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MULTIPLE GUT-LIVER AXIS ABNORMALITIES IN OBESE CHILDREN WITH AND WITHOUT HEPATIC INVOLVEMENT.

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MULTIPLE GUT-LIVER AXIS ABNORMALITIES IN OBESE CHILDREN WITH AND WITHOUT HEPATIC INVOLVEMENT.

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ABSTRACT

Background: Gut-liver axis (GLA) dysfunction appears to play a role in obesity and obesity-related hepatic complications (HC).

Aim: This study sought to concurrently explore several GLA components in a pediatric obese population with/without liver disease.

Methods: 32 children (mean age 11.2 years) were enrolled: 9 normal weight (NW) controls and 23 obese (OB+) patients. Of the 23 OB(+) patients, 13 did not have steatosis (ST-), and 10 did have steatosis (ST+) [associated (n=7) or not (n=3) with hypertransaminasemia (ALT +/-)]. Subjects were characterized using auxologic, ultrasonographic, and laboratory parameters. Moreover, a glucose hydrogen breath test (H2BT) was performed to test for small intestinal bacterial overgrowth (SIBO), a urinary lactulose/mannitol ratio (LMR) was obtained to assess intestinal permeability (IP), and tests for transaminases, blood endogenous ethanol endotoxin and fecal calprotectin were also conducted.

Results: 11 out of 23 OB(+) patients exhibited pathological LMR ($p<0.05$), with values paralleling the grade of liver involvement [NW < OB(+) < OB(+)ST(+)ALT(-) < OB(+)ST(+)ALT(+) ($p<0.05$)]. LMR was significantly correlated with ethanolemia ($r=0.38$ $p=0.05$) and endotoxemia ($r=0.48$ $p=0.015$) levels. LMR was a risk factor ($p<0.002$) for the development of steatosis. SIBO was present only in obese patients. Fecal calprotectin levels were within normal limits in all subjects.

Conclusions: IP, endogenous ethanol and systemic endotoxin levels appear to be associated with GLA dysfunction in obesity and its HC. Pending further results to establish potential causative roles of these factors, the modulation of the GLA appears to represent a possible target for the prevention and treatment of these conditions.

INTRODUCTION

The global pandemic of pediatric obesity is considered to be a substantial public health problem both in industrialized countries and in some developing countries (1-3). Equally worrying are its hepato-cardio-metabolic (HCM) complications (4), including nonalcoholic fatty liver disease (NAFLD), the most common cause of chronic liver disease at all ages (5). Its exact prevalence in children is not yet precisely known, although data have suggested that it affects approximately 10% of the pediatric population; the estimate increases to over 70% in obese children (6). This scenario represents how difficult it is today to prevent and effectively treat pediatric obesity and its HCM

complications with only lifestyle changes, such as a healthy diet and increased physical activity. Therefore, there remains a need to develop innovative therapeutic approaches based on the still poorly understood pathomechanisms underlying obesity and its complications.

This study focuses on alterations in the gut-liver axis (GLA), which increasingly appear to play a major role in obesity-associated health conditions (7). Numerous interrelationships among several components within this axis are finely adjusted by the intestinal mucosa, which often loses its barrier function in obese subjects with consequent alterations of intestinal permeability (IP). Here, we provide the first report of simultaneous testing for correlations between abnormal IP, bacterial products (ethanol and endotoxin), intestinal inflammation, and small intestinal bacterial overgrowth (SIBO) in a pediatric obese population *vs.* normal-weight healthy controls.

MATERIALS AND METHODS

Study population

Thirty-two Italian children (13 females and 19 males; mean age 11.2 ± 2.5 years) were recruited in this study at our teaching hospital and at local health service outpatient obesity clinics after parental agreement with written informed consent was obtained. Inclusion criteria were age between 7 and 14 years and body mass index (BMI) > 97th percentile for obese patients. Non-obese and non-overweight (BMI < 85th percentile) children with normal laboratory, anthropometric, clinical, and hepatic ultrasonography (US) parameters and no other known associated diseases were recruited as healthy controls. The presence of other comorbidities was considered to be an exclusion criterion. The study was approved by the institutional ethics committee.

Anthropometric measurements and clinical assessment of patients

For each patient, weight, height, BMI crude values and percentiles, waist circumference (WC) percentiles and the number of centimeters exceeding the 95th percentile, waist to height ratio (WtHR), blood pressure, and striae rubrae/stretch marks were recorded. The anthropometric measurements were obtained by trained staff members using calibrated instruments and standardized methods (8). The WCs were evaluated according to European percentiles (9). According to the value of the age- and gender-adjusted BMI percentile, the patients were allocated into 2 groups: cases (n=23 obese patients; 10F, 13M) and controls (n=9 normal-weight subjects; 3F, 6 M) (**Table 1**).

Ultrasound and laboratory evaluation

Liver and metabolic functions were assessed through laboratory tests (serum levels of transaminases, glucose, insulin, and insulin resistance [homeostatic model assessment (HOMA)].

All patients and controls underwent hepatic US to establish the presence/absence of steatosis. US examination was performed using a US apparatus (Hitachi Aloka, Wallingford, Connecticut, USA) with a convex pediatric probe. Liver steatosis (bright liver) was categorized as absent, mild, moderate or severe according to US criteria (10,11). As shown in **Table 1**, according to transaminase levels and US data, the obese patient group included:

- Patients without hepatic steatosis [OB(+)/ST(-)] and without hypertransaminasemia [OB(+)/ALT(-)] (n=12) (Subgroup IIa)

- Patients with hepatic steatosis [OB(+)/ST(+)] (n=11) [3 without hypertransaminasemia [OB(+)/ST(+)/ALT(-)] and 8 with hypertransaminasemia [OB(+)/ST(+)/ALT(+)](subgroup IIb and IIc)

Endotoxin, ethanol and fecal calprotectin assay

The endotoxin plasma concentration was measured using a commercially available endpoint limulus amebocyte lysate assay (Charles River Laboratories International, USA) with a concentration detection range of 0–1200 endotoxin units/L following the manufacturer's instructions (12).

Blood alcohol levels were determined using the alcohol dehydrogenase enzymatic method described by Bonnichsen and Lundgren (detection limit: 0.0125 µmol/µl) (13, 14).

Fecal calprotectin levels of stool samples kept on ice were measured with an ELISA kit (Kit Calprest®, Eurospital, Trieste, Italy) with a cut-off of 50 mg/kg of stool for a positive result.

Intestinal Permeability

IP was assessed by high performance liquid chromatography (HPLC) analysis of lactulose (L) and mannitol (M) urinary levels. Patients followed a lactulose- and mannitol-free diet for 24 h before the test. After an overnight fast, they voided their bladders and ingested a solution containing 5 g of lactulose and 1 g of mannitol in 120 mL of water. Urine samples were collected during the subsequent 5 h in refrigerated tubes. Total urine volume was measured, and a 10-mL aliquot was stored at −20˚C until HPLC analysis. The fractional excretion of lactulose was calculated from the following ratio: g lactulose excreted/g lactulose ingested. The number of grams of lactulose excreted was obtained from the equation: g/L lactulose × liters of urine. The same calculation was performed for mannitol. The results are expressed as the ratio of the fractional excretion of lactulose divided by the fractional excretion of mannitol (L/M ratio) (15,16).

Glucose Hydrogen (H2) Breath Test (BT)

Subjects fasted overnight (12 h) and during the H2BT. Subjects had not taken antibiotics during the previous 5 months and were asked to avoid eating fiber-containing foods during the previous

evening to avoid interference with H2 excretion. Exercise was not allowed for at least 2 h before and during the test. End-expiratory breath samples were obtained at 30-min intervals before and for 2 h after an oral load of 2 g/kg body weight (max 50 g) of D-glucose 33% solution. The results were expressed in parts per million (ppm). The test was considered positive when basal H2 values were > 20ppm or showed an increase of 10-12 ppm *vs*. basal values during the first 90 min following glucose ingestion for jejunal colonization and 110-120 min later for ileal colonization (17). H2 concentrations of expiratory gas samples were measured using an H2 portable apparatus (Gastrolyzer Bedfont 2, Kent, England). The minimum detectable H2 concentration was 1 ppm.

Statistical analysis

Statistical analysis was performed with STATA 12 (Stata Corp, Texas, USA). The differences in several variables among the four groups were assessed using the ANOVA test with Bonferroni correction because they were considered to be parametric. The difference between the two groups was calculated by Student's t-test. The correlations between GLA continuous variables were evaluated through Pearson's test because they were not parametric.

RESULTS

ANTHROPOMETRIC, CLINICAL AND HEPATOMETABOLIC CHARACTERISTICS

Anthropometric, clinical and laboratory data from the 23 enrolled obese patients are shown in **Table 1**. The averages of the parameters taken into consideration were significantly different ($p < 0.05$ ANOVA) for the BMI, WC, WtHR and alanine aminotransferase (ALT) groups. An increase in these values paralleled obesity measurements and, generally, the severity of hepatic involvement [OB(-)/ST(-)/ALT(-) < OB(+)/ST(-)/ALT(-) < OB(+)/ST(+)/ALT(-) < OB(+)/ST(+)/ALT(+)].

GUT-LIVER AXIS ABNORMALITIES

The results of the GLA tests in subgroups are summarized in **Table 2**. None of the controls were positive for *SIBO*, whereas obese subjects were positive in 50% of cases ($p = 0.006$). The group of obese patients with hepatic steatosis showed a moderate prevalence of SIBO vs. those without steatosis (p NS).

Intestinal permeability

LMR correlated significantly with WC, WTHR and systolic BP (Pearson's $r = 0.34$; 0.37; 038, respectively; $p < 0.05$).

The cut-off of the normal LMR values was calculated on the basis of the 90th percentile of the control group (0.026). Eleven of the 23 obese patients had a value of LMR above the upper limit; 8 of these had abnormal liver echogenicity.

As shown in **Table 2** and **Figure 1 Panel I**, LMR tended to increase in obese patients compared to normal weight controls ($p > 0.05$), with values that followed the trend of the increasing hepatic involvement from OB/ST(-)/ALT(-) to OB/ST(+)/ALT(-) and then to OB/ST(+)/ALT(+) ($p < 0.05$ ANOVA).

Logistic regression (**Figure 2**) showed that an altered L/M ratio was a risk factor (OR> 1) for the development of hepatic steatosis, with the probability of developing steatosis paralleling LMR values.

Endotoxemia, ethanolemia and fecal calprotectin

Endotoxemia and ethanolemia values tended to be higher in patients with more severe hepatic disease, but did not reach statistically significant differences among groups (ANOVA with Bonferroni correlation, p NS). The highest individual values of ethanolemia, endotoxemia and fecal calprotectin were all found in obese patients (**Figure 1**).

Ethanolemia ($p=0.05$ $r=0.38$) and endotoxemia ($p=0.015$ $r=0.48$) levels were positively correlated with LMR (**Figure 3**).

Fecal calprotectin values were within the normal range in all studied children (**Table 2; Figure 1 Panel 4**). The presence or absence of SIBO did not appear to influence intestinal permeability, fecal calprotectin, or levels of endotoxemia and ethanolemia (p NS).

DISCUSSION

In contrast to previous studies (18,19), *our data are the first obtained by concurrently examining several aspects of the GLA* in the same series of obese pediatric patients with and without liver involvement. The results corroborate the view that GLA abnormalities may represent an emerging risk factor for the onset of both conditions during childhood (20). The *most significant finding* of our study is the evidence of increased intestinal permeability in obese children, which paralleled the degree of hepatic involvement and predicted the development of steatosis. In addition, our study supports the possible role of endogenous ethanol in hepatic function via intestinal injury. ETOH produced by alcohol-producing bacteria of the intestinal microbiota has been proposed to be a pathogenic mechanism underlying the incidence of increased IP (21), most probably through a direct influence on tight junctions. The alteration of IP, in turn, is also strictly interconnected with

levels of circulating endotoxin (22), a potent inducer of hepatic inflammation in obese patients with or without NAFLD (23).

Despite the limited data available in the literature (which comprises only adult studies) suggesting a link between SIBO and the origin and subsequent worsening and transition from NAFLD into NASH (24, 25), we did not find correlations between SIBO and enzyme liver changes in our pediatric sample. One explanation may be that in most of our obese patients with liver abnormalities, hepatic echogenicity was mild. Another possible explanation is that some children present SIBO colonization by methane-producing bacteria, producing negative H2BT test results (false negatives) (26). Finally, the higher correlation with SIBO reported in adults has also been related to their greater frequency of having conditions that favor bacterial overgrowth, such as the use of pump inhibitor medications, gastric achlorhydria, and intestinal stasis due to longstanding diabetes (25); these conditions are rarely present in pediatric patients.

Although data from the literature have shown frequent chronic and systemic micro-inflammation in obese adult subjects with and without HC (27,28), fecal calprotectin levels were never abnormal in our pediatric study groups and did not correlate with alterations in the other components of the GLA. Our results are consistent with those of a pilot study in which the levels of fecal calprotectin, as well as alterations in intestinal permeability, were not statistically associated with the degree of obesity in a group of adult patients (29).

This study has some *limitations*: the small number of patients, their young age, and the mild degree of obesity-related liver and metabolic impairment. These factors might partially explain the lack of statistical significance in some of the studied parameters. Moreover, hepatic involvement was not proven by biopsy but was inferred on the basis of the presence of hepatic steatosis, as measured by US and serum liver enzyme elevation.

Future larger studies, including an analysis of serum levels of zonulin, fecal metabolomics, and microbiota signatures, should also be carried out to better characterize the gut liver-axis in this population.

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Guarantor of the article: Pietro Vajro

Salvatore Guercio Nuzio, Martina Di Stasi Luca Pierri performed most of the clinical studies on patients and controls;

Jacopo Troisi, Pierpaolo Cavallo, Massimo Boffardi, Doreen Ziegenhardt and Ina Bergheim managed several laboratory tests exploring the gut-liver axis;

Antonella Bisogno, Federica Belmonte, Marina Tripodi, Dario Di Salvio, Grazia Massa, Claudia Mandato collected data and biological samples and contributed to the thorough literature search;

Riccardo Savastano and Marco Poeta analysed statistical data;

Pietro Vajro and Salvatore Guercio Nuzio designed the research study and wrote the paper.

All authors contributed to improve specific parts of the manuscripts and approved the final agreed version of the manuscript.

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LEGENDS TO FIGURES

Figure 1:

Lactulose/Mannitol (L/M) ratio, blood endotoxin, ethanol and fecal calprotectin levels in our study population. * = $p<0.05$

Figure 2:

Logistic regression of Lactulose/Mannitol (L/M) ratio and Hepatic steatosis.

OR=5.97 p=0.002 CI [3.80 e^{08} – 9.40 e^{38}]

Figure 3:

Correlation between intestinal permeability, ethanol (panel A) and endotoxin levels (panel B).

Table 1. Demographic, anthropometric and hepatometabolic characterization

	TOTAL Group I	Sub-Group IIa	Sub-Group IIb	Sub-Group IIc	*TOTAL Group II*
	NW	OB/ST(-)/ALT(-)	OB/ST(+)/ALT(-)	OB/ST(+)/ALT(+)	*OB*
N of pts.	9	12	3	8	*23*
Boys/Girls	6/3	7/5	2/1	4/4	*13/10*
Age (yrs)	10.8±2.2	11.4±2.2	11.5±2.0	10.7±1.9	*11.4±2.0*
BMI	16.3±1.3*¥	27.9±4.4¥	27.6±4.1*	29.5±5.8*	27.6±4.9*
WC (cm)	59.8±7.5*¥	84.3±10.6¥	83.6±10,4*	85.3±11.5*	84.4±12.9*
WtHR	0.41±0.03*¥	0.56±0.05¥	0.54±0.05*	0.54±0.03*	0.57±0.05*
ALT (U/L)	11.2±2.6*¥	39.3±27.6¥	23.3±6.3*	28.6±10.7*	66.8±30.6*
AST (U/L)	25±3.3	28.65±10.2	23.1±3.9	34±26	34.2±10.2
Homa IR	2.4±1.3	3.7±2.6	2.8±1.3	3.7±1.7	4.8±3.7

Abbreviations. BMI: Body mass Index; WtHR: Weight to Height ratio; WC: waist circumference; HOMA IR: Homeostatic model assessment insulin resistance; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HOMA: homeostatic model assessment; NW: normal weight group; OB: all obese children; OB/ST(-)/ALT(-): obese group without steatosis; OB/ST(+)/ALT(-): obese group with steatosis/without hypertransaminasemia; OB/ST(+)/ALT(+): obese group with steatosis and hypertransaminasemia.

Statistical analysis were performed with ANOVA test with Bonferroni correction among four groups of patients. T test was performed between group I and II Total. It was statistically significant for BMI, WC, WtHR, ALT . ¥ = $p<0.05$.

Table 2. Urinary L/M ratio, Endotoxemia, Ethanolemia and Fecal Calprotectin levels

	Group I	Group II	Group IIa	Group IIb	Group IIc
	NW	OB	OB/ST(-)/ALT(-)	OB/ST(+)/ALT(-)	OB/ST(+)/ALT(+)
	9	23	12	3	8
LMR	0.017±0.005*¥	0.039±0.034¥	0.022±0.026*	0.055±0.015*	0.059±0.039*
Endotoxemia	0.049±0.012	0.054±0.030	0.049±0.012	0.038±0.019	0.068±0.044
Ethanolemia	0.016±0.070	0.020±0.012	0.018±0.005	0.016±0.007	0.025±0.018
Calprotectin	22.74±4.75	27.15±13.90	25.10±16.57	28.70±12.27	29.74±11.48

ANOVA with Bonferroni correction; * = p<0,05;

T-test was performed between Group I and Group II; ¥ = p>0,05

Legend: L/M: Lactulose/Mannitol; OB/ST(-)/ALT(-): obese group without steatosis; OB/ST(+)/ALT(-): obese group with steatosis without hypertransaminasemia; OB/ST(+)/ALT(+): obese group with steatosis and hypertransaminasemia.

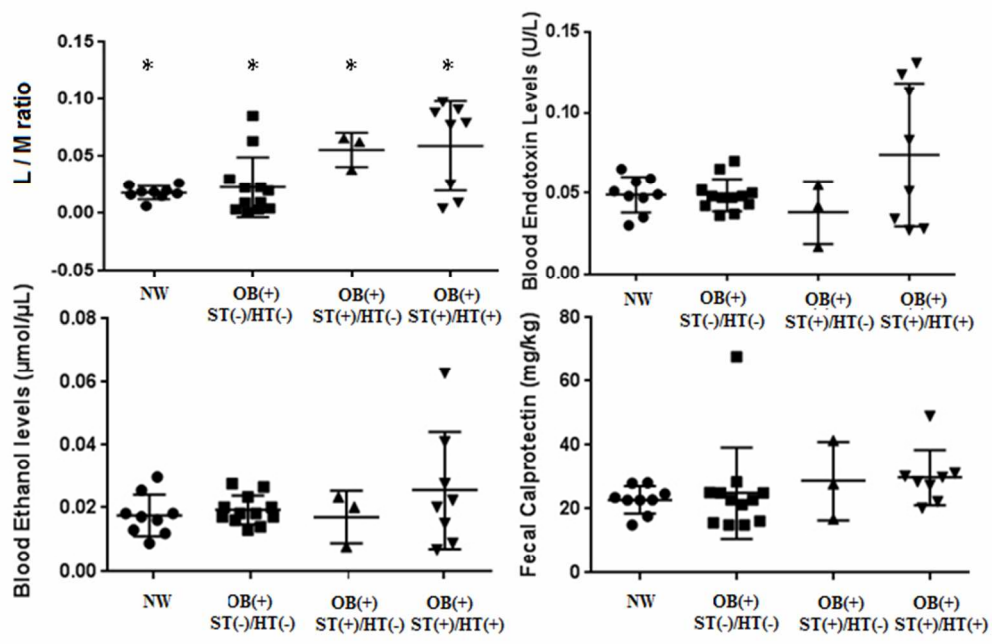

Figure 1:
Lactulose/Mannitol (L/M) ratio, blood endotoxin, ethanol and fecal calprotectin levels in our study population. * = $p<0.05$

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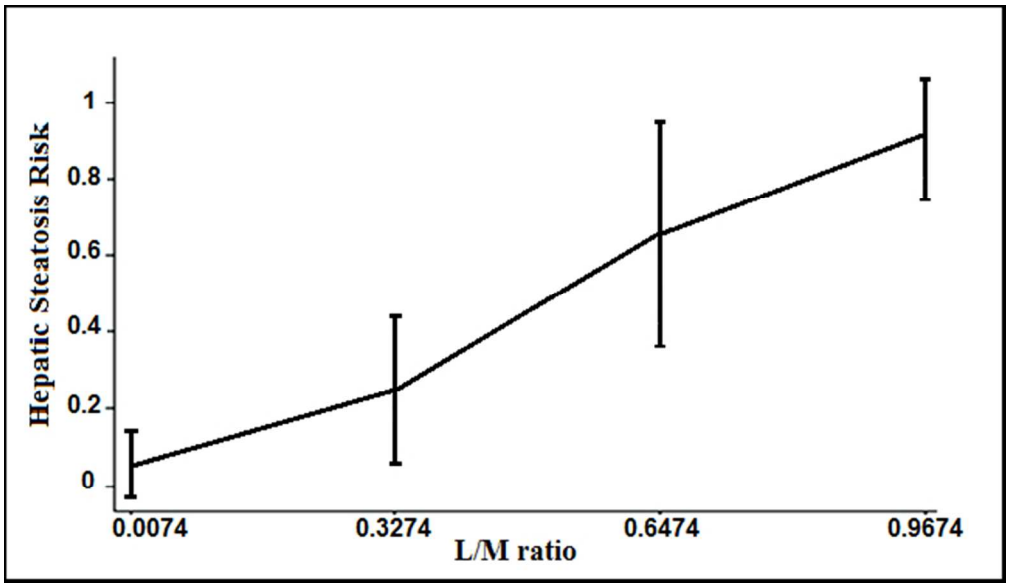

Figure 2:
Logistic regression of Lactulose/Mannitol (L/M) ratio and Hepatic steatosis.
OR=5.97 p=0.002 CI [3.80 e08 – 9.40 e38]

170x98mm (96 x 96 DPI)

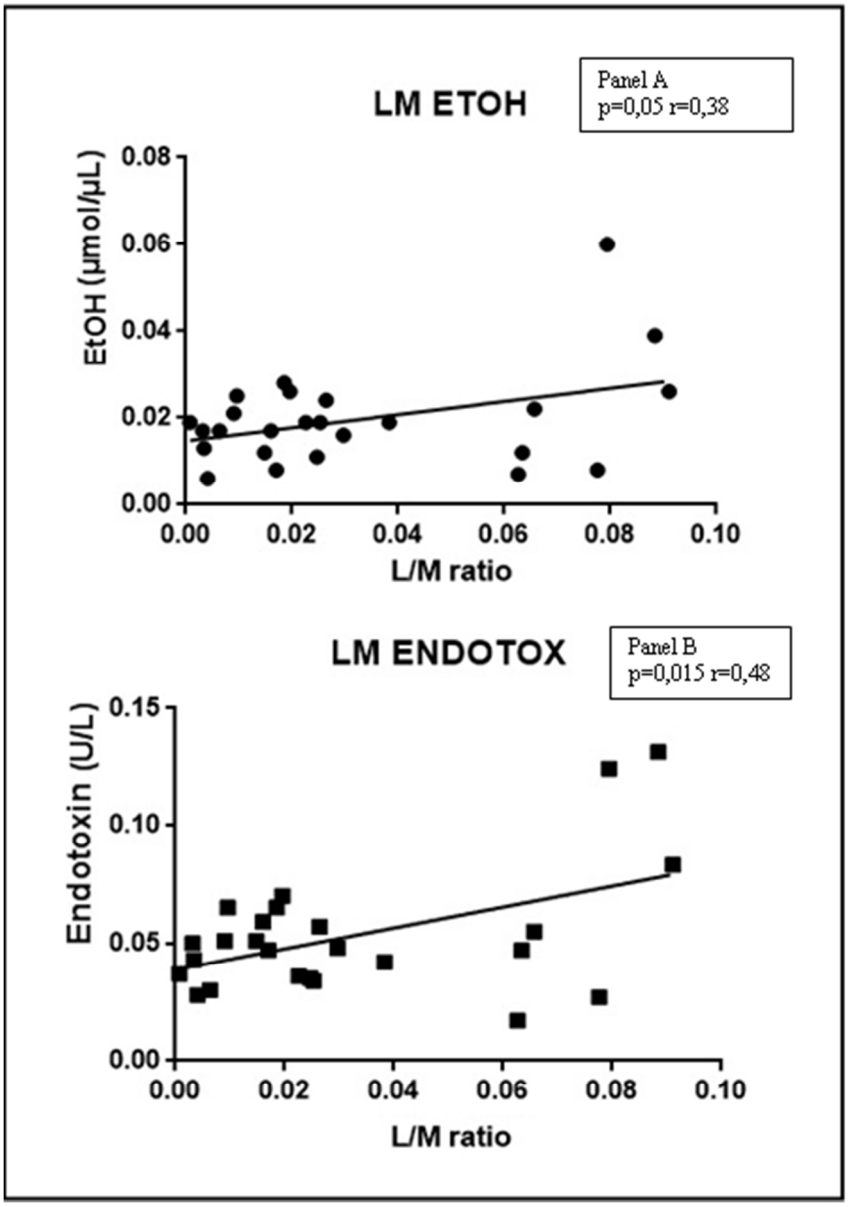

Figure 3:
Correlation between intestinal permeability, ethanol (panel A) and endotoxin levels (panel B).

118x169mm (96 x 96 DPI)

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