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RESEARCH LETTER

Relations of gut liver axis components and gut microbiota in obese children with fatty liver: A pilot study

The prevalence of metabolic syndrome and its hepatic component — non-alcoholic fatty liver disease (NAFLD) — has increased alarmingly, paralleling the worldwide obesity epidemics. The pathophysiology of NAFLD is not clearly understood, but it has been proposed to be the result of multiple ''hits''[1]. A number of studies increasingly supports the pathogenetic role also of the gut microbiota (GM) both in NAFLD onset and progression. In this respect, GM would exert its noxious effects through the dysfunction of the gut-liver axis (GLA), which includes some or all of the following components: increased intestinal permeability (IP), endogenous ethanol (ETOH) and systemic endotoxin (LPS) concentrations [2,3].

As these players have hitherto not been simultaneously investigated in the same patient [reviewed in reference 4], the aim of our study was to explore the possible existence of reciprocal influences of several GLA components and GM composition in the same group of well characterized obese (Ob) children with and without fatty liver compared to normal-weight (NW) control peers.

We studied 10 Ob Italian children, consecutively recruited at our center after parental agreement and written informed consent. The inclusion criteria were age 8—13 years, and a body mass index (BMI) > 97th percentile. Six non-Ob and non-overweight (BMI < 85th percentile), healthy normal-weight (NW) controls with normal anthropometric, clinical, laboratory and ultrasonographic (US) hepatic parameters and no other associated diseases were recruited among patients of the Pediatric Surgery Section listed for elective minor surgery.

Lifestyle including eventual medications or alcohol exposition and total daily fructose and caloric intake and food preferences were investigated by multiple-choice questionnaires [5]. Weight, height, BMI values and percentiles, waist circumference (WC) percentiles were recorded and obtained by trained staff members using calibrated instruments and standardized methods [5]. The patients were finally clustered into NW controls ($n = 6$) and 2 groups of 5 subjects each: Ob without fatty liver, Ob with fatty liver.

Main standard laboratory tests performed and respective values are shown in Table 1. Abdominal US examination to establish the presence/absence of hepatic steatosis was performed as described previously [6]. Common causes of fatty liver ± hypertransaminasemia other than obesity related fatty liver were excluded by appropriate tests [7].

IP was assessed by the high-performance liquid chromatography analysis of lactulose (L) and mannitol (M) urinary values 5 hours after sugars ingestion (L/M ratios normal values < 0.03) [8]. Endogenous serum alcohol was measured using the alcohol dehydrogenase (ADH) enzymatic method [9]. Serum LPS levels were assayed using the limulus amebocyte lysate (LAL) (Endochrome K; charge: C4452E) obtained from the arthropod *Limulus Polyphemus* (Charles River Laboratories (CRIVER), Inc., Massachusetts, USA) [10]. Total DNA was extracted from approximately 0.15 g of stool using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany); PCR amplification was performed with the primers 5′-CCTACGGGNGGCWGCAG-3′ (forward) and 5′-GACTACHVGGGTATCTAATCC-3′ (reverse) [11], targeting the hypervariable V3 and V4 regions of the *16S rRNA* gene. Each PCR reaction was assembled according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). Sequencing libraries were pooled to an equimolar amount of each index-tagged library, including the Phix Control Library. Pooled samples were sequenced on an Illumina MiSeq platform in 2×300 paired-end format. The generated raw sequence files (FASTQ files) were analyzed for taxonomic assignment with the Greengenes database [12] and sequence reads were grouped into operational taxonomic units (OTUs) at a sequence similarity level of 97%.

The study was carried out in accordance with the ethical principles of the declaration of Helsinki 2013 and approved by the local institutional ethics committee. The statistical analysis was performed using GraphPad Prism 7 software (CA, USA). The distribution model of data series was assessed with the Shapiro—Wilk test. The student's *t*-test and an analysis of variance (ANOVA) with the Bonferroni correction were used to compare the mean values between groups and to

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evaluate differences among groups, respectively. Fisher's exact test was used in the analysis of categorical data with contingency tables. Pearson's test was used to measure the linear relationship between two variables.

As shown by data summarized in Table 1:

- IP was more frequently pathologic in the Ob patients vs. NW controls (6/10 vs. 0/6; $P = 0.04$), with a prevalence of abnormal IP tests in subjects with fatty liver ($n = 4/5$) vs. Ob subjects without fatty liver ($n = 2/5$) (P = NS) and NW controls ($n = 0/6$) ($P = 0.01$). Furthermore, IP values were significantly correlated with systolic blood pressure, WC, and homeostasis model assessment—insulin resistance, HOMA-IR;
- serum LPS values were significantly higher in Ob patients vs. NW peers, with higher values tending to cluster in patients with fatty liver (3/5) compared to Ob patients without fatty liver (1/5) (P NS);
- although ETOH did not differ significantly between groups, Ob patients comprised those with the highest values of ETOH, mostly in the group with fatty liver (3/5) vs. that without fatty liver (1/5) (pNS);
- GM study showed that *Bacteroidetes* (48,5% in NW vs. 52,6% in Ob) and *Firmicutes* (41,4% in NW vs. 35,3% in Ob) were the most representative phyla, followed by *Proteobacteria* (5,4% NW vs. 8,0% Ob) and *Actinobacteria* (1,9% NW vs. 1,8% Ob) with no statistically significant differences among Ob patients and NW controls.

The phylum *Proteobacteria* analyzed per class however showed that G*ammaproteobacteria* (0,9% NW vs. 4,6% OB) was prevalent in Ob children ± fatty liver ($P < 0.05$), with an AUC (0.89) quite accurate for distinction between obese patients and NW subjects. This class correlated well with BMI and daily calories and fructose intake ($r = 0.70$, $P = 0.01$; $r = 0.54$, $P = 0.04$; and $r = 0.57$, $P = 0.02$, respectively).

The preliminary analysis per genera moreover showed that the H2-producing bacteria *Bacteroides* and *Prevotella* were present mostly in fatty liver patients (mean gene counts: 175,367.2 ± 183,717.5 in obese with fatty liver vs. 132.6 ± 120.99 in obese without fatty liver vs. 40.4 ± 14.32 NW; $P < 0.05$):

- IP showed a statistically significant correlation with endotoxinemia ($r = 0.56$, $P = 0.02$). Both parameters were correlated with the steatosis-related genus $\log_e$ *Prevotella* ($r = 0.48$, $P = 0.04$; and $r = 0.69$, $P = 0.003$, respectively);
- ETOH and LPS serum values were significantly correlated with $\log_e$ *Proteobacteria* and $\log_e$ *Gammaproteobacteria* ($r = 0.85$ $P = 0.01$; $r = 0.69$ $P = 0.02$; $r = 0.80$ $P = 0.01$; $r = 0.74$ $P = 0.01$ respectively). Moreover, blood LPS showed a sig-

Table 1 Demographic, anthropometric, hepatometabolic and gut liver axis components characterization.

	NW controls	All Ob pts	Ob [St−]	Ob [St+]
Number of patients	6	10	5	5
Gender (M/F)	3/3	4/6	2/3	2/3
Age (years)[a]	10.60 ± 3.10	11.5 ± 2.28	10.75 ± 2.59	12.26 ± 1.9
BMI[b]	18.36 ± 2.54	28.9 ± 4.45	26.63 ± 3.54	31.17 ± 4.38
WC (cm)[b]	65.00 ± 10.05	84.62 ± 12.57	77.4 ± 11.41	91.88 ± 9.69
Systolic BP mmHg[e1]	106.8 ± 8.4	123.8 ± 10.8	119.4 ± 12.6	128.2 ± 7.5
Diastolic BP mmHg	66.0 ± 5.47	62.9 ± 7.07	67.0 ± 4.47	65.0 ± 5.0
Glucose blood levels (mg/dL)[f1]	79.20 ± 1.30	89.5 ± 9.22	87.40 ± 0.64	91.60 ± 11.06
HOMA IR[e2]	1.91 ± 0.16	3.63 ± 2.11	2.58 ± 1.58	4.67 ± 2.19
ALT (U/L)	27.2 ± 6.30	38.5 ± 16.39	27.4 ± 6.58	49.60 ± 15.92
AST (U/L)	23.80 ± 3.49	30.8 ± 13.38	24.20 ± 4.60	37.40 ± 16.52
Caloric daily intake (kcal)	1200.4 ± 110.3	1685.3 ± 580.0	1438.4 ± 170.7	1932.3 ± 758.3
Total COH daily intake (g)	136.25 ± 45.45	184.81 ± 64.75	157.97 ± 30.77	211.66 ± 81.76
Sodium daily intake (mg)	626.20 ± 386.15[d]	1584.8 ± 960.5[d]	1473.2 ± 317.5	1696.4 ± 1390.0
Fructose daily intake (mg)	3.82 ± 1.64[c]	13.73 ± 11.52	8.19 ± 3.71[c]	20.66 ± 14.89[c]
Total fiber daily intake (g)	5.10 ± 2.39[c,d]	11.52 ± .87[d]	9.61 ± 1.38[c]	13.44 ± 6.50[c]
L/M ratio[e]	0.0171 ± 0.001	0.031 ± 0.029	0.019 ± 0.026	0.042 ± 0.030
Serum LPS (EU/mL)[d,f]	0.022 ± 0.003[d]	0.048 ± 0.028[d]	0.039 ± 0.013	0.058 ± 0.038
Serum ethanol (mmol/L)	15.0 ± 4.0	23.0 ± 13.0	17.0 ± 3.0	30.0 ± 17.0

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: Body mass Index; COH: carbohydrates; F: female; HOMA IR: homeostatic model assessment insulin resistance; M: male; NW: normal weight; Ob[St+]: obese with hepatic steatosis; Ob[St−]: obese without hepatic steatosis; WC: waist circumference.

[a] Age variability among the 3 groups, evaluated through ANOVA test, multiple comparisons ($P = 0.57$)

[b] BMI, WtHR and WC differences between NW and Ob were among inclusion criteria and showed a statistically significant difference (P value = 0.01; 0.02; and 0.02, respectively)

[c] ANOVA test, multiple comparison: statistical signif. difference only for total fiber, fructose daily intake ($P < 0.05$)

[d] t-test performed between NW and All obese groups showed statistical significant differences only for intake of total fiber, sodium. ($P = 0.01$, $P = 0.05$ respectively) and blood LPS levels (P value < 0.05).

[e] Pearson's test correlations between L/M ratio and ([e1]) Systolic blood pressure ($r = 0.52$; $P < 0.05$) and ([e2]) HOMA IR ($r = 0.49$; $P < 0.05$).

[f] Pearson's test correlation between Serum LPS and ([f1]) Glucose blood levels (r = 0.68; $P < 0.05$).

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