See corresponding editorial on page 3.

Gut permeability is related to body weight, fatty liver disease, and insulin resistance in obese individuals undergoing weight reduction[1,2]

*Antje Damms-Machado,[3,4] Sandrine Louis,[4] Anna Schnitzer, Valentina Volynets, Andreas Rings, Maryam Basrai, and Stephan C Bischoff**

Department of Nutritional Medicine, University of Hohenheim, Stuttgart, Germany

ABSTRACT

Background: Obesity and associated metabolic disorders are related to impairments of the intestinal barrier.

Objective: We examined lactulose:mannitol (Lac:Man) permeability in obese individuals with and without liver steatosis undergoing a weight-reduction program to test whether an effective weight-loss program improves gut barrier function and whether obese patients with or without liver steatosis differ in this function.

Design: Twenty-seven adult, nondiabetic individuals [mean ± SD body mass index (BMI; in kg/m^2): 43.7 ± 5.2; 78% with moderate or severe liver steatosis] were included in the follow-up intervention study (n = 13 by month 12). All patients reduced their weight to a mean ± SD BMI of 36.4 ± 5.1 within 12 mo. We assessed barrier functions by the oral Lac:Man and the fecal zonulin tests. Insulin resistance was assessed by the homeostatic model assessment index (HOMA), and liver steatosis by sonography and the fatty liver index (FLI).

Results: The Lac:Man ratio and circulating interleukin (IL) 6 concentration decreased during intervention from 0.080 (95% CI: 0.073, 0.093) to 0.027 (95% CI: 0.024, 0.034; $P < 0.001$) and from 4.2 ± 1.4 to 2.8 ± 1.6 pg/mL ($P < 0.01$), respectively. At study start, the Lac:Man ratio was higher in patients with moderate or severe steatosis than in those without any steatosis ($P < 0.001$). The Lac:Man ratio tended to correlate with HOMA (ρ = 0.55, P = 0.052), which correlated with FLI (ρ = 0.75, $P < 0.01$). A multiple-regression analysis led to a final model explaining FLI best through BMI, waist circumference, and the Lac:Man ratio.

Conclusions: Intestinal permeability is increased in obese patients with steatosis compared with obese patients without. The increased permeability fell to within the previously reported normal range after weight reduction. The data suggest that a leaky gut barrier is linked with liver steatosis and could be a new target for future steatosis therapies. This trial was registered at clinicaltrials.gov as NCT01344525. *Am J Clin Nutr* 2017;105:127–35.

Keywords: obesity, liver steatosis, insulin resistance, intestinal permeability, gut barrier

INTRODUCTION

The intestinal barrier is thought to play a major role in a variety of diseases, including gastrointestinal disorders such as inflammatory bowel diseases, irritable bowel syndrome, and gastrointestinal infections (1, 2). Metabolic disorders such as obesity, alcoholic and nonalcoholic fatty liver disease (NAFLD),[5] and insulin resistance have also been linked to the intestinal barrier (1–3). In all of these diseases, the barrier function seems to be impaired, resulting in enhanced translocation of commensal intestinal bacteria or bacterial products such as lipopolysaccharides to the host via the portal vein, the liver, and finally to the systemic level. Lipopolysaccharide is a most powerful inducer of tissue inflammation by activating the toll-like receptor 4 expressed on inflammatory cells such as monocytes, macrophages, and mast cells (4, 5). We and others have shown that lipopolysaccharide translocation causes liver inflammation and liver steatosis in experimental models of obesity (6, 7) and in obese humans (8–10). Because NAFLD is regarded as an early indicator of insulin resistance and the metabolic syndrome, it is tempting to speculate that such metabolic disorders could be at least in part induced by gastrointestinal barrier impairment (11–13).

In the present study, we therefore examined markers of gastrointestinal barrier function in obese individuals with and without liver steatosis. Two objectives were addressed. First, we aimed to understand whether changes in gastrointestinal barrier function are either related to obesity or to steatosis and other

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[2] Supplemental Figure 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[3] Present address: Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany.

[4] These authors contributed equally to the manuscript.

*To whom correspondence should be addressed. E-mail: bischoff.stephan@uni-hohenheim.de.

[5] Abbreviations used: AIC, Akaike information criterion; FLI, fatty liver index; GGT, γ-glutamyltransferase; Lac:Man, lactulose:mannitol; NAFLD, nonalcoholic fatty liver disease; PEG, polyethylene glycol; TJ, tight junction; WC, waist circumference.

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metabolic changes. Second, we wanted to know whether changes in gastrointestinal barrier function are reversible when body weight is reduced. We assessed the barrier by 2 established noninvasive means, the oral lactulose:mannitol (Lac:Man) permeability test and the fecal zonulin test.

METHODS

Study design

The study was performed in obese patients who underwent a weight reduction program for medical reasons. All patients who were included fulfilled the eligibility criteria within a given time and agreed to participate in the study. The patients were followed up for 1 y. During this time, permeability markers and other parameters were analyzed (**Supplemental Figure 1**). A control group was not included because it would not make sense to perform such a weight loss intervention on healthy individuals and because it would be ethically disputable to not perform it on obese individuals needing it. We believe such a control group without intervention would be highly similar to the baseline status of our study group.

Patients

Patients were recruited on a voluntary basis between 2009 and 2012 at the Metabolic Unit of the University of Hohenheim, Stuttgart, Germany. The study protocol was part of a multicenter clinical trial, research project "Obesity and the GI tract" (NCT01344525), approved by the ethics committee of the University Hospital of Tuebingen, Germany. Informed consent was obtained from every subject before participation.

Twenty-seven obese individuals (13 men, 14 women, age range: 24–63 y, mean ± SD age: 44 ± 8.2 y) were included. All patients who underwent a weight-reduction program were asked to participate and were recruited if they met the eligibility criteria and if they agreed to participate so that any bias in patient selection should be avoided. Eligibility criteria were *1*) age 18–65 y, *2*) BMI (in kg/m^2) ≥30, *3*) participation in a weight-reduction program, *4*) no patient history of chronic gastrointestinal disease, *5*) no diabetes, and *6*) informed consent. Mean body weight was 133 ± 19.6 kg, and mean BMI was 43.7 ± 5.20 at study start. In total, 97 obese individuals passed the weight-reduction program, and 27 of them (28%) met the eligibility criteria. Most of the patients (82%) had obesity grade III (BMI >40), only a minority (4%) presented with obesity grade I (BMI >30 to <35), and 14% were of obesity grade II (BMI >35 to <40). Further clinical data are summarized in **Table 1**.

Weight-reduction program

All study patients underwent an interdisciplinary 52-wk weight-loss program for obesity (Optifast52; Nestle Nutrition). This program, which is based on formula diet during the first 12 wk and a comprehensive lifestyle modification thereafter, has been described in detail elsewhere (14). Patient examinations were performed before program start (month 0), and 3, 6, and 12 mo after program start. Assessments included anthropometry [body height, body weight, waist circumference (WC)], measurement of blood pressure and heart rate, ultrasound of the liver, routine laboratory parameters, and permeability markers measured in urine or feces. In addition, metabolic syndrome was assessed according to the criteria of the International Diabetes Federation, which defined increased WC as prerequisite for the presence of the metabolic syndrome (15).

Laboratory analyses

Routine laboratory analyses in serum included the liver enzymes alanine-aminotransferase and γ-glutamyltransferase (GGT); C-reactive protein; the glucose metabolism parameters fasting glucose, insulin, and hemoglobin A1c; and the lipid metabolism parameters total, HDL, and LDL cholesterol and triglycerides. All blood samples were collected in the morning in a fasting state, and parameters were measured in a certified medical laboratory (Laborärzte Sindelfingen). The HOMA index was calculated according to the formula HOMA index = [insulin (μU/mL) × glucose (mg/dL)] ÷ 405. If the HOMA index was >2.5, the presence of insulin resistance was assumed (16).

Assessment of liver steatosis

All patients underwent sonography with the use of the LOGIQ P6 device (GE Healthcare). All sonography examinations were performed by the same experienced clinician. Grade of steatosis was assessed according to liver size, liver shape and caudal angle, and liver parenchyma based on hyperechogenic liver tissue, the increased discrepancy of echo amplitude between liver and kidney (increase in echogenicity compared with renal tissue) and the loss of echoes from the walls of the portal system. Moreover it was assessed quantitatively by measurement of the hepatorenal index as described by Webb et al. (17) by using the cutoffs 1.49 for >5%, 1.86 for 5–25%, and 2.23 for >60% fat infiltration. Results were scored into 4 grades (none, mild, moderate, and severe fatty liver) as described (18, 19). However, because hepatorenal index assessment is technically difficult to perform in severely obese individuals, the index only served as a complementary instrument for grading liver steatosis besides the classical and well-established semiquantitative grading into 4 categories. Liver fibrosis was not analyzed in this study because ultrasound is not accurate enough for differentiating fibrosis from steatosis (20) and FibroScan cannot be used precisely in severely obese individuals (21).

Steatosis was also assessed by clinical and laboratory means such as BMI, WC, GGT, and triglycerides, from which the fatty liver index (FLI) was calculated according to the formula:

$$\mathrm{FLI} = \left[e^{0.953 \times \log(\mathrm{TC}) + 0.139 \times \mathrm{BMI} + 0.718 \times \log(\mathrm{GGT}) + 0.053 \times \mathrm{WC} - 15.745}\right] \div \left[1 + e^{0.953 \times \log(\mathrm{TC}) + 0.139 \times \mathrm{BMI} + 0.718 \times \log(\mathrm{GGT}) + 0.053 \times \mathrm{WC} - 15.745}\right] \times 100 \quad (1)$$

This algorithm allows the prediction of liver steatosis in the general population (22). The FLI can vary between 0 and 100. An FLI value <30 (negative likelihood ratio = 0.2) rules out and an FLI value ≥60 (positive likelihood ratio = 4.3) rules in fatty liver (22).

TABLE 1
Patient characteristics at study start[1]

	Steatosis grade[2]				
	0–1	2	3	All	P^3
Patients, *n*	6 (22)	13 (48)	8 (30)	27 (100)	
BMI, kg/m^2	41.4 ± 2.5[4]	44.1 ± 6.7	44.8 ± 3.4	43.7 ± 5.2	NS
Waist circumference, cm	115 ± 6	128 ± 16	137 ± 8	128 ± 15	0.01
Systolic blood pressure, mm Hg	126 ± 13	129 ± 14	145 ± 13	133 ± 15	0.02
Diastolic blood pressure, mm Hg	83 ± 6	88 ± 11	89 ± 9	87 ± 9	NS
Alanine aminotransferase,[5] U/L	22 ± 6	56 ± 37	48 ± 24	45 ± 29	0.09
γ-glutamyltransferase,[5] U/L	38 ± 30	47 ± 28	49 ± 21	44 ± 24	NS
C-reactive protein,[5] mg/dL	6.3 ± 3.0	10.8 ± 5.7	8.6 ± 4.1	9.1 ± 4.9	NS
Fasting glucose,[5] mg/dL	102 ± 6.4	105 ± 20	119 ± 23	109 ± 19	NS
Glycated hemoglobin,[5] %	5.57 ± 0.19	6.02 ± 0.52	6.11 ± 0.57	5.95 ± 0.51	NS
Insulin,[5,6] μU/L	11.5 ± 4.0	22.9 ± 12.3	28.5 ± 8.8	21.0 ± 11.5	0.03
HOMA[6]	2.90 ± 1.09	5.97 ± 3.36	6.81 ± 3.83	5.39 ± 3.34	0.09
Insulin resistance[6]	4 (67)	9 (82)	5 (100)	18 (82)	NS
Total cholesterol,[5] mg/dL	210 ± 37	192 ± 34	208 ± 62	201 ± 44	NS
HDL cholesterol,[5] mg/dL	61 ± 18	47 ± 11	43 ± 8	49 ± 14	0.03
LDL cholesterol,[5] mg/dL	131 ± 20	123 ± 28	138 ± 42	130 ± 31	NS
Triglycerides,[5] mg/dL	132 ± 25	151 ± 61	223 ± 181	168 ± 110	NS
Metabolic syndrome[7]	3 (50)	7 (54)	8 (100)	18 (67)	0.06

[1] Values are *n* (%) unless otherwise indicated.
[2] Measured by ultrasound as described in Methods.
[3] Data were compared by ANOVA (continuous data) or chi-square test, $P \geq 0.1$.
[4] Mean ± SE; all such values.
[5] Normal values: alanine aminotransferase, <50/35 U/L (male/female); γ-glutamyltransferase, <60/40 U/L; C-reactive protein, <0.5 mg/dL; fasting glucose, <100 mg/dL; glycated hemoglobin, 3.4–6.0%; insulin, 2.0–22 μU/L; total cholesterol, <200 mg/dL; HDL cholesterol, >40/50 mg/dL; LDL cholesterol, <155 mg/dL; triglycerides, <150 mg/dL.
[6] Insulin resistance was estimated according to the HOMA index [(fasting serum insulin expressed in μU/mL × fasting serum glucose expressed in mg/dL) / 405]. A HOMA value of ≤2.5 was considered no/unlikely insulin resistance, and a HOMA value of >2.5 was considered a likely insulin resistance index.
[7] Metabolic syndrome was defined according to the criteria of the International Diabetes Federation.

Assessment of intestinal permeability

Intestinal permeability was assessed by the oral Lac:Man test after a 12-h fasting and nicotine-abstinence phase as described (23, 24) with slight modifications. Briefly, after collecting a sample of spontaneous urine, the test solution containing 5 g lactulose syrup (Ratiopharm), 2 g mannitol, 2 g polyethylene glycol (PEG) 400 (both Sigma-Aldrich), 2 g PEG 1500 (Carl Roth), and 16 g PEG 4000 (Merck) was administered in the morning. Urine was collected over 6 h after sugar administration. Samples were first treated with chloroform to avoid interference with PEG. Of the watery urine extract, 3.6 g was evaporated in a vacuum centrifuge and resolved in 500 μL eluent (sulphuric acid 0.003N). All measurements were performed in duplicate. Proteins were removed through perchloric acid (7%) precipitation. The supernatants were filtered by using a 0.2-μm filter and samples (injection volume 40 μL) were analyzed by HPLC (flow rate 0.40 mL/min at 70°C) as described (24) with a Resex ROA Organic Acid H+ 8%, 300 × 7.8–mm column (Phenomenex) and Security Guard Cartridge Kit with guard column corresponding to the main column. The ratio of the urinary concentration of both molecules measured after 6 h, which is supposed to be <0.03 under normal conditions (23), reflects increased paracellular passage across the small intestinal barrier if higher ratios are measured (25). PEGs were not analyzed in the present study because during our study a report was published according to which PEG and the Lac:Man test demonstrate equivalent performance for the assessment of permeability changes (26).

Assessment of fecal zonulin

Zonulin is the main physiologic modulator of tight junctions (TJs) in the intestinal epithelial layer (27). Intestinal epithelial cells, on challenge with specific luminal molecules such as wheat-derived gliadin or microbial products, secrete zonulin into the lumen, which induces the opening of TJ. Zonulin has been implicated in the pathogenesis of celiac disease and other autoimmune diseases related to intestinal barrier dysfunction (27). We measured zonulin in stool samples using an ELISA kit (Immundiagnostik) following the manufacturer's instructions (detection limit 0.1 ng/mL). Zonulin measurements were performed in duplicate.

Assessment of circulating IL6 and endotoxin

To assess the inflammation status of the patients during the intervention, we measured IL6 in serum samples using a high-sensitive ELISA kit (Abcam,) following the manufacturer's instructions (detection limit 0.8 pg/mL). In parallel pyrogens were quantified from plasma samples (diluted 1:50) by using a LAL-based kinetic kit (Charles River, Ecully, France) as recommended by the manufacturer and described previously (28). The recovery of the endotoxin spikes at 0.5 endotoxin unit/mL was,

on average, ~40%. These measurements were performed at 4 time points for the 13 patients who finished the study (no dropouts).

Statistical analysis

All data are shown as means ± SDs if not indicated otherwise. The required number of participants was calculated by using data from a previous study (23) showing a higher Lac:Man ratio (primary outcome) in patients with obesity and NAFLD (0.05 ± 0.025) than in healthy controls (0.025 ± 0.024). Assuming a similar difference in Lac:Man ratio between the beginning and ending of intervention, we calculated the patient numbers needed in the present study using the paired *t* test, a statistical power ($1 - \beta$) of 90% and a type I error rate α of 0.05 resulting in a group size of 10 using the software GPower3.1 (29). The group size might sound small; however, the paired study design allows such numbers. Loss to follow-up was kept to a minimum by regular visits every week; however, because of the cumbersome examinations, particularly collection of urine for 5 h and fecal sampling, we expected a dropout rate of 60% during the follow-up and therefore included slightly >25 patients. For time course analysis, only datasets for which all time points were available were included into the analysis. Test for normal data distribution were performed by using the Kolmogorov-Smirnov test. If normally distributed, paired data were compared by the paired *t* test, otherwise by the Wilcoxon matched-pair test. Data from multiple patient groups were compared by ANOVA/Bonferroni test and follow-up data by repeated measure ANOVA/Tukey's, provided that data were normally distributed, or by the Kruskal-Wallis test/Dunn test and the Friedman/Dunn comparison, respectively, if normal distribution was not confirmed. Data from 2 patient groups were compared by *t* test or by the *U* test if not normally distributed. Correlations between 2 parameters were calculated on the mean of each individual (over the 4 time points) and performed by calculating the Spearman correlation coefficient suitable for nonparametric parameters. A probability $P < 0.05$ was considered statistically significant. Most analyses and figure presentations were performed by using the SPSS version 21 software (IBM) and the graph pad Prism version 5 software (Graph Pad Inc.). Multiple regression analyses were performed in R (http://www.r-project.org/) by using the function lm and stepAIC from the packages stats and MASS (30).

RESULTS

Body weight reduction by the intervention program

As expected, the established multidisciplinary weight-reduction program, which is based on a low-calorie diet that uses formula during the first 12 wk and a comprehensive lifestyle modification over 52 wk, caused a marked weight reduction of 23.5 ± 12.3 kg on average after 1 y corresponding to a relative weight loss [calculated according to the formula: 1 − (body weight/initial body weight)] of 16.3% ± 9.0% after 12 mo. This weight loss was already achieved after 3 mo when exclusive consumption of low-calorie formula diet ended (relative weight loss 16.3% ± 4.5%) and reached a maximum after 6 mo, when the time period of calorie restriction ended (19.6% ± 7.6%). Correspondingly, the BMI was significantly reduced within this time period from 43.7 ± 5.2 to 36.4 ± 5.1, which means a reduction of 16.7% (**Figure 1**). The WC was reduced by 16.3% within this time period. Weight reduction was most pronounced during the first 3 mo of intervention, when patients' energy intake was restricted to 800 kcal/d administered only via formula diet, and continued during the second 3 mo, when patients were still on energy restriction. Within the second half of the program, weight did not change significantly.

Changes in fatty liver disease

Steatosis was assessed by ultrasound (grades 0–3, where grades 2 and 3 were rated as steatosis) and by the FLI (scale 0–100, where >60 means steatosis). According to ultrasound, 78% of the patients had steatosis defined as grade 2 or 3 (Table 1). The results derived from ultrasound and FLI calculation were related ($P = 0.003$, chi-square test after Pearson), whereas FLI was more sensitive than ultrasound (data not shown). In contrast, ultrasound might be more specific because all cases rated as steatosis according to ultrasound had an FLI value >60 (99 ± 1), but two-thirds of the cases rated as no substantial steatosis (grade 0 or 1) also had an FLI value >60 (86 ± 14). In the course of the 12-mo weight-reduction program, the prevalence of steatosis could be reduced from 78% to 7.4% according to ultrasound. Within the same time period, the mean FLI value decreased from 97 to 75 points (Figure 1D).

Results from the Lac:Man test

For this analysis we included only patients from whom Lac:Man test results were available at study start and at all consecutive time points ($n = 13$). Based on these data sets the median Lac:Man ratio decreased in the course of intervention from 0.080 (95% CI: 0.073, 0.093) to 0.027 (95% CI: 0.024, 0.034; **Figure 2**A). If all data sets were analyzed ($n = 27$ at study start, $n = 18$ after 3 and 6 mo), the mean Lac:Man ratio decreased significantly (data not shown). Apart from the Lac:Man ratio, we separately analyzed lactulose and mannitol recovery in urine and found, despite a considerable variability of values, that only lactulose recovery decreased as expected, whereas mannitol recovery did not change (Figure 2B, C). Interestingly, the Lac:Man ratio continued to decrease between time point 6 and 12 ($P = 0.009$, Wilcoxon's rank test), whereas anthropometric parameters stayed stable or increased slightly.

At study start we found a higher Lac:Man ratio in patients with steatosis grade 2 and 3 compared with the ratio in patients with no steatosis [Lac:Man ratio medians of 0.080 (95% CI: 0.070, 0.091) and 0.079 (95% CI: 0.074, 0.088) vs. 0.035 (95% CI: 0.029, 0.038), **Figure 3**A]. The relation between the permeability marker Lac:Man ratio and the steatosis grade assessed by sonography existed in a similar way for the other time points at month 3, 6, and 12 (data not shown).

In patients with metabolic syndrome at baseline, the median Lac:Man ratio tended to be higher than in those without metabolic syndrome [0.079 (95% CI: 0.064, 0.088) compared with 0.062 (95% CI: 0.042, 0.074) $P = 0.06$, Figure 3B].

Results from the zonulin test

For this analysis we included only patients from whom zonulin data were available at study start and at all consecutive measurements ($n = 11$). Based on these data sets, the median zonulin

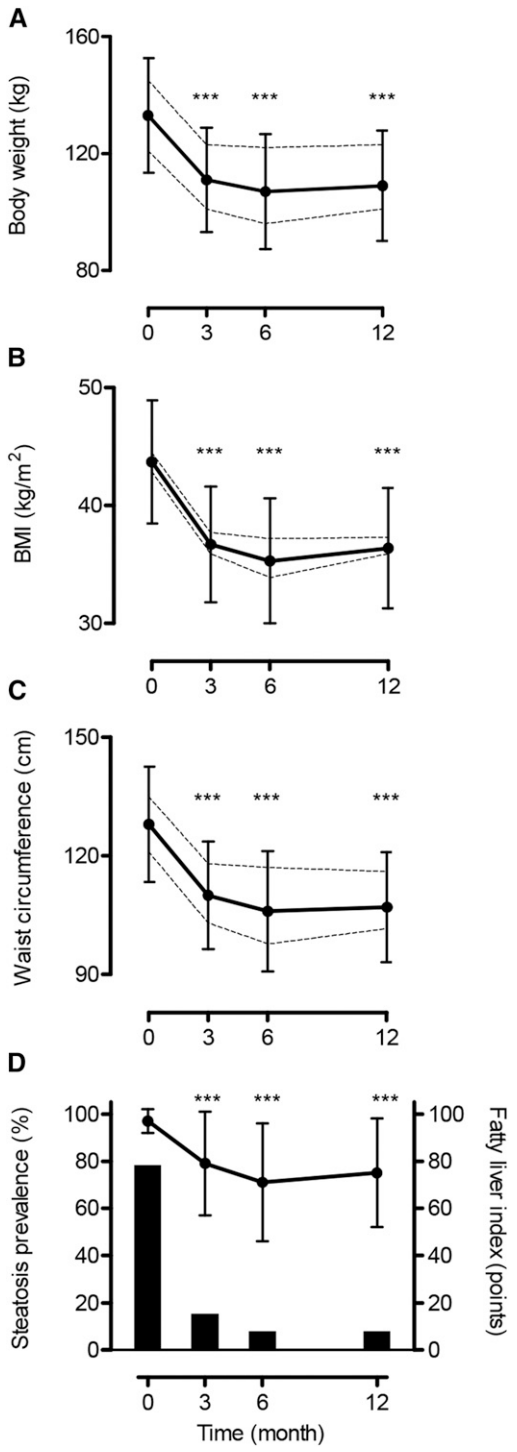

FIGURE 1 Effect of the weight-reduction program. The 12-mo program induced a significant reduction in body weight (A), BMI (B), waist circumference (C), and steatosis assessed by ultrasound or fatty liver index (D) over time. Means ± SDs are shown and connected by a continuous bold line for all individuals ($n = 27$) and for panels A, B, and C separately for men ($n = 13$, upper dotted line) and women ($n = 14$, lower dotted line). ***$P < 0.001$ compared with month 0 (ANOVA, Bonferroni test).

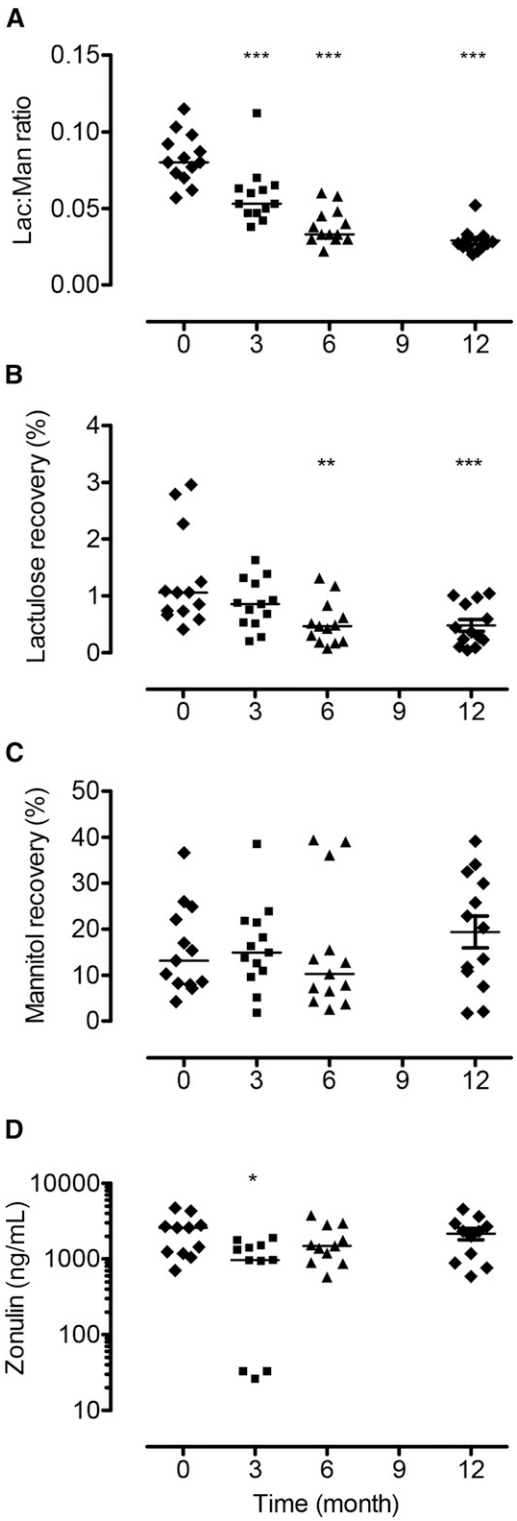

FIGURE 2 Change of intestinal permeability in the course of weight reduction. Results from each individual (plotted points, $n = 11$–13) and medians (horizontal lines) are shown for the time points indicated in the x axis. Not only the Lac:Man ratio (A) but also lactulose and mannitol recoveries (B and C) and fecal zonulin concentrations (D) are shown. *·**·***Compared with month 0 (repeated-measures ANOVA or Friedman, Tukey's, or Dunn's multiple comparison): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. No measurements were performed at month 9. Lac:Man, lactulose:mannitol.

concentrations only slightly decreased at month 3 but not at month 6 or 12 (Figure 2D). If all data sets were analyzed ($n = 24$ at study start, $n = 18$ after 3 and 6 mo), the zonulin concentrations did not change significantly (data not shown).

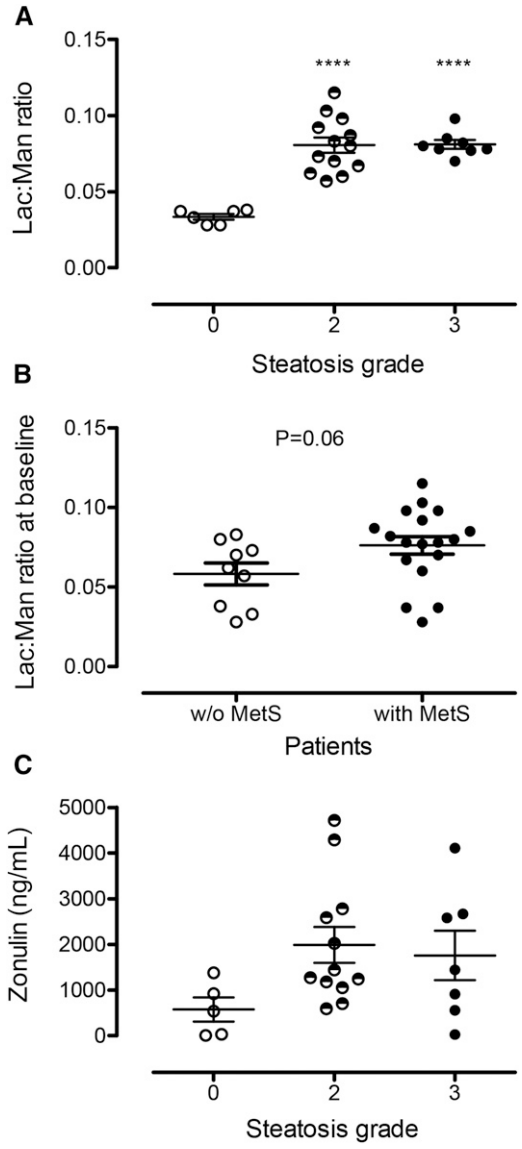

FIGURE 3 Intestinal permeability is related to liver steatosis and the metabolic syndrome. Lac:Man ratio (A) and fecal zonulin concentrations (C) at study start from each individual (plotted points) and means ± SEs are shown for 3 patient subgroups (steatosis grade 0, n = 6; grade 2, n = 13; and grade 3, n = 8; no patient had a grade 1 steatosis at baseline). ****$P < 0.0001$ compared with grade 0 (ANOVA with Bonferroni's multiple comparison). The Lac:Man ratio tended to be related to the metabolic status of the patients at baseline (B). Scatter plots (means ± SEs) of the Lac:Man ratio are shown for patients with (n = 18) and without (n = 9) MetS (MetS defined according to the criteria of the International Diabetes Federation, t test). Lac:Man, lactulose:mannitol; MetS, metabolic syndrome.

At study start, we found slightly elevated median zonulin concentrations in patients with steatosis grade 2 and 3 compared with the ratio in patients with no steatosis [1355 ng/mL (95% CI: 1127, 2850 ng/mL) (n = 12) and 1444 ng/mL (95% CI: 433, 3079 ng/mL) (n = 7) compared with 541 ng/mL (95% CI: 0, 1305 ng/mL) (n = 5), Figure 3C]; however, the differences were not statistically significant (P = 0.07, Kruskal-Wallis test). A similar trend was found at the end of the study at which patients with a steatosis (grade 1 and 2 pooled, n = 7) had higher fecal zonulin concentrations than patients without steatosis [n = 6; 2941 ng/mL (95% CI: 1388, 3989 ng/mL) compared with 1028 ng/mL (95% CI: 521, 2156 ng/mL), Mann-Whitney U test P = 0.03, data not shown].

Results from the IL6 and endotoxin measurements

The plasma concentrations of lipopolysaccharide did not change significantly during intervention, although the circulating IL6 concentrations significantly decreased after 6 and 12 mo compared with initial values (**Figure 4**A and B). IL6 concentration paralleled the Lac:Man ratio, as both parameters decreased during intervention; however, the decrease of IL6 concentration was less pronounced and occurred later than the decrease of the Lac:Man ratio.

Among the 13 nondropout patients, all had liver steatosis at baseline. Patients with a grade 2 steatosis had IL6 and lipopolysaccharide concentrations slightly lower than those with a grade 3 steatosis (4.3 ± 1.3 compared with 4.8 ± 1.8 pg/mL and 0.07 ± 0.03 compared with 0.08 ± 0.02 endotoxin unit/mL, respectively), but these differences were not statistically significant (data not shown).

Correlations and regressions

Considering the mean values for each individual (n = 13), we found that liver steatosis, as assessed by the FLI, did not correlate with the BMI but did correlate positively with the HOMA index (ρ = 0.75, P = 0.003; **Figure 5**A). This correlation resulted from a close correlation of FLI with serum insulin (ρ = 0.73, P = 0.005,

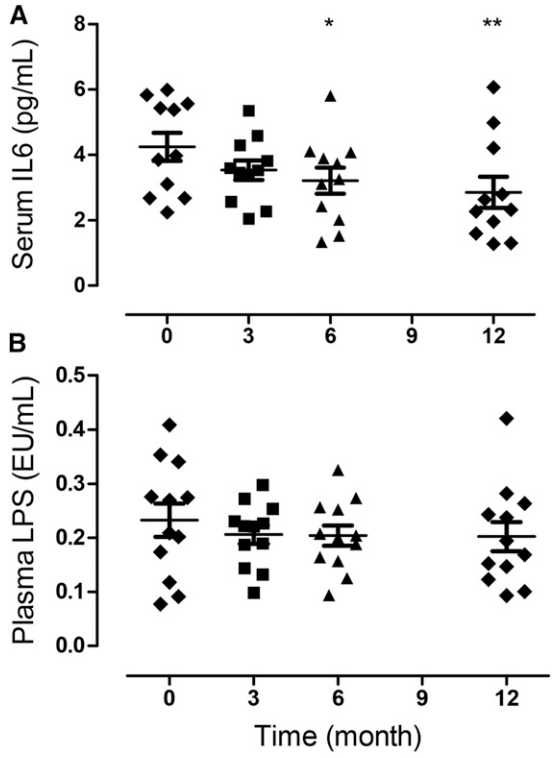

FIGURE 4 Changes of inflammation and endotoxemia in the course of weight reduction. Circulating IL6 concentrations (A) decrease along the intervention, whereas LPS concentrations do not change significantly (B). Scatter plot: *·**Compared with month 0 (means ± SEs; n = 13, repeated-measures ANOVA with the Bonferroni multiple-comparison test), *$P < 0.05$, **$P < 0.01$. No measurements were performed at month 9. LPS, lipopolysaccharide.

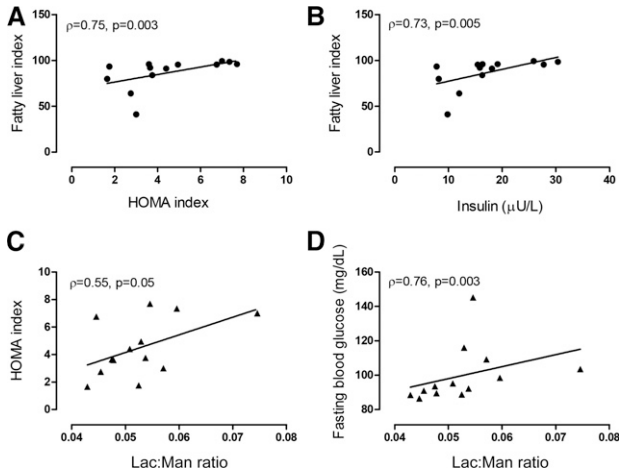

FIGURE 5 The correlation of the HOMA index with FLI (A) is related to a correlation between FLI and blood insulin concentrations (B). Lac:Man ratio tends to correlate with the HOMA index (C) through a correlation with the fasting blood glucose concentrations (D). Data are means over the intervention for each subject (n = 13). The Spearman correlation coefficient ρ is indicated. FLI, fatty liver index; Lac:Man, lactulose:mannitol.

Figure 5B) because no correlations could be observed between FLI and the other HOMA variable, fasting blood glucose. HOMA also tended to correlate with the Lac:Man ratio ($\rho = 0.55$, $P = 0.052$, Figure 5C). This correlation resulted from a strong association between the Lac:Man ratio and fasting blood glucose concentrations ($\rho = 0.76$, $P = 0.003$, Figure 5D) because no correlations could be detected between the ratio and insulin.

No correlations between zonulin and anthropometric or laboratory parameters, respectively, were found (data not shown).

To test whether, beside anthropometric parameters, gut permeability, inflammation, or endotoxemia could predict liver steatosis, we performed a multiple-regression analysis. The following x variables were tested for effects on the y variable FLI: individuals, as dummy variable; time; WC; IL6; lipopolysaccharide; BMI; and Lac:Man ratio (LM). The complete model (i.e., with all 7 x variables) showed significant influence of BMI, and WC (as expected) had an adjusted R^2 of 0.68 and an Akaike information criterion (AIC) of 253.5. Using the function "stepAIC" (selecting the best model according to its AIC value removing stepwise one x variable so that at each step the lowest AIC is reached) led to the final model explaining FLI with the 2 expected parameters BMI and WC but also Lac:Man ratio, leading to following equation: FLI = $-36.64 + 1.20$ BMI + 0.72 WC $-$ 186.6 Lac:Man ratio. All estimates were statistically significant; adjusted R^2 was 0.70 and AIC was 248.5.

Similarly, we tested whether the BMI could be explained with individuals, time, WC, lipopolysaccharide, IL6, and Lac:Man ratio. The complete model (i.e., with all 6 x variables) showed a significant role for WC only and had an adjusted R^2 of 0.71 and an AIC of 131.4. The final model, selected by stepAIC, included WC, Lac:Man ratio, and IL6: BMI = 4.77 + 46.3 Lac:Man ratio + 0.29 WC $-$ 0.29 IL6. It had an AIC of 126 and an adjusted R^2 of 0.73.

DISCUSSION

Metabolic endotoxemia caused by overfeeding, particularly by high-fat or high-fructose diets, is a phenomenon inducible in lean (31, 32) and obese individuals (9, 33). It has been recognized as a major mechanism promoting the development of NAFLD and other metabolic disorders (3, 34–36). However, the underlying mechanisms are unclear to a large extent. An impairment of the intestinal barrier function must be assumed, resulting in increased permeability for endotoxin and possibly other luminal factors, but how nutrients cause such impairment or trigger it in individuals with a genetic susceptibility is unknown. It is also unclear whether permeability changes are associated with obesity or with associated metabolic diseases or both and whether the permeability changes are reversible after weight reduction.

In humans, the Lac:Man test has been used widely to assess intestinal permeability in different settings (37–39). It is a cumbersome test because it requires urine collections, but it is likely one of the best established tests to assess intestinal permeability in humans (1). Therefore, we chose the Lac:Man test to assess intestinal permeability in the obese with or without associated metabolic disorders before and after successful weight reduction.

We found a highly significant decrease of intestinal permeability assessed by the Lac:Man ratio in the course of intervention. While mean body weight was reduced from 133 $\pm$ 20 to 109 $\pm$ 19 kg within the 1-y intervention period, the Lac:Man ratio dropped to one-third (from 0.08 to 0.027). By assuming a ratio of $<$0.030 is normal (12), most of the patients returned to normal levels after intervention. These data strongly suggest that successful weight reduction can normalize the increased intestinal permeability in the obese. This effect is mainly owing to modifications of the paracellular permeability, as revealed by our finding that lactulose recovery was changing, whereas mannitol recovery indicating transcellular permeability remained largely unchanged in the course of intervention.

In addition, we measured stool zonulin concentrations because they can be analyzed noninvasively in feces and because zonulin is a unique and relevant endogenous regulator of TJ (27). We preferred the fecal measurement from serum measurements because zonulin is secreted into the intestinal lumen. Its secretion was shown to coincide with an increase in gut permeability in prediabetic rats (40). However, our data revealed no correlation between fecal concentrations of zonulin and the Lac:Man ratio. Moreover, zonulin stool concentrations were not influenced by the weight-reduction therapy and did not correlate with anthropometric variables. We cannot exclude, however, that obese individuals have elevated stool zonulin concentrations because we did not analyze a lean control group in the present study. Others indeed showed an increase of serum zonulin concentrations with increased BMI (41), but whether this can be transferred to fecal measurements needs to be confirmed. The lack of fecal zonulin changes after intervention in our study suggests that a zonulin-independent regulation of TJ occurs in the course of weight reduction that leads to the documented changes in gut permeability. Elevated paracellular permeability, such as the one observed in the obese before intervention, was shown to be associated with decreased expression of TJ proteins in the intestinal mucosa (1). Animal studies suggested that components of the Western-style diet might reduce the expression of TJ proteins (6, 7, 42) resulting in endotoxemia and fatty liver disease (43).

Although animal models clearly showed enhanced intestinal permeability in obesity, previous human studies that use the Lac:Man test yielded less-clear results (44, 45). In these studies, no significant change in the Lac:Man ratio was found in obese

compared with lean individuals, probably because obesity was less pronounced (grades I–II, no comorbidities) than in our study (grade III, 78% with steatosis). Increased intestinal permeability was found in patients with NAFLD, in whom permeability correlated with the severity of steatosis but not with presence of steatohepatitis (12). In one of our previous studies, we could show that intestinal permeability assessed by the Lac:Man test is markedly altered in mild to moderately obese patients with NAFLD (23). Our present data show that not only body weight but in particular the extent of liver steatosis is related to intestinal permeability. Although obese patients with a mean BMI of 41 and no significant steatosis had largely normal Lac:Man ratios, those patients with only a slightly higher BMI of 44, but a moderate to severe steatosis, were all above the normal range. Thus, the Lac:Man ratio seems to discriminate between the obese with and without steatosis. Previous animal experiments suggest increased intestinal permeability and subsequent lipopolysaccharide translocation are major mechanisms of fatty liver disease and other metabolic disorders accompanying obesity (6, 7, 42). Our data confirm some link between steatosis and gut permeability as our regression analysis showed an influence of the Lac:Man ratio on FLI, but did not confirm a role of lipopolysaccharide. Lipopolysaccharide measurements in peripheral blood might be limited by the diluting effect of the liver preventing a clear assessment of differences related to the intervention.

Our data show that the increased intestinal permeability can be reversed to normal levels by successful obesity therapy. A similar finding has been reported recently in mildly obese individuals undergoing a dietetic intervention based on whole grains, traditional Chinese medicinal foods, and prebiotics (46). NAFLD was not assessed in this trial.

The elevated Lac:Man ratio we measured in the obese individuals is not only related to the presence or absence of liver steatosis but also by trend of the metabolic syndrome (according to the International Diabetes Federation criteria). Moreover, the HOMA index correlated with FLI and by trend with intestinal permeability measured by the Lac:Man test. During intervention, we saw a decrease not only in the BMI and the Lac:Man ratio but also in the HOMA index (from 5.4 $\pm$ 3.3 at study start to 2.7 $\pm$ 1.7 after 12 mo, $P < 0.001$, data not shown) and serum IL6 concentrations. However, the kinetics of changes were slightly different. The Lac:Man ratio and IL6 concentrations, but not the FLI and anthropometric parameters, decreased not only within the first half of the year but also between months 6 and 12. This could be because of a delayed effect of weight reduction on the intestinal barrier because the BMI change was almost complete already after month 3, whereas the Lac:Man ratio continuously decreased over the whole observation period. Another explanation for the different kinetics could be that intestinal barrier function is related not only to BMI and caloric intake but also to food quality. Indeed, from month 3 on, the patients returned stepwise from formula diet to a normal diet and all underwent diet counseling for balanced healthy eating. This hypothesis is supported by previous observations that revealed that an unhealthy diet, e.g., a high-fat diet, leads to intestinal barrier impairment measured by increased lipopolysaccharide translocation from the gut into the systemic circulation (47). Our data suggest that not only weight reduction but also weight maintenance promotes improvement of intestinal barrier function.

Our data suggest that the interrelated pieces of the metabolic disease puzzle, such as fatty liver disease, high values of HOMA, high WC, and subclinical inflammation, are all associated with intestinal permeability. Indeed, according to our regression analysis, both BMI and FLI are related to intestinal permeability assessed by the Lac:Man ratio. In contrast with the sugar test, fecal zonulin concentrations were not related to clinical variables. By trend, fecal zonulin concentrations were higher in patients with steatosis but were hardly changed in the course of weight reduction. These findings suggest that the Lac:Man ratio could be a valuable marker of intestinal permeability in patients with obesity and metabolic disorders.

In conclusion, our data show that intestinal permeability assessed by the Lac:Man test is increased in obese patients with liver steatosis compared with obese patients without. The increased permeability is associated with high values of the HOMA index and can be reversed by successful and substantial weight reduction.

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