 This work indicates that the use of a minimally-absorbed, broad spectrum antibiotic is not  
324 associated with consistent changes in the stool microbiota at the phylum or genus level, but  
325 suggests a metabolic effect, illustrated by the urinary hippurate levels. Nevertheless, such  
326 an intervention has not led to detectable changes in ALT, insulin sensitivity and hepatic  
327 steatosis, nor is it associated with a robust pattern of inflammatory cytokines. This study  
328 does not support the use of antibiotics as a therapeutic intervention in NASH, but suggests a  
329 possible adverse metabolic effect which needs further evaluation. The variable effect of this  
330 intervention at a genus level between patients indicates that future studies should focus on  
331 functional niches rather than the abundance of the microbiota to direct therapy. Future  
332 therapies targeting the gut microbiota will need to be more nuanced to result in beneficial  
333 metabolic and inflammatory modulation.

334 Administration of Rifaximin for 6 weeks to subjects with non-alcoholic steatohepatitis was  
335 not associated with changes in markers of hepatocellular damage, hepatic triglyceride  
336 content, insulin sensitivity or systemic inflammation at 6 weeks, although an increase in  
337 serum ALT levels was noted at 12 weeks, associated with increased HOMA-IR and HDL. On  
338 the basis of the evidence presented in this study, Rifaximin cannot be recommended as a  
339 potential therapy in NAFLD/NASH, but further studies are warranted to investigate the  
340 hepatic and metabolic consequences of enteric antibiotic therapies.

341

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350

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467 synthesizing capacity in surgical patients with liver disease: a metabolic tolerance test. J Lab  
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471 **Table 1. Baseline Characteristics.**

Characteristic	Total cohort
Number	15
Gender, M/F	13/2
Age, yrs	46 (32-63)
Weight, kg	83.8 (66.3-116.0)
BMI, kg/m <sup>2</sup>	27.2 (22.9-35.3)
Waist, cm	101.9 (86.9-127.3)
Diabetes, Y/N	7/8
Abdominal Obesity <sup>†</sup> , Y/N	14/1
Dyslipidaemia <sup>†</sup> , Y/N	11/4
Hypertension <sup>†</sup> , Y/N	9/6
Metabolic syndrome <sup>†</sup> , Y/N	9/6
ALT, IU/L	55 (33-191)
AST, IU/L	35 (20-100)
Triglyceride, mmol/L	1.69 (0.94-2.94)
HDL, mmol/L	1.07 (0.73-1.45)
HOMA -IR	3.65 (1.52- 8.18)
Histology <sup>‡</sup>	
Steatosis, 0/1/2/3	0/4/8/3
Ballooning, 0/1/2/3	0/12/3/0
Lobular inflammation, 0/1/2	0/12/3
Fibrosis, 0/1/2/3/4	1/6/4/4/0

472 Data expressed as numbers or median (range) as appropriate. <sup>†</sup>IDF criteria 2005. <sup>‡</sup> Kleiner

473 et al. 2005

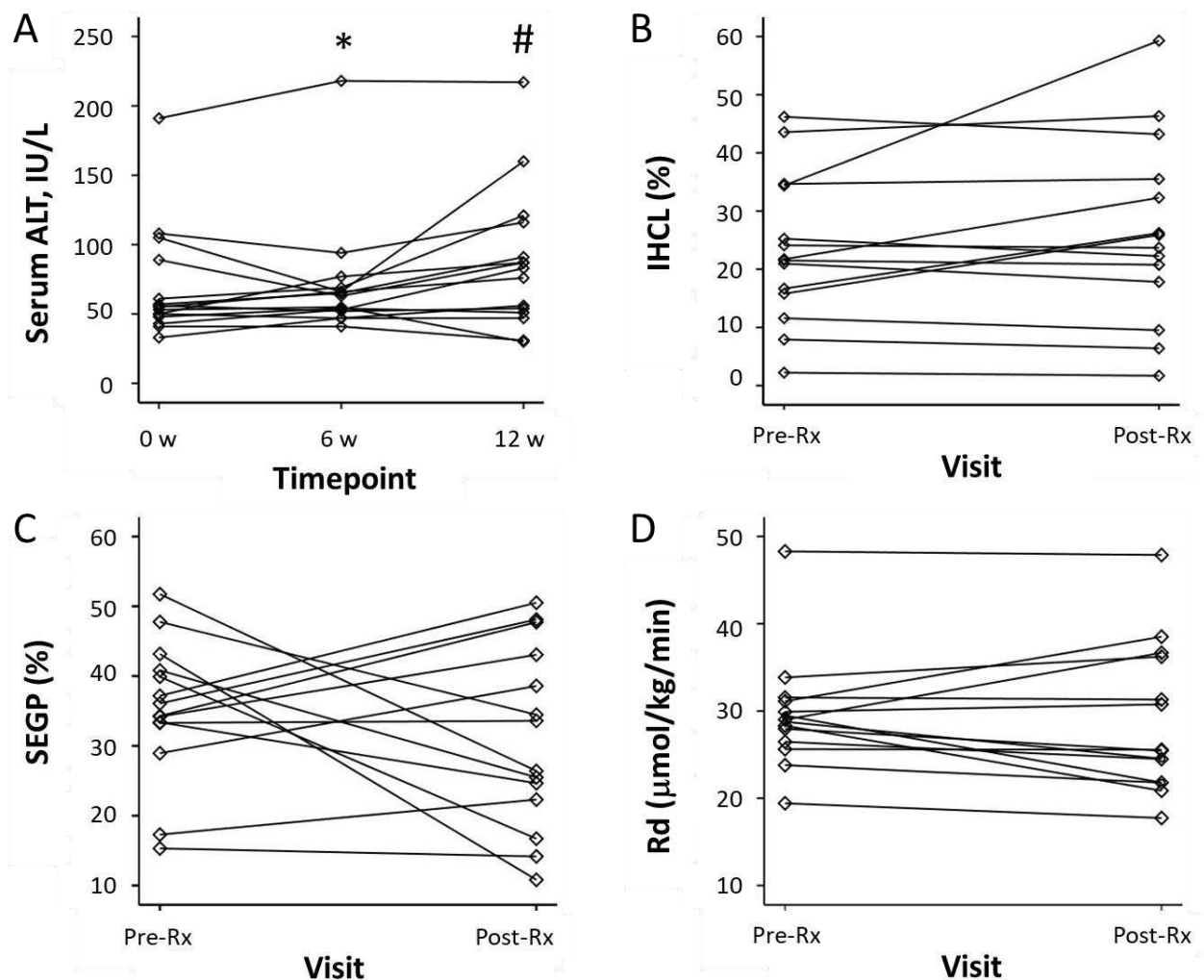
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475 **Table 2. Anthropometrics and metabolic clinical chemistry**

	Pre-Rifaximin (0 weeks)	Post-Rifaximin (6 weeks)	†P=	Post-Rifaximin (12 weeks)	†P=
BMI, kg/m <sup>2</sup>	27.15 (22.86-35.27)	27.84 (22.92- 35.59)	0.14	28.08 (22.73- 35.59)	0.47
Waist, cm	101.9 (86.9- 127.3)	100.6 (87.7-125.5)	0.58	101.5 (87.0-126.0)	0.27
HOMA-IR	3.65 (1.52-8.18)	4.31 (1.25-8.54)	0.08	4.29 (2.04-15.71)	<b>0.05</b>
Total Cholesterol, mmol/L	4.68 (2.52-5.98)	4.65 (2.58-7.37)	0.14	4.44 (2.75-7.10)	0.33
HDL, mmol/L	1.07 (0.73-1.45)	1.11 (0.80-1.45)	0.18	1.19 (0.77-1.62)	<b>0.004</b>
Triglycerides, mmol/L	1.69 (0.94-2.94)	1.47 (0.81-3.17)	0.73	1.47 (0.76-5.23)	0.89

476 †compared to baseline

477

478 **FIGURE LEGENDS**

479

480 **Fig. 1. Primary and secondary study endpoints before and after Rifaximin therapy. (A)**

481 Serum ALT values at baseline, 6 weeks (end of treatment) and 12 weeks (6 weeks after end

482 of treatment). Individual patient data (n=15). \* P=0.41 vs baseline, # P=0.02 vs baseline,

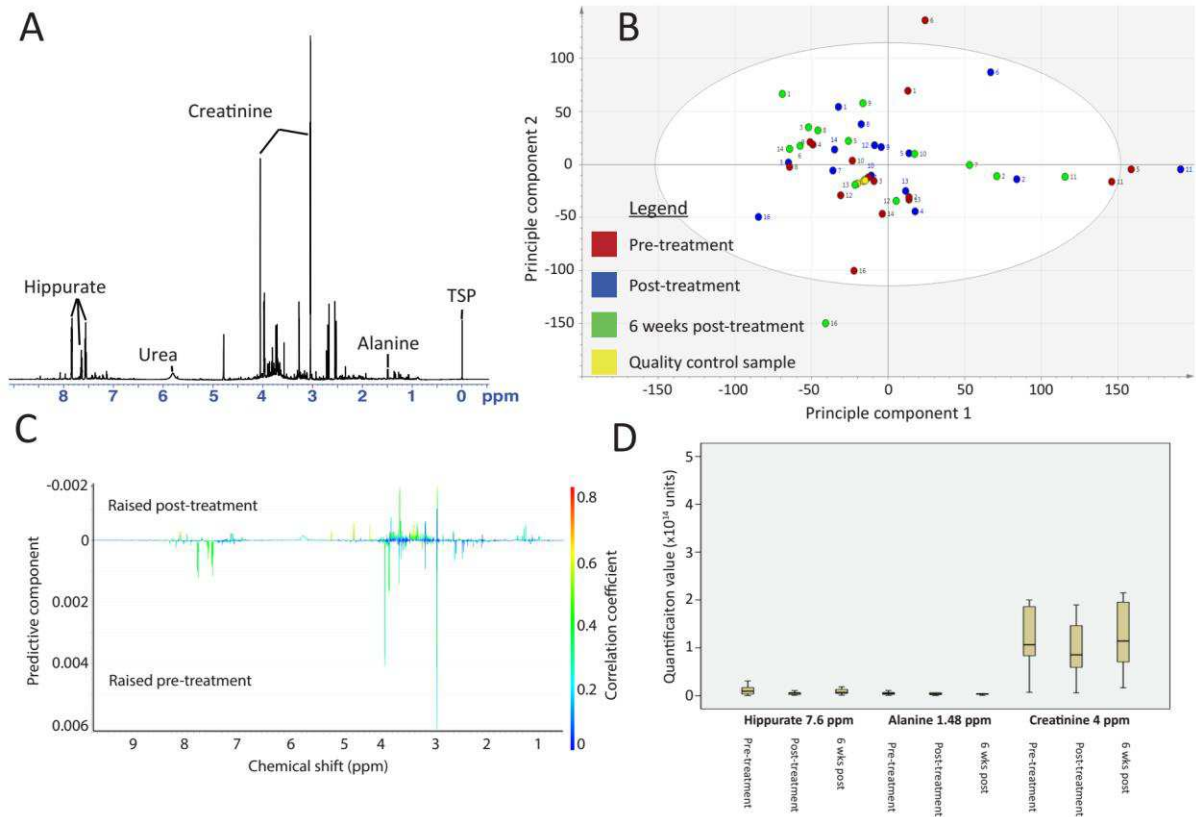
483 P=0.04 vs 6 wks. **(B)** Intrahepatocellular lipid content (IHCL), expressed as a percentage,484 before and after Rifaximin therapy. Individual patient data (N=14). **(C)** Hepatic insulin

485 sensitivity (% suppression of endogenous glucose production, SEGP) before and after

486 Rifaximin therapy. Individual patient data, (N=14). **(D)** Peripheral insulin sensitivity (Rd)

487 before and after Rifaximin therapy. Individual patient data (N=14).

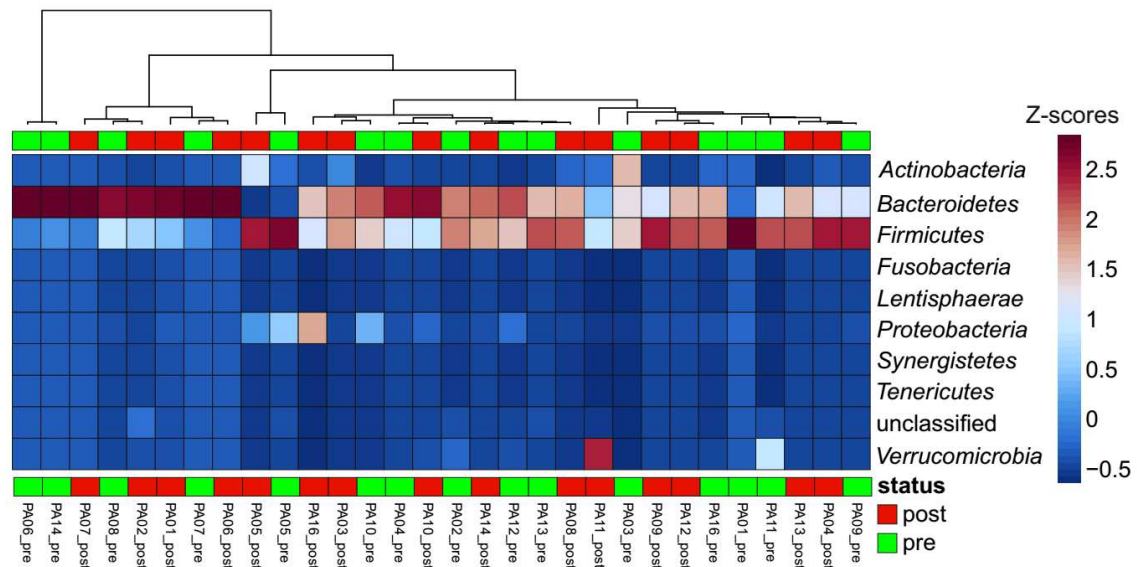
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490 **Fig. 2. Urinary metabonomic analysis. (A)** Typical 1D-NOSEY NMR spectrum of urine. **(B)**  
 491 Scores plot from unsupervised principal components analysis, coloured by timepoint and  
 492 labelled by patient identification number. **(C)** S-line loadings plot from OPLS-DA for pre- and  
 493 post- treatment timepoints. **(D)** Boxplots of quantified metabolites of interest at each study  
 494 timepoint.

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496

497 **Fig. 3. Effect of Rifaximin on the phylum level composition of faeces.** The heatmap shows  
 498 the abundance of the phylum-level 16S rRNA gene sequences for each patient pre and post  
 499 Rifaximin. Relative abundances of the sequence reads plotted are colour coded from less  
 500 (blue) to more abundant (red). The colour value shows log<sub>10</sub> fold changes.

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503 Cobbold et al.  
 504 Supplementary Material  
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RiFL

506 **RIFAXIMIN IN NON-ALCOHOLIC STEATOHEPATITIS: AN OPEN-LABEL PILOT**  
 507 **STUDY**

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 509 Wai<sup>1</sup>, Julie Stove<sup>1</sup>, Fariba Shojaee-Moradie<sup>5</sup>, Nicola Jackson<sup>5</sup>, A Margot Umpleby<sup>5</sup>,  
 510 Julie Fitzpatrick<sup>6</sup>, E Louise Thomas<sup>6</sup>, Jimmy D Bell<sup>6</sup>, Elaine Holmes<sup>3</sup>, Simon D  
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 512 Thursz<sup>1</sup>

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515 **Table of contents**

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516

517

518 **METHODS**519 **Hyperinsulinaemic euglycaemic clamp**

520 Patients consumed nothing but water orally after eating a low-fat pre-prepared meal  
521 (identical before and after intervention) 10 hours prior to the clamp study. Upon  
522 arrival intravenous cannulae were inserted into both antecubital fossae for blood  
523 sampling and infusion of the glucose isotope, insulin and glucose. A primed infusion  
524 of [6,6-<sup>2</sup>H<sub>2</sub>] glucose (170 mg; 1.7 mg.min<sup>-1</sup>) was administered for 7 hours. Samples  
525 were taken from 100-120 min to measure baseline hepatic glucose production  
526 (HGP). A 2-step hyperinsulinaemic–euglycaemic clamp was commenced at 120 min:  
527 insulin infusion at 0.3 mU.kg<sup>-1</sup>.min<sup>-1</sup> (low-dose) for 120 min to measure the insulin  
528 sensitivity of HGP, then at 1.5 mU.kg<sup>-1</sup>.min<sup>-1</sup> (high-dose) for 180 min to measure  
529 insulin sensitivity of peripheral glucose uptake (Rd). Euglycaemia was maintained by  
530 adjusting a 20% glucose infusion, spiked with [6,6-<sup>2</sup>H<sub>2</sub>] glucose (7 mg.g<sup>-1</sup> glucose for  
531 low-dose, 10 mg.g<sup>-1</sup> high dose) according to 5 minute plasma glucose measurements  
532 to keep the patient's blood glucose level at a target concentration of 5 ± 0.5 mmol/L.

533 Plasma glucose concentration and enrichment time-courses were smoothed using  
534 optimal segments analysis(1). HGP and Rd (μmol.kg<sup>-1</sup>.min<sup>-1</sup>) were calculated using  
535 non-steady-state equations(2), assuming a volume of distribution of 22% body  
536 weight. Hepatic insulin sensitivity was calculated as (basal HGP (90-120 min) – HGP  
537 during low-dose insulin (210-240 min)/basal HGP) x 100. Glucose Rd was calculated  
538 at steady-state following high-dose insulin (390-420 min)

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540

**541 Urinary metabolomics**

542 Urine was collected in a sterile container at each assessment visit, centrifuged to  
543 remove sediment and frozen at -70<sup>0</sup>C within 30 minutes. For analysis, 540 $\mu$ l of urine  
544 was centrifuged at 14,000 rpm for 15 minutes and buffered with 60 $\mu$ l NMR buffer  
545 (containing TSP), then plated into 96-rack NMR plate. Control samples comprising  
546 an equal mixture of all case samples were generated and prepared in the same  
547 manner. An NPC NMR machine (Bruker, Billerica, USA) with a refrigerated robotic  
548 sample-handling unit was used. Two NMR experiments (NOSEY and J-resolved)  
549 were conducted on each sample. All spectra were referenced to trimethylsilyl  
550 propionic acid (TSP) and a sharp and appropriately sited water peak. A Bruker  
551 automated script was used to perform Fourier transforming, phasing and baselining.  
552 Resulting spectra were aligned and subsequently trimmed and the water peak  
553 removed prior to normalisation using in-house Matlab scripts (Mathworks, Natick,  
554 USA). Resulting spectral data were imported into Simca (Umetrics, San Jose, USA).

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563 **Supplementary Table 1.** Serum Cytokine and Adipokine values before and after

564 Rifaximin

Cytokine	Pre-Rifaximin	Post-Rifaximin	P
IL1beta*, pg/mL	0.55 (0.01-3.41)	0.57 (0.12-3.07)	1.00
IL6, pg/mL	4.52 (1.65-43.32)	3.09 (1.50-56.23)	0.39
IL10, pg/mL	0.76 (0.18-5.64)	0.95 (0.12-6.31)	0.96
IL18, pg/mL	318.4 (140.1-504.7)	336.6 (193.7-650.7)	0.65
CD14, ug/mL	2.22 (1.12-6.50)	2.34 (0.89- 10.95)	1.00
TNFalpha#, pg/mL	1.20 (0.35-15.18)	1.06 (0.17-22.67)	0.59
Leptin, ng/mL	14.81 (4.63-956.00)	14.79 (5.06-1094.26)	0.10
Resistin, ng/mL	19.61 (9.76-80.00)	21.08 (9.07-107.69)	0.73
Adiponectin, ug/mL	2.26 (0.90-8.33)	2.34 (0.62- 15.47)	0.57

565 (n=15, \*n=7, #n=9)

566

567 **Supplementary Fig. S1. Effect of Rifaximin on the genus level composition of**  
568 **faeces in individual patients.** Significant changes, >5%, at the genus level were  
569 plotted for each patient pre (blue bars) and post (orange bars) antibiotic  
570 administration. Significance of genus change was estimated in STAMP using  
571 Fisher's exact test and multiple testing was controlled for using a Benjamini-  
572 Hochberg FDR test. Sample PA07 was not included as changes were <5%.

573

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