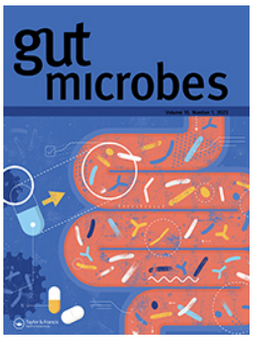

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Gut microbiota in nonalcoholic fatty liver disease: a PREDIMED-Plus trial sub analysis

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ABSTRACT

To evaluate the changes in the gut microbiota associated with changes in the biochemical markers of nonalcoholic fatty liver disease (NAFLD) after a lifestyle intervention with the Mediterranean diet. Participants ($n = 297$) from two centers of PREDIMED-Plus trial (Prevención con Dieta Mediterránea) were divided into three different groups based on the change tertile in the Hepatic Steatosis Index (HSI) or the Fibrosis–4 score (FIB–4) between baseline and one year of intervention. One-year changes in HSI were: tertile 1 (T1) (−24.9 to −7.51), T2 (−7.5 to −1.86), T3 (−1.85 to 13.64). The most significant differences in gut microbiota within the year of intervention were observed in the T1 and T3. According to the FIB–4, participants were categorized in non-suspected fibrosis (NSF) and with indeterminate or suspected fibrosis (SF). NSF participants showed higher abundances of *Alcaligenaceae*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Peptostreptococcaceae*, *Verrucomicrobiaceae* compared to those with SF. Then, participants were divided depending on the FIB–4 tertile of change: T1 (−89.60 to −5.57), T2 (−5.56 to 11.4), and T3 (11.41 to 206.24). FIB–4 T1 showed a decrease in *Akkermansia* and an increase in *Desulfovibrio*. T2 had an increase in *Victivallaceae*, *Clostridiaceae*, and *Desulfovibrio*. T3 showed a decrease in *Enterobacteriaceae*, and an increase in *Sutterella*, *Faecalibacterium*, and *Blautia*. A relation between biochemical index changes of NAFLD/NASH (HSI and FIB–4) and gut microbiota changes were found. These observations highlight the importance of lifestyle intervention in the modulation of gut microbiota and the management of metabolic syndrome and its hepatic manifestations.

PLAIN LANGUAGE SUMMARY

What You Need to Know

What is the context:

Obesity and metabolic syndrome have been associated with nonalcoholic fatty liver disease (NAFLD). Gut microbiota and its interaction with the environment may play a key role in NAFLD.

What is new:

Mediterranean diet and physical activity can modify the scores for liver steatosis (HSI) and liver fibrosis (FIB–4) in only one year. A relation between the changes in these scores and gut microbiota changes was found.

What is the impact:

The discovery of microbiota-based biomarkers for NAFLD and the development of strategies to modulate gut microbiota in the treatment of NAFLD.

ARTICLE HISTORY

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KEYWORDS

Microbiome; metabolic liver disease; hepatic steatosis index; the Fibrosis-4 score; Mediterranean diet

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the main cause of chronic liver disease nowadays, with a global prevalence of 25%.[1] NAFLD begins with simple macrovesicular steatosis and might progress to nonalcoholic steatohepatitis (NASH) which is characterized by steatosis and ballooning and inflammation with or without fibrosis. NASH implies a higher risk of progression to cirrhosis, hepatocellular carcinoma, and end-stage liver disease. There are many risk factors associated with NAFLD, most of them associated with obesity, type 2 diabetes mellitus (T2DM), and other components of metabolic syndrome (MS).[2] NAFLD is considered a hepatic manifestation of MS, but also has a role in the rest of its components.[3] Recently, the term metabolic dysfunction associated with fatty liver disease (MAFLD) has been proposed, though there is no consensus on the generalization of this new nomenclature.

It has been demonstrated a relationship between a western lifestyle and NAFLD, but there are also some genetic risk factors such as some single nucleotide polymorphisms (SNPs), including patatin-like phospholipase domain-containing protein 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), or membrane-bound O-acyltransferase domain containing 7 (MBOAT7), among others.[2]

Some studies in mice have shown that differences in microbiota composition influenced the development of NAFLD after a high-fat diet, with an increased abundance of *Firmicutes* phylum, *Barnesiella* and *Roseburia* genera, *Lachnospiraceae* and *Barnesiella intestinihominis*.[4] In addition, several human studies have also shown a role of dysbiosis in the development and progression of NAFLD, with increased *Proteobacteria* phylum[5;] increased *Enterobacteriaceae*, and decreased *Rikinellaceae* and *Rummunoccaceae* at the family level[6;] or increased *Escherichia* and *Dorea* and decreased *Coprococcus*, *Faecalibacterium*, and *Prevotella* at the genus level.[7] In patients with advanced fibrosis, some specific alterations have been also described, predominantly an increase in Gram-negative bacteria and *Proteobacteria*, and a decrease in *Firmicutes*.[6] Finally, some microbial metabolites and endotoxins also seem to play a role in NAFLD pathophysiology, such as increased bile acids circulation, acetate, and butyrate short-chain fatty acids, ethanol, or choline-related metabolites. Some of them have been shown to be able in increasing gut permeability, inducing inflammation, or promoting hepatic lipogenesis.[4]

However, there is important heterogeneity in the results reported to date, probably due to the use of different methodologies, study designs, and ethnic population differences among others. Therefore, further studies are warranted in the future to clarify the role of gut microbiota and their metabolites, interacting with environmental factors such as diet or lifestyle in the development and progression of NAFLD. In the present study, we evaluated the changes in the microbiota associated with changes in biochemical markers of NAFLD/NASH after an intervention aiming to promote weight loss with an energy-reduced Mediterranean diet and physical activity promotion, a lifestyle intervention with probed benefits for metabolic syndrome.

Results

Changes in the HSI steatosis index during the intervention

Participants were categorized into three groups depending on HSI tertile of change after 1-year of the intervention: T1 ($n = 99$; −24.9 to −7.51), T2 ($n = 99$; −7.5 to −1.86) and T3 ($n = 99$; −1.85 to 13.64); being T1 the group with the more favorable change and T3 with the less favorable change across the first year of intervention. In Table 1, we represent the anthropometric and biochemical variables at baseline, 1-year of intervention, and their changes over the first year of follow-up. Significant differences between extreme tertiles were shown for body weight, waist circumferences, and BMI. Compared to T2 and T3, a higher percentage of participants with type 2 diabetes diagnosis and metformin treatment was observed in T1. In addition, T1 participants had a higher baseline HSI and showed a higher decrease in this index after 1-year of intervention, like those in T2. Regarding 1-year changes, some significant differences were found in weight in T1 participants compared to those in T2 and T3 (−5.86 ± 4.14 kg in T1 *vs.* −3.04 ± 3.43 kg in T2 and 0.41 ± 3.18 kg in

Table 1. Clinical and laboratory characteristics at baseline and 1-year of intervention according to the change HSI tertiles.

Variables		T1 ($n = 99$)	T2 ($n = 99$)	T3 ($n = 99$)	*p-value* across tertiles[&]
Age (years)		64.29 ± 5.25	64.80 ± 5.14	65.13 ± 4.63	0.643
Sex (male/female)		60/39	51/48	45/54 [a]	0.099
HSI, median (IQR)	Baseline	46.87 (42.93–50.33)	44.75 (40.25–47.95)[a]	43.91 (40.21–46.51) [a]	<.001
	1-year	41.20 (37.51–44.87)*	42.47 (38.50–45.60)*	44.38 (41.62–48.06)[*, a, b]	<.001
	Change	−11.88 ± 3.48	−4.74 ± 1.60[a]	2.39 ± 3.73[a, b]	<.001
Weight (kg), mean±SD	Baseline	88.78 ± 11.46	88.86 ± 14.56	87.71 ± 12.66	0.764
	1-year	83.53 ± 11.36*	86.10 ± 14.01*	88.03 ± 12.43[a]	0.049
	Change	−5.86 ± 4.14	−3.04 ± 3.43[a]	0.41 ± 3.18[a, b]	<.001
Waist circumference (cm), mean±SD	Baseline	110.46 ± 8.68	109.65 ± 10.66	108.90 ± 9.98	0.565
	1-year	104.81 ± 9.41*	106.53 ± 10.50*	109.37 ± 10.06[a]	0.006
	Change	−5.03 ± 4.73	−2.80 ± 4.29[a]	0.47 ± 4.09[a, b]	<.001
Waist-Hip ratio, mean±SD	Baseline	1.0 ± 0.07	0.99 ± 0.07	0.99 ± 0.07	0.352
	1-year	0.98 ± 0.06*	0.98 ± 0.07*	0.98 ± 0.07	0.944
	Change	−2.08 ± 4.59	−1.12 ± 4.56	−0.57 ± 4.34[a]	0.092
BMI (kg/m^2, median (IQR)	Baseline	32.66 (30.14–35.68)	32.81 (29.59–36.24)	33.15 (30.78–36.15)	0.771
	1-year	31.01 (29.18–33.35)*	31.66 (29.16–35.35)*	33.48 (31.06–36.09)[a, b]	<.001
	Change	−5.50 ± 4.38	−2.85 ± 3.33[a]	0.45 ± 3.18[a, b]	<.001
Glucose (mg/dL), median (IQR)	Baseline	105 (95–122.75)	102.50 (92–117)	102 (93–114.50)	0.340
	1-year	104 (89.25–124)*	101 (93.25–114.75)	99 (93–115.50)	0.898
	Change	−3.13 ± 14.95	−0.18 ± 13.90	3.34 ± 20.83[a]	0.015
Triglycerides (mg/dL), median (IQR)	Baseline	138 (116–202)	173.50 (124.50–206)	151 (118–216)	0.422
	1-year	132 (95.25–171.50)*	150.50 (115–190.50)[a]	151 (111–191)[a]	0.029
	Change	−5.82 ± 39.38	4.79 ± 68.13	1.59 ± 38.78	0.190
Total cholesterol (mg/dL), mean±SD	Baseline	194.20 ± 36.60	205.07 ± 35.21[a]	198.84 ± 35.49	0.062
	1-year	191.25 ± 35.74	203.53 ± 38.88 [a]	199.09 ± 41.82	0.081
	Change	−0.20 ± 13.31	−0.41 ± 14.27	0.99 ± 17.66	0.928
HDL (mg/dL), median (IQR)	Baseline	45 (39.25–55)	46 (39–55)	48 (43–54.50)[a]	0.099
	1-year	47.50 (40–58.75)*	49 (42–58)*	51 (44–58)*	0.372
	Change	8.85 ± 15.39	5.07 ± 15.76	5.14 ± 15.50	0.112
LDL (mg/dL), median (IQR)	Baseline	110.50 (91–136.25)	116 (106–141.50)[a]	116 (93–137)	0.036
	1-year	109 (94–130.25)	115.50 (100–143)	113 (91–144)	0.262
	Change	2.15 ± 22.30	−1.15 ± 20.91	2.55 ± 28.64	0.468
HbA1c (%)	Baseline	6.33 ± 1.15	6.01 ± 0.78[a]	5.96 ± 0.59[a]	0.070
	1-year	5.98 ± 0.90*	5.95 ± 0.83*	6.12 ± 1.03*	0.442
	Change	−4.59 ± 8.13	−0.75 ± 8.83[a]	2.55 ± 9.66[a, b]	<.001
AST (U/L), median (IQR)	Baseline	21 (16.25–26)	21 (17–26)	23 (19–27)[a]	0.067
	1-year	20 (16–24)	21 (18–25.75)	21 (17–26)*	0.122
	Change	1.20 ± 28.84	4.60 ± 30.71	−2.03 ± 23.47	0.464
ALT(U/L), median (IQR)	Baseline	27.50 (22–38)	25 (20–31)[a]	26 (19–31)[a]	0.097
	1-year	23 (17–28)*	23 (17–28)*	26 (19–35)[a]	0.017
	Change	−20.68 ± 23.48	−2.73 ± 28.27 [a]	11.13 ± 38.84[a, b]	<.001
GGT (U/L), median (IQR)	Baseline	32 (23–46)	26 (22–38.75)	29 (20–44.50)	0.263
	1-year	25 (19–34.75)*	25.50 (19.25–35.75)	29 (20–46.50)	0.131
	Change	−10.28 ± 45.86	3.80 ± 77.54	10.63 ± 46.35 [a]	<.001
Albumin (g/dL), median (IQR)	Baseline	4.22 (4.01–4.48)	4.34 (4.06–4.48)	4.29 (4–4.47)	0.858
	1-year	4.16 (3.87–4.42)*	4.24 (4–4.43)*	4.26 (4.09–4.42)	0.242
	Change	−2.02 ± 5.25	−1.79 ± 8.25	−0.57 ± 6.46	0.216
SBP (mm Hg), median (IQR)	Baseline	139 (130.33–149.58)	138.50 (128.08–150.33)	139 (127.33–148.16)	0.988
	1-year	134.33 (124.91–150.25)*	132.16 (122.50–144.25)*	137 (128.83–145.33) [b]	0.110
	Change	−2.41 ± 10.11	−3.72 ± 10.40	−0.52 ± 10.82	0.097
DBP (mm Hg), mean±SD	Baseline	79.40 ± 9.86	79.05 ± 10.28	79.88 ± 10.27	0.936
	1-year	77.76 ± 10.56*	75.74 ± 9.22	79.02 ± 9.03[b]	0.052
	Change	−1.79 ± 10.61	−3.56 ± 11.08	−0.30 ± 9.56[b]	0.091
Metformin treatment (%)	Baseline	32.32 (32/99)	15.15 (15/99)[a]	11.11 (11/99)[a]	<.001
	1-year	20.20 (20/99)	13.13 (13/99)	10.10 (10/99)[a]	0.051
Type 2 Diabetes (%)	Baseline	34.34 (34/99)	17.17 (17/99)[a]	12.12 (12/99)[a]	<.001
	1-year	37.37 (37/99)	20.20 (20/99)[a]	17.17 (17/99)[a]	0.001
Hypercholesterolemia (%)	Baseline	50.50 (50/99)	48.48 (48/99)	52.52 (52/99)	0.851
	1-year	48.48 (48/99)	49.49 (49/99)	51.51 (51/99)	0.741
Hypertension (%)	Baseline	77.77 (77/99)	76.76 (76/99)	85.85 (85/99)	0.214
	1-year	75.75(75/99)	87.87 (87/99)	85.85 (85/99)	0.208

ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; GGT, gamma-glutamyl transferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HSI, hepatic steatosis index; IQR, interquartile range; LDL, low-density lipoprotein; SBP, systolic blood pressure; SD, standard deviation. The groups were HSI tertile of change at one year after lifestyle of intervention: T1 (−24.9 to −7.51); T2 (−7.50 to −1.86) and T3 (−1.85 to 13.64). *$p \leq .05$ baseline vs. 1-year of intervention value, according to paired Student's tests or Wilcoxon tests. & One-way ANOVA, Pearson's chi-square test or Kruskal–Wallis test used to calculate differences across tertiles; Pearson's chi-square test, Student's t-test or Mann–Whitney test used to calculate differences between tertiles; a $p \leq .05$ *vs*. T1; b $p \leq .05$ *vs*. T2.

T3; $p < .001$) and in a similar way for the waist circumference and BMI, with greater reductions in T1 compared to T2 and T3. An increase in fasting glucose was observed in T3 participants compared to a reduction in those in T1 (−3.13 ± 14.95 mg/dL in T1 *vs.* 3.34 ± 20.83 mg/dL in T3, $p = 0.015$). Similar changes were observed in the case of HbA1c, with a greater reduction in T1 compared to T2 and an increase in T3. Significant differences in changes were also observed for liver enzymes: ALT showed a higher reduction in T1 compared to T2 and an increase in T3 compared to T1 and T2 ($p < .001$). GGT increased in T2 and T3, whereas decreased in T1 ($p < .001$) (Table 1).

Biochemical changes with the HSI steatosis index were accompanied by changes in gut microbiota populations

Significant changes in Weighted UniFrac distances (beta diversity), but not in the alpha diversity indexes, were found between baseline and 1-year of intervention in those participants in T1 ($p = .013$), and a tendency in case of T2 ($p = .054$), while no differences were shown in T3 (Supplementary Table S1).

Regarding particular 1-year changes within each tertile, those participants in T1 were characterized by a decrease in *Proteobacteria* ($p = .001$, q = 0.010) and *Lentisphaerae* phyla ($p = .005$, q = 0.015). In addition, a significant decrease in the *Enterobacteriaceae* family ($p < .001$, q < 0.001) was observed. At the genus level, we found a significant decrease in *Blautia* ($p = .008$, q = 0.043), and an increase in *Coprococcus* ($p < .001$, q = 0.008), *Lachnospira* ($p = .002$, q = 0.021) and *Oscillospira* ($p = .003$, q = 0.021) (Figure 1a). Participants in T2 did not show any significant differences at the level of phylum, family, or genus within the year of intervention. Finally, those participants in T3 were characterized by a significant increase in the *Alcaligenaceae* ($p < .001$, q = 0.002) and *Bifidobacteriaceae* ($p < .001$, q = 0.002) families, as well as, an increase in *Desulfovibrio* ($p < .001$, q < 0.001), *Bifidobacterium* ($p = .001$, q = 0.011), *Blautia* ($p < .001$, q < .001), *Faecalibacterium* ($p < .001$, q = 0.002), and *Sutterella* ($p = .006$, q = 0.03) genera (Figure 1a).

Gut microbiota shows different populations according to the levels of the FIB–4 index.

To deepen into characteristics of the gut microbiota populations associated with the FIB–4, the study participants were categorized into those with non-suspected fibrosis (NSF: F0-F1, $n = 205$), and those with indeterminate or suspected fibrosis (SF: F2-F6, $n = 92$) at baseline. We found some statistically significant differences at baseline. Waist circumference was higher in NSF ($p = .048$), but no differences were found in the waist/hip ratio. BMI was also higher in NSF group ($p = .001$) as well as glucose ($p = .004$), total cholesterol ($p = .012$) and HbA1c (0 < .001) though both groups exhibited a well metabolic control in terms of HbA1c (<6.5%). On the other hand, age ($p < .001$) and AST levels were higher at baseline in SF ($p < .001$) (Table 2).

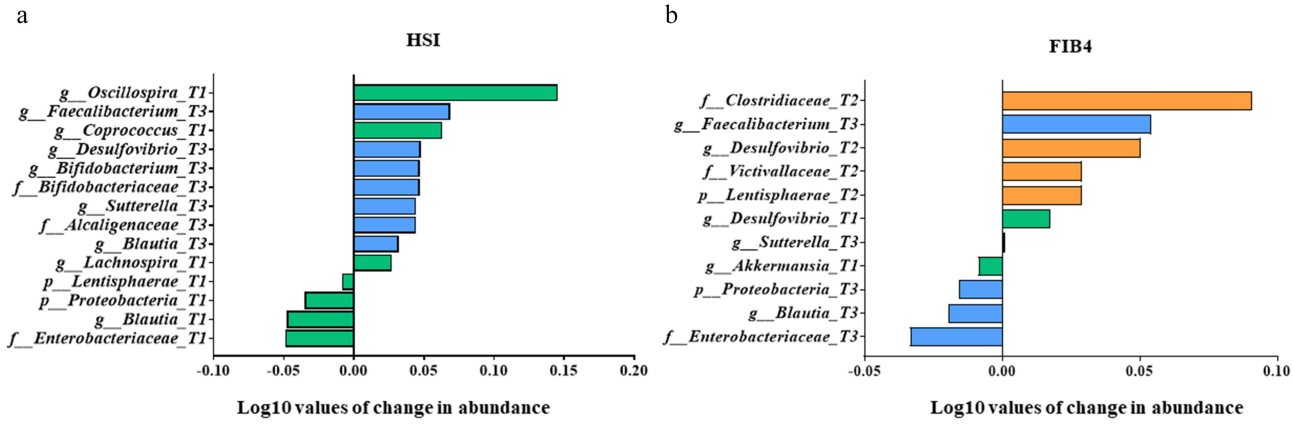

Figure 1. Graphs show the log10 values of the fold change in the abundance in phylum, family, and genera of gut microbiota found statistically significant between time-points ($p < .05$, q < 0.05). a. Significant changes in the abundance of gut microbiota in T1, T2 and T3 groups of HSI score. b. Significant changes in the abundance of gut microbiota in T1, T2 and T3 groups in FIB – 4 score.

Table 2. Anthropometric and biochemical baseline characteristics. Comparison between patients without fibrosis (NSF) and patients with indeterminate or suspected fibrosis (SF).

	NSF (205)	SF (92)	*p-value*
Sex, male/female	100/105	56/36	0.054
Age, years	64.05 ± 4.86	66.26 ± 5.01	<.001
Weight (kg), mean±SD	89.08 ± 13.19	87.08 ± 12.44	0.222
Waist circumference (cm), mean±SD	110.42 ± 9.55	107.99 ± 10.25	0.048
Waist/Hip (ratio), mean±SD	0.99 ± 0.07	0.99 ± 0.07	0.887
BMI (kg/m2), median (IQR)	33.39 (31.17–36.25)	31.47 (29.48–34.96)	0.001
Glucose (mg/dL), median (IQR)	104 (94–120.50)	99.50 (91–110.50)	0.004
Triglycerides (mg/dL), median (IQR)	156.50 (124.25–201)	151 (100.25–221.75)	0.300
Total cholesterol (mg/dL), mean±SD	203.04 ± 36.37	191.68 ± 33.79	0.012
HDL (mg/dL), median (IQR)	47 (41–55)	45 (39–51.50)	0.112
LDL (mg/dL), median (IQR)	116 (96.50–141)	110.50 (96.25–130)	0.063
HbA1c (%), mean±SD	6.21 ± 0.94	5.84 ± 0.67	<.001
AST (U/L), median (IQR)	20 (16–24.50)	25 (22–29)	<.001
ALT(U/L), median (IQR)	26 (20.50–34)	25 (20–31.75)	0.525
Fosfatase (U/L), median (IQR)	73 (60.50–89)	66 (57–81)	0.013
GGT (U/L), median (IQR)	29 (22–39)	28 (20–48)	0.928
Albumin (g/dL), mean±SD	4.24 (3.97–4.46)	4.36 (4.16–4.54)	0.017
SBP (mm Hg), median (IQR)	139 (129–149.33)	138.83 (126.33–149.58)	0.738
DBP (mm Hg), mean±SD	79.58 ± 10.46	79.13 ± 9.36	0.724
Metformin treatment (%)	22.43 (46/205)	13.04 (12/92)	0.059
Type 2 Diabetes (%)	23.90 (49/205)	15.21 (14/92)	0.090
Hypercholesterolemia (%)	52.19 (107/205)	46.73 (43/92)	0.385
Hypertension (%)	80.48 (165/205)	79.34 (73/92)	0.820

ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; GGT, gamma-glutamyl transferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; SBP, systolic blood pressure; SD, standard deviation.

Concerning gut microbiota results, although no differences were found in alpha or beta diversity (Supplementary Table S2), NSF participants showed a higher increase in the levels of *Proteobacteria* ($p < .001$, $q < 0.001$) and *Verrucomicrobia* ($p < .001$, $q < 0.001$) phyla compared to SF participants. At family level, compared to SF a significant higher increase was observed in the families *Alcaligenaceae* ($p < .001$, $q < 0.001$), *Bacteroidaceae* ($p = 0.001$, $q = 0.004$), *Bifidobacteriaceae* ($p < .001$, $q < 0.001$), *Clostridiaceae* ($p = .001$, $q = 0.004$), *Enterobacteriaceae* ($p < .001$, $q < 0.001$), *Peptostreptococcaceae* ($p < .001$, $q < 0.001$), *Verrucomicrobiaceae* ($p < .001$, $q < 0.001$) in those participants with NSF, while the *Porphyromonadaceae* family showed a higher decrease ($p < .001$, $q = 0.002$) (Figure 2). At the genus level, the genus *Bifidobacterium* ($p < .001$, $q = 0.01$) was found to increase in NSF compared to SF, and the abundance of the unknown genus g_ ($p < .001$, $q < 0.001$) was found to be significantly lower in NSF compared to SF. Of those participants included in NSF, 29.1% were in T1, 33% in T2, and 37.9% in T3, while those included in SF 45.7% were in T1, 31.3% in T2, and 20,7% in T3 ($p = .004$), respectively (Figure 2).

Changes in the FIB–4 fibrosis index during the intervention

In the same way as the HSI index, the sample was categorized in three tertiles of FIB–4 changes after 1-year of intervention: T1 ($n = 99$; −89.60 to −5.57), T2 ($n = 99$; −5.56 to 11.4), and T3 ($n = 99$; 11.41 to 206.24). Like the steatosis index, those participants in T1 showed the most favorable changes in anthropometric and biochemical variables, and those in T3 had the less favorable changes across the first year of intervention (Table 3).

FIB–4 score tertiles of change showed fewer differences than in the case of steatosis. At baseline, AST levels were lower in T3 compared to T1 and T2 ($p < .001$) in the same manner with respect ALT, with lower baseline levels in T3 compared to T1 ($p = .012$). Albumin levels were lower in T3 compared to T1 ($p = .008$) and T2 ($p = 0.002$) and after 1-year of intervention, albumin levels decreased in T3 compared to T2 ($p = .006$) and T1 ($p = .017$). Regarding changes after 1-year, we found an increase in AST in T3 compared to a reduction in T1 and T2 ($p < .001$). On the other hand, we observed a significant reduction in HbA1c in T3 and T2 compared to a mild increase in T1 ($p = .002$ and $p = .025$, respectively) as shown in Table 3.

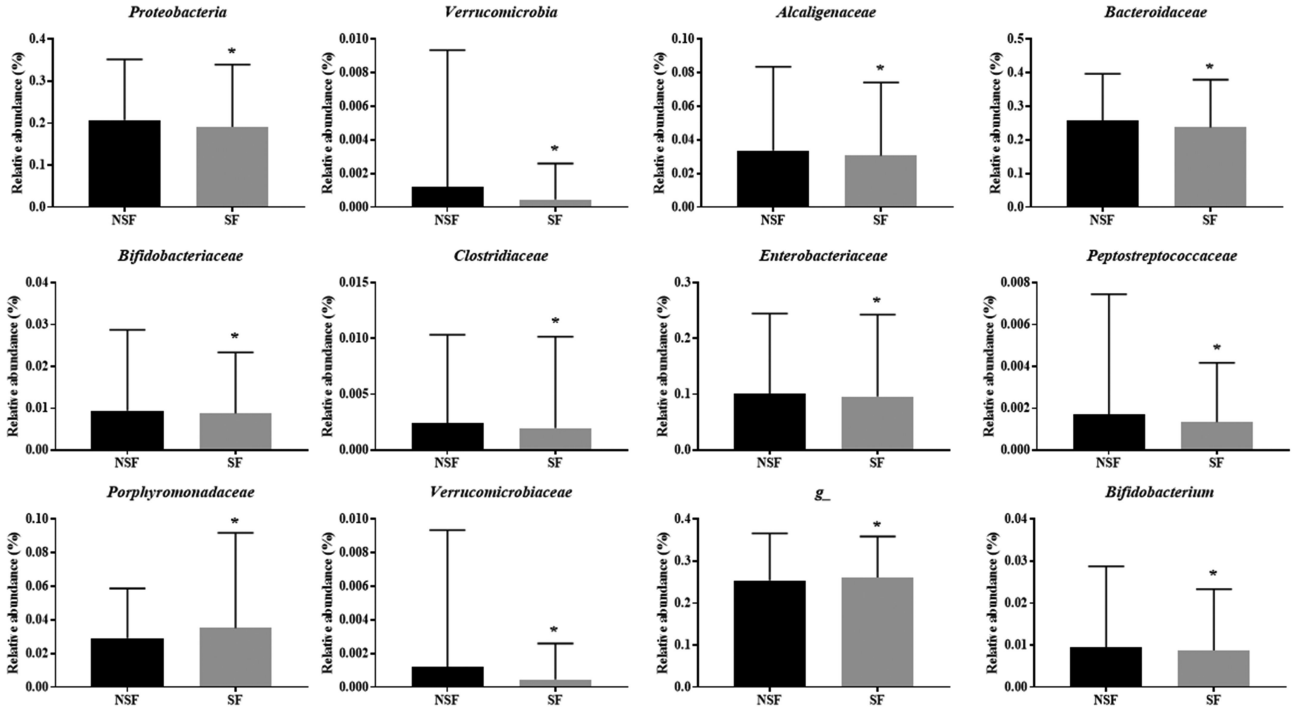

Figure 2. Comparison among participants with non-suspected fibrosis (NSF) and paticipants with indeterminate or suspected fibrosis (SF). Relative abundance (%) of phyla, families and genera that have been found significant between fibrosis groups. Significantly differences *$p \leq .05$.

Biochemical changes with fibrosis index were accompanied by changes in gut microbiota populations

Regarding beta diversity, gut microbiota populations changed within T1 according to the Weighted UniFrac index ($p = .016$), as well as in T2 ($p = .010$), but with no differences within the T3 group. Regarding alpha diversity parameters, participants in T1 showed a significant increase in the baseline Evenness index compared to the 1-year time-point ($p = .024$). Participants in T2 and T3 did not show any difference in these alpha diversity indexes (Supplementary Table S3). Figure 1B shows the log10 values of change in abundance of the particular phyla, families, and genera that differed within each tertile. Participants in T1 were characterized by a decrease in the *Akkermansia* genus ($p = 0.002$, $q = 0.043$) and an increase in the *Desulfovibrio* ($p < .005$, $q = 0.004$) genus. T2 participants showed an increase in the phylum *Lentisphaerae* ($p < .001$, $q = 0.002$). At the family level, an increase in *Victivallaceae* ($p < .001$, $q = 0.007$) and *Clostridiaceae* ($p < .001$, $q = 0.012$) was observed. At the genus level, we found an increase in *Desulfovibrio* ($p < .001$, $q < 0.001$). And finally, T3 participants were characterized by a significant decrease in the *Proteobacteria* phylum ($p < .001$, $q < 0.001$), a significant decrease in its family *Enterobacteriaceae* ($p < .001$, $q < 0.001$), and a significant increase in *Sutterella* ($p = 0.002$, $q = 0.017$), *Faecalibacterium* ($p < .001$, $q = 0.001$), and *Blautia* ($p < .001$, $q < 0.001$) genera.

Metabolic pathways associated with the microbiota.

Figure 3 depicts the routes found according to the HSI tertile changes (Supplementary Table S4). Compared to T2 and T3, T1 was characterized by a decrease in the menaquinol biosynthesis (PWY−5838 to PWY−5899), and in the biosynthesis of fatty acids and lipids (PWY−5989 to PWY0−862). We found routes involved in heme group biosynthesis, HEME-BIOSYNTHESIS-II and HEMESYN2-PWY, PWY−5918, and PWY0−1415, which were decreased in group T1 to T2 and T3. Participants in T1 showed an increase in the biosynthesis of methionine, an amino acid with important cellular functions such as the initiation of protein synthesis, DNA methylation, rRNA, the biosynthesis of

Table 3. Clinical and laboratory characteristics at baseline and 1-year of intervention according to the change of FIB–4 tertile groups.

Variables		T1 ($n = 99$)	T2 ($n = 99$)	T3 ($n = 99$)	*p-value* across tertiles [&]
Age (years)		64.14 ± 4.69	64.56 ± 5.22	65.53 ± 5.04	0.125
Sex (male/female)		60/39	43/56[a]	53/46	0.052
FIB–4, median (IQR)	Baseline	1.34 (1.04–1.83)	1.14 (0.93–1.53)[a]	1.05 (0.84–1.39)[a]	<.001
	1-year	1.07 (0.83–1.48)*	1.18 (0.96–1.58)*	1.42 (1.08–1.74)[*, a, b]	<.001
	Change	−17.39 ± 13.38	3.61 ± 4.91[a]	34.24 ± 26.66[a, b]	<.001
Weight (kg), mean±SD	Baseline	89.46 ± 13.79	88.39 ± 12.21	87.47 ± 12.89	0.455
	1-year	87.23 ± 13.57*	86 ± 12.31*	84.38 ± 12.34*	0.292
	Change	−2.49 ± 4.11	−2.57 ± 4.68	−3.42 ± 4.42	
Waist circumference (cm), mean±SD	Baseline	110 ± 10.28	109.32 ± 9.47	109.67 ± 9.76	0.753
	1-year	108.06 ± 10.02*	106.43 ± 9.99*	106.17 ± 10.46*	0.362
	Change	−1.75 ± 4.45	−2.43 ± 4.79	−3.16 ± 5.41[a]	
Waist-Hip ratio, mean±SD	Baseline	1 ± 0.07	0.98 ± 0.07	1 ± 0.06	0.142
	1-year	0.99 ± 0.07*	0.97 ± 0.07[*, a]	0.98 ± 0.07*	0.070
	Change	−1.26 ± 4.52	−1.39 ± 4.04	−1.12 ± 5.01	
BMI (kg/m^2, median (IQR)	Baseline	32.93 (30.43–35.33)	33.97 (30.58–36.48)	32.21 (30.05–35.30)	0.169
	1-year	31.75 (29.92–34.64)*	33.22 (29.72–36.06)*	31.18 (29.33–34.23)[*, b]	0.096
	Change	−2.45 ± 4.04	−2.40 ± 4.75	−3.04 ± 4.40	
Glucose (mg/dL), median (IQR)	Baseline	103 (92–120)	102 (93–122)	102 (93–114)	0.884
	1-year	101 (88–120.50)	101 (92–114)	102 (95–114)	0.934
	Change	1.26 ± 21.32	−1.20 ± 15.87	−0.04 ± 12.74	
Triglycerides (mg/dL), median (IQR)	Baseline	153 (112.75–230.50)	171 (124–201)	151 (118–192)	0.667
	1-year	140 (111.75–195.25)	139 (110–181)*	146.50 (105.50–191.75)	0.881
	Change	0.62 ± 38.49	0.66 ± 69.09	−0.72 ± 38.74	
Total cholesterol (mg/dL), mean±SD	Baseline	200 ± 37.86	198.28 ± 31.73	200.28 ± 38.14	0.857
	1-year	195.62 ± 38.79	199.35 ± 37.98	199.48 ± 40.83	0.732
	Change	−1.14 ± 15.38	1.49 ± 14.47	0.02 ± 15.64	
HDL (mg/dL), median (IQR)	Baseline	45 (38–51)	46 (42–55)	49 (40–56.50)	0.060
	1-year	47 (41–57)*	51 (44–58)*	50.50 (41.25–60)*	0.250
	Change	6.82 ± 16.04	6.64 ± 15.23	5.61 ± 15.65	
LDL (mg/dL), median (IQR)	Baseline	116 (93.75–138)	116 (98–138)	112.50 (93.75–138.50)	0.897
	1-year	112.50 (92.75–135)	113 (97–139)	115 (94–143.75)	0.774
	Change	−0.66 ± 26.75	2.95 ± 21.51	1.26 ± 24.04	
HbA1c (%)	Baseline	6.04 ± 0.73	6.19 ± 0.91	6.06 ± 0.99	0.264
	1-year	6.12 ± 1.07	6.07 ± 0.72*	5.85 ± 0.92*	0.091
	Change	1.32 ± 10.22	−1.23 ± 7.35 [a]	−2.88 ± 9.78[a]	
AST (U/L), median (IQR)	Baseline	24 (20–29)	22 (18–27)[a]	18 (15–23) [a, b]	<.001
	1-year	21 (16–25) *	21 (17–25)*	21.50 (18–26) *	0.653
	Change	−13.60 ± 22.00	−3.82 ± 17.39[a]	21.21 ± 30.17[a, b]	
ALT(U/L), median (IQR)	Baseline	27 (22–40.25)	26 (20–31)	24 (19–31.75)[a]	0.021
	1-year	24 (19–31)*	22 (18–29)*	23.50 (18–30)	0.325
	Change	−4.53 ± 40.42	−8.51 ± 25.71	0.75 ± 32.17[b]	
GGT (U/L), median (IQR)	Baseline	30 (22–50.25)	27 (21–43)	29 (22–38)	0.325
	1-year	28.50 (20–40.25)*	25 (19–34)*	27.50 (19.25–39)	0.343
	Change	−4.02 ± 32.17	−2.51 ± 33.15	10.90 ± 91.03	
Albumin (g/dL), median (IQR)	Baseline	4.36 (4.05–4.51)	4.36 (4.11–4.49)	4.17 (3.96–4.41)[a, b]	0.005
	1-year	4.26 (4.05–4.43)*	4.27 (4.05–4.44)*	4.16 (3.8–4.34)[*, a, b]	0.012
	Change	−1.83 ± 8.72	−1.52 ± 5.92	−1.03 ± 5.20	
SBP (mm Hg), median (IQR)	Baseline	142.33 (130.83–151.33)	138.33 (126.33–150.33)	136.66 (130.08–146.25)	0.232
	1-year	136.83 (129.16–149.50)	134 (123–145.33)*	134.16 (124.66–146.83)*	0.228
	Change	−2.24 ± 10.38	−2.06 ± 10.93	−2.36 ± 10.27	
DBP (mm Hg), mean±SD	Baseline	79.47 ± 10.31	79.87 ± 10.36	78.95 ± 9.74	0.918
	1-year	78.15 ± 9.84	77.17 ± 9.50*	77.04 ± 9.75*	0.680
	Change	−1.24 ± 10.42	−2.53 ± 10.23	−1.89 ± 10.87	
Metformin treatment (%)	Baseline	19.19 (19/99)15.15 (15/99)	25.25 (25/99) 16.16 (16/99)	14.14 (14/99)	0.142
	1-year			12.12 (12/99)	0.377
Type 2 Diabetes (%)	Baseline	22.22 (22/99)	26.26 (26/99)	15.15 (15/99)	0.154
	1-year	27.27 (27/99)	31.31 (31/99)	16.16 (16/99)[b]	0.055
Hypercholesterolemia (%)	Baseline	43.43 (43/99)	55.55 (55/99)	52.52 (52/99)	0.207
	1-year	47.47 (47/99)	54.54 (54/99)	47.47 (47/99)	0.476
Hypertension (%)	Baseline	76.76 (76/99)	82.82 (82/99)	80.80 (80/99)	0.553
	1-year	80.80 (80/99)	86.86 (86/99)	81.81 (81/99)	0.259

ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; FIB–4: Fibrosis–4 index for liver fibrosis; GGT, gamma-glutamyl transferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; SBP, systolic blood pressure; SD, standard deviation. The groups were FIB–4 tertile of change at one year after lifestyle of intervention: T1 (−89.68 to −5.57); T2 (−5.56 to 11.40) and T3 (11.41 to 206.24). *$p < .05$ baseline *vs*. 1-year of intervention value, according to paired Student's tests or Wilcoxon tests. & One-way ANOVA, Pearson's chi-square test or Kruskal–Wallis test used to calculate differences across tertiles; Pearson's chi-square test, Student's t-test or Mann–Whitney test used to calculate differences between tertiles; a $p \leq .05$ *vs*. T1; b $p \leq .05$ *vs*. T2.

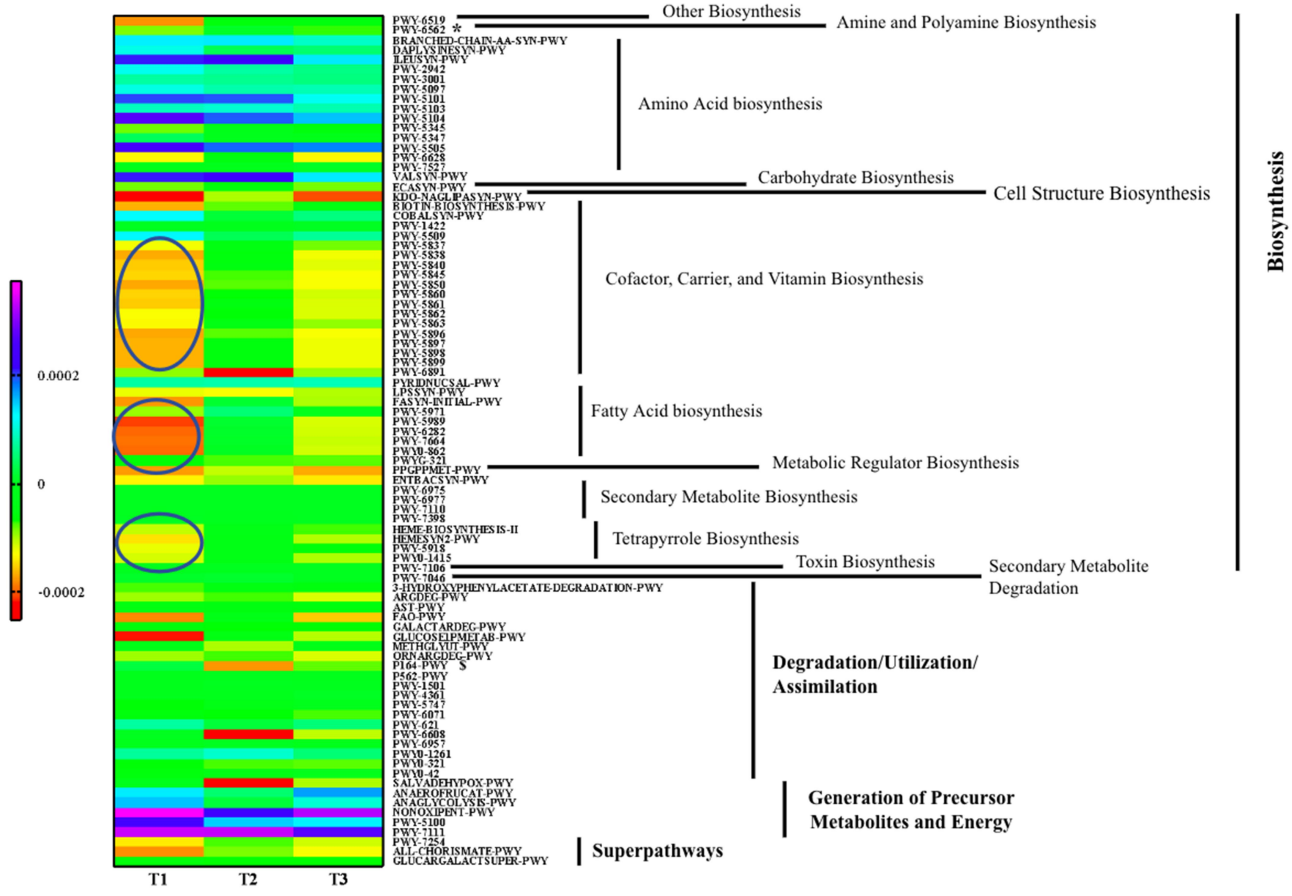

Figure 3. Heatmap showing the means of changes in predicted pathways between the three HSI score tertiles. *Indicates significant differences between tertiles ($p \leq .05$) and, $ indicates $p < .1$ in multiple group tests using the Kruskal–Wallis test.

cysteine, phospholipids, and polyamines as well as in purine degradation (P164-PWY; $p = .057$), compared to those in T2 and T3. Participants in T2 showed an increase in the biosynthesis of the polyamine norspermidine (PWY−6562; $p = .019$) pathway, compared to those in T1 and T3.

No great pattern differences between FIB−4 score tertiles were observed for the theoretically metabolic pathways involved, although some routes especially showed significant changes among tertiles. In the HEXITOLDEGSUPER-PWY pathway ($p = .035$), hexitol degradation pathway was increased in T2 to T1 and T3. Compared to T1 and T2, participants in T3 had an increase in the P122-PWY pathway ($p = .014$) involved in lactate fermentation, and in the P124-PWY pathway ($p = .011$), called "Bifidobacterium shunt", that was involved in the fermentation of short-chain fatty acids (SCFAs) such as acetate and lactate. Finally, the P461-PWY ($p = .04$), a fermentation pathway from hexitols to lactate, formate, ethanol, and acetate, was increased in the T2 to T1 and T3 (Figure 4 and Supplementary Table S5).

Discussion

In recent years, the evidence regarding the impact that microbiota has on NAFLD has grown, although the results of studies are heterogeneous and difficult to generalize. In the present study, we reported that two noninvasive scores for liver steatosis (HSI) and liver fibrosis (FIB−4) usually used in clinical practice, could differentiate gut microbiota populations. In addition, we have shown the effect of a lifestyle intervention based on the Mediterranean diet and physical activity on these noninvasive indexes, indicating a possible interplay between steatosis/fibrosis, gut microbiota, and lifestyle.

An increase in the prevalence of liver metabolic diseases has been reported in the last decades[8], in parallel to the obesity and metabolic syndrome

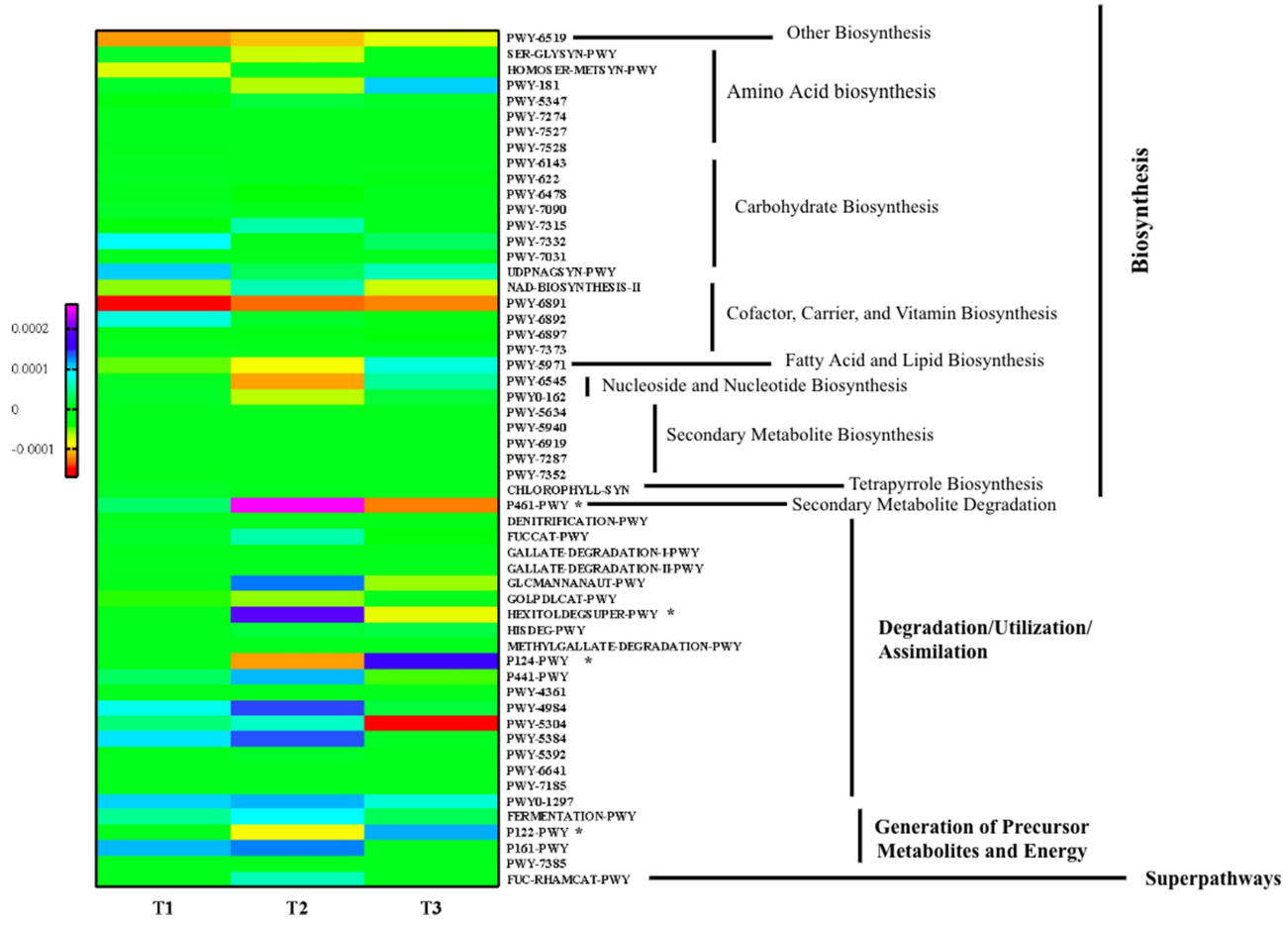

Figure 4. Heatmap showing the means of changes in predicted pathways between three FIB–4 score tertiles. *Indicates significant differences between tertiles ($p \leq .05$) in multiple group tests using the Kruskal–Wallis test.

epidemic. Although NAFLD is not considered a diagnostic criterium for metabolic syndrome, it is a common hepatic manifestation of this syndrome, and it has been claimed to be introduced as a sixth metabolic syndrome criterium.[9] In last years, some interesting study results have pointed out the relationships between gut microbiota and liver axis.[10] Indeed, gut microbiota could serve as a potent biomarker for this condition.[11] Therefore, we investigated this possibility within the frame of the well-characterized PREDIMED-Plus cohort in which possible reversion with a lifestyle intervention based on a Mediterranean diet can be explored.

First, regarding steatosis measured with the HSI, as per PREDIMED-Plus inclusion criteria, our participants had overweight/obesity and metabolic syndrome, and 21% of them were diagnosed with diabetes, most of them had a high HSI and an elevated clinical suspicion of steatosis, precluding the categorization of participants in different grades at baseline. However, we observed that HSI was modifiable with a lifestyle intervention based on a Mediterranean diet, and therefore.[12], we categorized our patients by their HSI response to the intervention. Patients with the worst metabolic change after the lifestyle intervention showed the lowest HSI values at baseline. However, no special differences were noticed between HSI tertiles regarding anthropometric or biochemical variables. Regarding gut microbiota, the most prominent changes were found in HSI T1 and T3 participants. In those in T1 (most favorable change), the genus *Oscillospira* in the gut increased, meanwhile, *Proteobacteria* and its family *Enterobacteriaceae* decreased. These changes are consistent with those previously reported in NAFLD/NASH. Many studies have shown higher levels of *Proteobacteria* in subjects with steatosis.[5,13,14] In addition, a reduction in

Proteobacteria abundance was detected in individuals after weight loss interventions such as exercise programs,[15] contrary to *Oscillospira* which increases, together with short-chain fatty acids (SCFAs) concentrations, with physical activity.[16] *Oscillospira* has been reported that also increases after a Mediterranean diet,[17] and has been related to a BMI reduction after weight loss induced by diet.[18] In the same way as *Oscillospira*, other SCFAs producers such as *Coprococcus* and *Lachnospira* were also increased in participants in T1. In our study, participants in T1 seem to have had a better response to the Mediterranean lifestyle intervention, with an increase in the SCFAs producers and a reduction in pro-inflammatory bacteria.

However, in those with the least favorable change in HSI (T3), other microbiota feature composition was shown in gut. *Blautia* was the only bacterium shared by both HSI tertiles, T1 and T3, although with changes in opposite directions. *Blautia* is a genus of *Firmicutes* that has been linked positively or negatively to obesity-related diseases.[19] In the context of NAFLD/NASH, its action could be related to the bioconversion of primary bile acids into secondary bile acids which have been reported as harmful and even carcinogens.[20] Participants in T3 showed an increase in *Faecalibacterium*, this increase in a bacterium that has been usually related to beneficial effects on health[21] could be controversial. However, a reduction in the abundance of *Faecalibacterium* has been reported after bariatric surgery and the abundance was higher in obese subjects compared to lean subjects[21,22] suggesting a possible negative influence of acute weight loss in the abundance of this genus, as *Faecalibacterium* has been reported to be decreased in patients with NAFLD/NASH.[15] This is the same pattern found in our present study with the family *Bifidobacteriaceae* and its genus *Bifidobacterium*. Interestingly, participants in HSI T1 had a significant weight loss compared to those in T3. Therefore, changes in the gut microbiota profile in T1 may be related to weight loss and higher adherence to physical activity after 1-year of intervention.

Regarding fibrosis, our results concerning FIB−4 differ from those observed in the case of HSI. Moreover, FIB−4, in its equation, does not consider BMI or the presence of diabetes, being these factors discriminating in our participants. Changes in weight and BMI were non-statistically significant among our three study groups, so changes observed in gut microbiota did not seem to be influenced by adiposity changes. This fact also permitted us to study the characteristics of patients without suspected fibrosis or suspected fibrosis at baseline. According to the data of our participants, which must be corroborated in future studies, patients without suspected fibrosis displayed higher abundance in phyla *Proteobacteria* and *Verrucomicrobia*, and in families *Alcaligenaceae*, *Bacteriodaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Enterobacteriaceae* and *Verrucomicrobiaceae*, and lower abundance in the family *Porphymonadaceae* and high levels of *Bifidobacterium* genus. In our study, fibrosis has not been ascertained by biopsies or elastography, therefore we must take our data with caution, although some of our results are consistent with the intervention period. Indeed, participants in FIB−4 T3 (worst response to the lifestyle intervention) showed a decrease in *Proteobacteria* and its family *Enterobacteriaceae*. *Proteobacteria* and *Enterobacteriaceae* abundances have been found higher in patients after bariatric surgery (especially after Roux-en-Y-gastric bypass) and lower in lean subjects.[23,24] Some explanations for this increase in a bacterium that is usually considered proinflammatory are the increase in oxygen availability in the large intestine after surgery, favoring the presence of facultative anaerobes,[25] but also as a consequence of a reduced gastric acid secretion after surgery, the decreased caloric intake or the changes produced in the intake of nutrient.[26] Again, diet composition and caloric restriction may play an important role in the changes observed in our participants, since those in FIB−4 T3 were also those with lower improvements or in some cases worsening evolution in metabolic parameters. However, FIB−4 T1 participants – who had a better response to the lifestyle intervention – showed a decrease in *Akkermansia* levels. Although *Akkermansia* has been reported to have beneficial effects on health, it has been observed to be decreased in patients with NAFLD/NASH,[15,21] and has been reported to be protective against hepatic inflammation.[27,28] This decrease could be related to the age of our

volunteers, inasmuch as aging induces a decrease in the abundance of *Akkermansia*,[29] although the lifestyle intervention would have attenuated a higher reduction in this genus. On the other hand, an increase in the levels of *Desulfovibrio* was displayed, which is in line with the findings of Hong and collaborators who described a depletion of this genus in high-fat diet-fed mice, being the *Desulfovibrio vulgaris* a potent acetic acid producer that contributes to attenuate the effects of high-fat diet-induced body weight gain and hepatic steatosis. Hong *et al.* also found a negative correlation between *Desulfovibrio* and liver triglycerides levels, fasting serum insulin, and proinflammatory cytokines in the liver and white adipose tissue.[30]

Trying to find some shared profiles between HSI and FIB−4 changes under a Mediterranean lifestyle intervention, the only group that shared characteristics was in group T3 of both indexes, where an increase in the *Sutterella* and *Faecalibacterium* levels was registered. *Faecalibacterium* is increased after the Mediterranean diet.[31] *Sutterella* (from the *Alcaligenaceae* family) has been previously related to liver steatosis and fibrosis,[32] but no explanation was given for its involvement. However, *Sutterella* has been classified as a proinflammatory bacterium and has been related to ulcerative colitis for its capacity of degrading IgA[33] and this could give some clues, although further research is needed.

Our study had some important limitations. First, we evaluated changes in gut microbiota according to clinical indexes of hepatic steatosis and fibrosis, then we only had a clinical suspicion of NAFLD and NASH but not a certain diagnosis by biopsy or elastography. On the other hand, all participants included in this analysis received Mediterranean diet advice and the changes observed in our sample may be difficult to replicate in different populations, with different dietary patterns.

Material and methods

Study design and participants

This substudy was conducted in the frame of the PREDIMED-Plus (Prevención con Dieta Mediterránea-Plus) study, a 6-year, multicentre, randomized clinical trial for primary prevention of cardiovascular disease (CVD) conducted in men aged 55–75 years and women aged 60–75 years with overweight or obesity (body mass index (BMI) ≥27 and <40 kg/m^2) and MS. Briefly, exclusion criteria included a previous history of CVD, any chronic medical condition, acute infectious processes, psychiatric disorders, alcohol, and drug abuse, institutionalization, use of specific medications, relevant recent weight loss, any food allergy to Mediterranean diet food ingredients and the use of antibiotics, probiotic or prebiotic supplements in the previous three months. Eligible participants were randomized either to an energy-reduced traditional Mediterranean diet, physical activity promotion, and behavioral support (intervention group) or an energy-unrestricted Mediterranean diet and usual care intervention (control group). All participants provided written informed consent, and the study protocol and procedures were approved according to the ethical standards of the Declaration of Helsinki by all the participating institutions. More details of the PREDIMED-Plus study protocol are fully described and available at http://predimedplus.com.[34] The study was registered at the International Standard Randomized Controlled Trial (ISRCT; http://www. Isrctn.com/ISRCTN89898870) with the number 89,898,870 and the date of 24 July 2014. For this descriptive substudy, we included 297 participants with blood and stool samples from two PREDIMED-Plus centers (Reus and Málaga), recruited between 2013 and 2016 and with all the variables needed for the calculation of the NAFLD/NASH indexes as well as quality sequences in the baseline and 1-year timepoints.

For the present analysis, participants were categorized into three different groups based on changes in the Hepatic Steatosis Index (HSI) or the Fibrosis−4 score (FIB−4) between baseline and after one year of intervention. For the calculation of the scores, pertinent analytical and anthropometric data were considered.

Hepatic steatosis and fibrosis scores

The equation for HSI is: HSI = 8×Alanine transaminase (ALT)/Aspartate transaminase (AST)+ body mass index (BMI) (+2 if type 2 diabetes yes, + 2 if female). HSI values below 30 rules out NAFLD; above 36 indicate high probabilities of

NAFLD. The positive predictive value (PPV) was 85.9% (83.9–87.6) and the negative predictive value (NPV) was 84.3% (82.1–86.2).[35] The equation for FIB−4 is: (FIB−4 Score = (age*xAST)/(number of platelets x√ALT). A FIB−4 value <1.45 indicates the absence of fibrosis with a NPV of 90%, between 1.45 and 3.25 is considered inconclusive, and >3.25 indicates fibrosis with a PPV of 65%.[36]

Anthropometric and biochemical variables

At baseline and 1-year follow-up, waist circumference (midway between the lowest rib and the iliac crest using an anthropometric tape), weight (using high-quality electronic-calibrated scales), and height (using a wall-mounted stadiometer) were measured. BMI was calculated as weight/height.^{2} (kg/m2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in triplicate using a validated semiautomatic oscillometer (Omron HEM−705CP, Kyoto, Japan). After overnight fasting, peripheral venous blood samples were collected from each participant, at both time points. Serum glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, ALT, AST, gamma-glutamyl transferase (GGT), and albumin were measured by standard laboratory enzymatic methods and following validated protocols.[37] Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula.[38] Glycated hemoglobin was measured by a chromatographic method.

Microbiota analysis

Stool samples were collected at baseline and 12-month timepoint and immediately stored at −80°C until posterior analysis. DNA extraction from stools was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined by absorbance at 260 nm (A260) and purity was estimated by determining the A260/A280 ratio in a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Ribosomal 16S rRNA gene sequences were amplified from DNA using the Ion 16S Metagenomics Kit (Thermo-Fisher Scientific Inc., Waltham, MA, USA). The kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. Libraries were created using the Ion Plus Fragment Library Kit (Thermo-Fisher Scientific). Barcodes were added to each sample using the Ion Xpress Barcode Adapters kit (Thermo-Fisher Scientific). Emulsion PCR and sequencing of the amplicon libraries were performed on an Ion 530 chip (Ion 530^{TM} Chip Kit) using the Ion Torrent S5^{TM} system and the Ion 510/520^{TM}/530^{TM} Kit-Chef (Thermo-Fisher Scientific) according to the manufacturer's instructions. After sequencing, the individual sequence reads were filtered using Ion Reporter Software V4.0 to remove low-quality and polyclonal sequences.

The open-source Quantitative Insights into Microbial Ecology QIIME2 (version 2020.8)[39] was used to analyze the data. Sequencing reads were denoised and clustered into amplicon sequence variants (ASVs) with DADA2, with adapted parameters for Ion Torrent data.[40] QIIME2 was also used for diversity analysis with the diversity plugin. Alpha diversity was assessed through different indexes (Shannon, Faith_pd, Pielou's evenness, and Observed features) and beta diversity was measured using UniFrac distances in its unweighted and weighted versions, and permutational multivariate analysis of variance (PERMANOVA) was used to look for differences in group compositions. Taxonomic assignment was performed through clustering with VSEARCH and the reference base Greengenes version 13_8 at 97% of identity. ASV counts and taxonomic information generated with QIIME2 were imported into the MicrobiomeAnalyst web tool,[41] where the data was filtered and the trimmed mean of M-values (TMM) normalization was performed. Differential abundance analyses were assessed with edgeR within MicrobiomeAnalyst with the default parameters of the developer.[42]

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin (PICRUSt2)[43] was used to predict metagenome function within QIIME2 with the DADA2 output. MetaCyc pathways[44] were normalized within QIIME2 and further analyzed with the open-source software STAMP (Statistical Analysis of Metagenomics Profiles) with Welch's t-test option.[45]

Statistical analysis

Normality was analyzed using the Kolmogorov–Smirnov test. Quantitative variables were expressed as mean±standard deviation (SD) for normally distributed data, as median±interquartile range (IQR) for non-normally distributed data, and percentages for categorical variables. The bivariate analysis was performed using paired Student's tests for continuous data or Wilcoxon test for non-normally distributed data. Differences across tertiles were evaluated through one-way analysis of variance (ANOVA) for continuous data or Kruskal – Wallis's test for non-normally distributed data. Categorical data were analyzed using Pearson's chi-square test. Student's t-test or Mann–Whitney U test were used to calculate differences between tertiles for numerical variables, Pearson's chi-square test was used for categorical variables.

In all cases, the null hypothesis was rejected for an alpha ≤ 0.05 for two tails. Statistical analysis was performed with SPSS (15.0 version for Windows: SPSS, Chicago, IL, USA).

Conclusion

NAFLD is a growing problem related to metabolic syndrome. Though the diagnosis requires imaging techniques and confirmation of a certain diagnosis only can be made by liver biopsy, some biochemical indexes may be useful in the clinical setting. In this study, we found a relationship between liver disease biochemical indexes changes and gut microbiota changes within a context of a Mediterranean lifestyle. In addition, we reported that this Mediterranean lifestyle intervention can modify these indexes in only one year, something that gives us a clue to continue exploring the importance of lifestyle interventions to fight non-communicable diseases. This comprehension may enable the development of future research to find strategies to modulate the gut microbiota in the integrated management of NAFLD.

Abbreviations

ALT	Alanine aminotransferase.
ANOVA	one-way analysis of variance.
AST	Aspartate aminotransferase.
ASVs	amplicon sequence variants.
BMI	body mass index.
CVD	cardiovascular disease.
DBP	Diastolic blood pressure.
FIB–4	fibrosis 4 score.
GGT	gamma-glutaril transferase.
HDL	high-density lipoproteins.
HIS	hepatic steatosis index.
IQR	Interquartile range.
ISRCT	International Standard Randomized Controlled Trial.
LDL	low-density lipoproteins.
MAFLD	metabolic liver disease.
MBOAT7	membrane-bound O-acyltransferase domain containing 7.
MS	metabolic syndrome.
NAFLD	nonalcoholic fatty liver disease.
NASH	nonalcoholic steatohepatitis.
NSF	non-suspected fibrosis.
NPV	negative predictive value.
PCR	polymerase change reaction.
PERMANOVA	permutational multivariate analysis of variance.
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin.
PNPLA3	patatin-like phospholipase domain-containing protein 3.
PPV	positive predictive value.
PREDIMED-Plus	Prevención con Dieta Mediterránea-Plus.
QIIME2	Quantitative Insights into Microbial Ecology.
SBP	systolic blood pressure.
SCFAs	short-chain fatty acids.
SD	standard deviation.
SF	suspected fibrosis.
SNPs	single nucleotide polymorphisms.
STAMP	Statistical Analysis of Metagenomics Profiles.
TM6SF2	transmembrane 6 superfamily member 2.
T2DM	type 2 diabetes mellitus.

Authors' contributions

JV, DC, MF, JV, JS-S, and FJ. T designed the study. AMG-P, PR-L, AA, LT-C, AA-S, MAM, AG, DB, JG-G, and MRB-L provided sample collection and processing. AMG-P, PR-L, IM-I, and FJ. T conducted the statistical analysis. JS-S, FJ. T and IM-I provided supervision. AMG-P, PR-L, IM-I, and FJ. T wrote the manuscript. All authors read and approved the final manuscript.

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Disclosure statement

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Data sharing

According to the data regulations and ethical considerations, the datasets generated and analyzed during the study are not publicly available, because our participants only provided their consent to the original team of investigators to use their data, so this information might compromise their consent for the study. Nevertheless, collaboration for data analyses can be requested by sending a letter to the PREDIMED-Plus steering Committee (predimed_plus_s-committee@googlegroups.com). All members of the PREDIMED-Plus Steering Committee will be notified of the request for their consideration.

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