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ABSTRACT

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Obesity and its metabolic complications are characterized by subclinical systemic and tissue inflammation. In rodent models of obesity, inflammation and metabolic impairments are linked with intestinal barrier damage. However, whether intestinal permeability is altered in human obesity remains to be investigated. In a cohort of 122 severely obese and non-obese patients, we analyzed intestinal barrier function combining *in vivo* and *ex vivo* investigations. We found tight junction impairments in the jejunal epithelium of obese patients, evidenced by a reduction of occludin and tricellulin. Serum levels of zonulin and LPS-Binding Protein, two markers usually associated with intestinal barrier alterations, were also increased in obese patients. Intestinal permeability *per se* was assessed *in vivo* by quantification of urinary lactitol/mannitol (L/M) and measured directly *ex vivo* on jejunal samples in Ussing chambers. In the fasting condition, L/M ratio and jejunal permeability were not significantly different between obese and non-obese patients, but high jejunal permeability to small molecules (0.4 kDa) was associated with systemic inflammation within the obese cohort. Altogether, these results suggest that intestinal barrier function is subtly compromised in obese patients. We thus tested whether this barrier impairment could be exacerbated by dietary lipids. To this end, we challenged jejunal samples with lipid micelles and showed that a single exposure increased permeability to macromolecules (4 kDa). Jejunal permeability after the lipid load was two-fold higher in obese patients compared to non-obese controls and correlated with systemic and intestinal inflammation. Moreover, lipid-induced permeability was an explicative variable of type 2 diabetes. In conclusion, intestinal barrier defects are present in human severe obesity and exacerbated by a lipid challenge. This paves the way to the development of novel therapeutic approaches to modulate intestinal barrier function or personalize nutrition therapy to decrease lipid-induced jejunal leakage in metabolic diseases.

Key words: Intestinal permeability; intestinal barrier function; Obesity; Jejunum; Tight Junction Proteins; Lipids; Inflammation; Type 2 diabetes; Ussing chamber

INTRODUCTION

Intestinal barrier impairment is associated with local and systemic inflammation in inflammatory bowel diseases [1, 2]. Barrier defects, including increased epithelial permeability, may promote the transfer of dietary and bacterial antigens across the intestinal mucosa, thereby contributing to imbalanced responses of mucosal immune cells and induction of inflammation [3]. In return, pro-inflammatory cytokines are able to induce dysregulation of tight junctions involved in the control of paracellular flux, leading to a local vicious cycle of inflammation [2, 4]. In Crohn's disease, healthy first-degree relatives of patients display higher intestinal permeability than unrelated controls, suggesting that intestinal barrier defects could be early events contributing to disease pathogenesis [3]. This "leaky gut" concept has been extended to extra-digestive diseases also associated with perturbed inflammatory responses, such as HIV/AIDS and type 1 diabetes [1, 2].

Obesity and associated metabolic disorders, such as type 2 diabetes, are also characterized by systemic inflammation but at subclinical levels [5, 6]. These metabolic diseases are associated with tissue inflammation in the liver [7], adipose tissue [8, 9] as well as small intestine, for which we recently demonstrated a link between activated intestinal T cells and impaired insulin signaling in enterocytes [10]. These results denote the importance of intestinal inflammation in the pathogenesis of obesity-related metabolic alterations. In this context, relationships with intestinal barrier dysfunction must be addressed in depth.

In mice, increased gut permeability has been linked to inflammation and metabolic alterations by the pioneering work of P. Cani [11]. These results were confirmed in multiple studies using rodent models of high-fat diet-induced obesity [12]. It has been proposed that endotoxemia, i.e. the passage of bacterial LPS into the systemic circulation, is a consequence of an altered intestinal barrier and plays a crucial role in the low-grade inflammation that is

triggered by diet-induced obesity [13]. By contrast, only few studies to date have investigated gut permeability in human obese patients. Based on the lactitol-mannitol intestinal permeability test performed in small cohorts, two studies showed an association between increased gut permeability and metabolic alterations such as insulin-resistance and hepatic steatosis [14, 15], while another one failed to show any disturbance of intestinal barrier function [16]. Similarly, analysis of endotoxemia gave rise to discordant results [17, 18]. Thus, the impairment of intestinal barrier function in human obesity remains an open question, especially in severe obesity.

Excessive consumption of energy dense foods has undoubtedly contributed to the obesity epidemic. In rodents, high-fat feeding is associated with changes in the gut microbial profile and decreased bacterial diversity [12], with similar findings described in human obesity [12, 19]. In mice, changes in gut microbiota have been involved in the increase of intestinal permeability after 4 weeks of high-fat diet (HFD) since effects were alleviated upon antibiotic treatments [11]. However, recent studies show that increased intestinal permeability is an early response to HFD and precedes weight gain and the onset of type 2 diabetes [20-22]. Eventhough microbiota modifications could partly mediate barrier alterations [11], direct effects of dietary lipids on the intestinal epithelium must be considered. The hypothesis of short-term deleterious effects of lipids on the intestinal barrier is also supported by the observation that a single lipid bolus can trigger a post-prandial endotoxemia in mice [23] and in humans [18, 24, 25]. It is currently admitted that endotoxemia reflects an increased intestinal permeability since LPS can cross an epithelial monolayer by the paracellular route [26]. However, post-prandial endotoxemia can also result from the transcellular passage of LPS and its secretion in association with chylomicrons [23, 25]. Therefore, a post-prandial endotoxemia does not necessarily reflect an increased intestinal permeability and effects of lipids on the intestinal barrier remain to be elucidated.

The current study addresses in humans the relationships between obesity, alterations of intestinal permeability, endotoxemia, systemic and intestinal inflammation as well as metabolic alterations, in the fasting state and after a lipid challenge.

METHODS

Study population

A total of 78 severely obese candidates for Roux-en-Y gastric bypass (RYGB) surgery were prospectively recruited at the Nutrition department of Pitié-Salpêtrière University Hospital, Paris, France. Forty-two patients (54%) had type 2 diabetes, defined according to ADA criteria [27]. Among them, 29 (69%) were treated with metformin, 16 (38%) with sulfonylureas, 12 (29%) with GLP-1 agonists and 14 (33%) with insulin. Jejunum samples were collected during RYGB. Jejunum characteristics (permeability and tight junction analysis) were compared to 14 non-obese controls C1 which consisted of patients who underwent pancreaticoduodenectomy or gastrectomy allowing access to proximal jejunal samples. Most importantly, we excluded non-obese patients with diabetes, renal- cardiac- or hepatic failure or patients with personal or familial history of inflammatory bowel disease or under treatment that could interfere with intestinal permeability (i.e. antibiotics, anti-inflammatory drugs, and chemotherapy). Their levels of white blood count were under $10.10^9/mm^3$ and of CRP below 5 mg/l.

For *in vivo* assays (i.e. Lactitol/Mannitol test, LPS-transporters and zonulin), an independent control group of 30 healthy patients C2 and 28 obese patients were investigated. This subgroup of obese patients was not different from the rest of the cohort according to age and BMI (ANOVA statistical test with p=0.98 and p=0.07 for age and BMI respectively).

The study was conducted in accordance with the declaration of Helsinki, received approval from the local ethics Committee (CPP Ile de France I), and was registered in the ClinicalTrials.gov Web site (https://clinicaltrials.gov/ct2/show/NCT02292121). Informed written consent was obtained from all patients prior to study inclusion.

Clinical, anthropological and biological characteristics

Medical history and clinical variables were recorded for the two groups of controls (C1 and C2) and for obese patients before surgery. The percentage of total fat mass was evaluated by dual energy X-ray absorptiometry (Discovery DXA Hologic, Hologic Discovery W, Bedford, MA, USA) in obese patients as described [28] and by bioimpedance in C2 patients. The percentage of visceral fat mass was measured in obese patients at the L4 spinal segment level using computed tomography. Venous blood samples were collected after a 12-hour fast for routine assessment of biological metabolic and inflammatory variables as described [5]. Calprotectin is a cytoplasmic protein released from activated neutrophil granulocytes; in feces, it is considered as a marker of intestinal inflammation. Fecal calprotectin was quantified using ELISA (Calprest®, Eurospital, Trieste, Italy). The quantification limit of the assay is 15 µg/g stool. Insulin resistance was assessed using the HOMA-IR index (insulinemia (mUI/L) × fasting blood glucose (mmol/L)/22.5). Insulin and HOMA-IR were not considered for patients treated by insulin, GLP-1 agonists or sulphonylureas.

Serum levels of LPS-binding protein (LBP), soluble CD14 (sCD14) and zonulin were measured using ELISA kits (respectively: R&D systems, BioTechne, Lille, France; Hycult Biotechnology, Uden, The Netherlands; Immundiagnostik,) as described [18].

***In vivo* Lactitol/ Mannitol urinary excretion ratio test**

The Lactitol/Mannitol (L/M) urinary excretion test was performed during the month preceding the surgery. Patients were given a mannitol (M) and lactitol (L) mix in aqueous solution (4 g of each sugar in 40 mL water) after a 12-hour fast. Urine concentration of both

sugars was determined as described in supplementary material, Supplementary materials and methods and expressed as L/M ratio.

Tight junction analysis and *ex vivo* permeability assays in Ussing chambers

Proximal jejunum samples from obese and non-obese C1 patients were collected during surgery, conditioned and transported as described [10].

Tight junction proteins occludin (Invitrogen™, ThermoFisher Scientific, Illkirch, France, catalog number #71-1500, dilution 1:200) and tricellulin (Invitrogen™, #700191, dilution 1:200) and NaK ATPase (Abcam, Paris, France, #ab2871, dilution 1:500) were analyzed by immunofluorescence in paraffin embedded jejunum from obese and non-obese C1 patients, as described in supplementary material, Supplementary material and methods. Occludin and tricellulin total levels were determined in jejunum samples using a WES capillary electrophoresis system (ProteinSimple, San Jose, CA, USA) as described in supplementary material, Supplementary material and methods.

For jejunal permeability assays in Ussing chambers, mucosa was dissected from the submucosa-muscle plane. Explants were mounted on inserts exposing 0.5 cm² of mucosa between Ussing chambers (World Precision Instruments, Hitchin, UK). Luminal and serosal chambers were filled with 2 mL of Dulbecco's Modified Eagle's Medium at 37 °C and saturated with 95% oxygen and 5% CO_2 gas flow. After equilibration for 30 min, fluorescein isothiocyanate (FITC)-labeled sulfonic acid (FITC-SA, 0.4 kDa, 500 µM; Invitrogen) or FITC-Dextran (4 kDa, 500 µM or 10 kDa, 200 µM; TdB consultancy, Uppsala, Sweden) was added to the luminal chamber. Intestinal permeability was derived from serosal-chamber fluorescence values, measured at 15 min intervals from 60 to 105 min following addition of the fluorescent molecule, using a microplate fluorometer (BMG Labtech®, Champigny s/

Marne, France). Baseline permeability (i.e. measured in jejunum samples of fasting patients) was determined by the slope obtained by plotting the increase in fluorescence intensity over time, using a linear regression fit model (Microsoft Excel, Microsoft Office). The slope value was adjusted to initial fluorescence intensity in each corresponding luminal reservoir. Permeability was calculated as the average values of at least three Ussing chambers per subject.

The impact of lipids on jejunal permeability was assessed by measuring permeability to FITC-Dextran 4 kDa in the same jejunal samples before and after addition of lipid micelles prepared as described in supplementary material, Supplementary material and methods. After measuring baseline permeability up to 105 min, lipid micelles were added to luminal chambers and fluorescence was monitored every 15 min until time point 180 min.

Permeability measurements in Caco-2/TC7 cells treated with lipid micelles

We used the TC7 clone of the human intestinal epithelial cell line Caco-2 as a recognized model of well-differentiated enterocytes [29]. Caco-2/TC7 cells were cultured on Transwell® filters (Corning, VWR International, Strasbourg, France). Lipid micelles, prepared as described in supplementary material, Supplementary material and methods, were added to the apical medium on the last day of culture.

To assess paracellular permeability, FITC-labeled Sulfonic Acid (400 Da) or Dextran tracers (4 or 10 kDa) were added to the apical medium. Samples of basal medium were collected at indicated times and fluorescence was determined (BMG Labtech®). Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS apparatus (MilliporeTM, Molsheim, France).

Immunofluorescence analysis of tricellulin was performed as described in supplementary material, Supplementary material and methods.

Statistics

Data are displayed as means ± standard error of the mean (SEM) unless otherwise indicated. We used a generalized linear model (GLM) with binomial family classification for categorical variables and Gaussian family classification for quantitative variables, adjusting for age, sex and BMI (age and sex only when comparing obese and non-obese patients). The relevant p-values were obtained using ANOVA on the full models. Partial correlation (Pearson's family) including correction for age, sex and BMI was used to determine correlation of *in* and *ex vivo* intestinal parameters with clinical data and biological parameters. GLM and partial correlation were computed using R software (R Foundation for Statistical Computing, Vienna, Austria) using *glm* and *ANOVA* functions in package *car* for GLM and *pcor.test* in package *RVAideMemoire* for partial correlation. For inflammation parameters, Principal Component Analysis (PCA) was performed to avoid multiple testing and to detect relevant variables. Kinetics experiments in Caco-2/TC7 cells were analyzed using 2-way ANOVA followed by Tukey's *post hoc* test. Figures were created with Graphpad® Prism 6.0 (Ritme Informatique, Paris, France). $P < 0.05$ was considered as statistically significance.

RESULTS

Tight junctions are altered in the jejunum of severely obese patients

The characteristics of 122 patients included in the study are provided in Table 1. As expected, severely obese patients showed a high prevalence of comorbidities. They exhibited impaired metabolic parameters such as increased fasting glycemia, insulinemia, triglycerides, and decreased HDL-c values.

We evaluated the organization of tight junctions in the jejunum epithelium collected in the fasted state (supplementary material, Figure S1). Fluorescence intensity of occludin and tricellulin labelling in the respective bicellular and tricellular tight junctions were significantly decreased in obese patients as compared with non-obese patients (Figure 1, occludin: -43%, $P<0.0001$, tricellulin: -27%, $P<0.0001$), without modification in the total amount of these proteins (supplementary material, Figure S2). Contrariwise, ZO-1 was unchanged (data not shown).

Intestinal permeability is not increased in fasting obese patients but permeability to small molecules is linked to subclinical inflammation

To assess whether these tight junction impairments were associated with intestine barrier dysfunction, we performed an *ex vivo* measure of intestinal permeability in Ussing chambers using tracers of different molecular weights: 0.4 kDa (representative of disaccharides) and 4 or 10 kDa (representative of larger size components such as dietary or bacterial molecules) [30]. For the three tracers, the distributions of the jejunal permeability values were more heterogeneous in obese patients than in non-obese control group, but no significant difference was found (Figure 2A-C). In agreement with these *ex vivo* data, urinary lactitol/Mannitol (L/M) ratios were not statistically different between obese and non-obese

patients (Figure 3A).

We examined a series of serum markers that have been shown to be associated with impaired barrier function. Fasting serum levels of zonulin and lipopolysaccharide-binding protein (LBP) were increased in obese patients (+13%, P=0.03 and +25%, P<0.0001 respectively, Figure 3B-C). Soluble CD14 (sCD14), another LPS co-receptor, was comparable between obese and non-obese groups (Figure 3D).

No difference between diabetic and non-diabetic obese patients was observed, regardless of the method of intestinal barrier evaluation considered (Figures 1, 2 and 3). We did not find any relevant relationship between the permeability parameters and anti-diabetic treatments (supplementary material, Table S1). Moreover, none of the *in vivo* markers of intestinal permeability or altered barrier function was correlated with the values obtained by direct measurement of jejunal permeability in Ussing chambers (supplementary material, Table S2).

Taking advantage of the heterogeneous distribution in jejunal permability to FITC-SA 0.4 kDa and FITC-Dextran 4 kDa within the obese group, we analyzed whether links could be found between intestinal permeability and subclinical inflammation. Indeed, patients with severe obesity displayed varying levels of systemic low-grade inflammation markers (Table 2) and of fecal calprotectin, a surrogate marker of the intestinal micro-inflammation (Figure 3E). Using partial correlation analysis, we examined the relationships between different parameters of barrier function and a large set of clinical and biological markers (supplementary material, Table S3). Several positive correlations were found between permeability values measured in Ussing chamber and inflammation markers. Permeability to 0.4 kDa tracer was positively correlated with haptoglobin (rho=0.65; P<0.001) and CRP (rho=0.47; P=0.009) (Figure 4A). The distribution of the jejunal permeability values allowed delination of two groups with low or high permeability to 0.4 kDa tracer (Figure 2A). To

assess further the link between inflammation and permeability, we performed Principal Component Analysis (PCA) including 6 different serum markers of inflammation (Table 2) and fecal calprotectin. The first two principal components suggest an important impact of only three markers -CRP, haptoglobin and fibrinogen- which drive the clustering of patients as a function of their jejunal permeability (Figure 4B). Taking into account each marker individually, we confirmed higher levels of haptoglobin and CRP in the high permeability group ($P<0.001$ and $P=0.002$ respectively, Figure 4C) but no statistical difference was observed for fibrinogen levels ($P=0.12$). No link was observed between inflammation and permeability to large molecules (FITC-Dextran 4 kDa) or L/M ratio. Regarding serum biomarkers of altered barrier function, the only correlation with inflammation was found for LBP, whose level was positively correlated with fibrinogen level (Figure 4A and supplementary material, Table S3).

To summarize, no statistical difference was found between fasted obese and non-obese patients concerning their intestinal permeability measured either *in vivo* by the Lactitol/Mannitol test or directly in Ussing chambers. However, altered tight junctions, increased serum levels of two potential markers of altered barrier function (zonulin and LBP), as well as association between jejunal permeability and inflammation suggested that the intestinal barrier function is weakened in severe obesity.

Increased jejunal permeability to large molecules is revealed by a lipid challenge and linked with inflammation and type 2 diabetes

We hypothesized that the subtle impairment of the intestinal barrier observed in the fasting state could be exacerbated by a lipid challenge mimicking a meal.

In a first step, we aimed to test wether dietary lipids exert direct effects on intestinal permeability. For this purpose, we exposed the Caco-2/TC7 human intestinal cells to lipid micelles with a composition reflecting that of micelles in the intestinal lumen after a lipid-rich meal [31]. Whereas transepithelial electrical resistance (TEER) was not modified (supplementary material, Figure S3A), lipid micelles significantly increased Caco-2/TC7 permeability to different tracers, with a stronger effect on permeability to large-size molecules (≥4 kDa), compared to small size molecules (0.4 kDa) (Figure 5A). This increased permeability was associated with a decreased intensity of tricellulin staining (Figure 5B). Micelles containing digestion products of triglycerides ("postprandial micelles") significantly increased paracellular permeability, but micelles containing only biliary products ("interprandial micelles") had no effect (Figure 5A), nor did taurocholic acid or a combination of taurocholic and oleic acid (supplementary material, Figure S3B).

We then evaluated the effect of such an *ex vivo* lipid challenge on the permeability of human jejunum samples. As lipids impacted mostly permeability to macromolecules in Caco-2/TC7 cells, we focused on passage of FITC-dextran 4 kDa, which was measured before and after the addition of postprandial micelles in the luminal reservoir of Ussing chambers. When considering all patients, including non-obese and obese, lipid micelles significantly increased the passage of FITC-Dextran 4 kDa (Figure 6A; $P<0.001$). However, postprandial micelles had a potent effect in obese patients (89.9±9.4 AU at baseline versus 151.2±15.9 AU with micelles; mean fold increase +68%, $P<0.0001$) and a lower impact in non-obese patients (54.1±16.6 AU at baseline versus 78.5±17.2 AU with micelles; mean fold increase +45%, $P<0.001$). Strikingly, the permeability to FITC-Dextran 4 kDa after the lipid challenge was 1.9-fold higher in obese than in non-obese patients (Figure 6B; $P=0.03$). Exposing obese patients' jejunal explants to lipid micelles resulted in a significant decrease in tricellulin fluorescence intensity (-17%), as compared to control explants without micelles (Figure 6C;

P=0.012).

In the obese patients, *ex vivo* jejunal permeability to FITC-Dextran 4 kDa after the micelle challenge showed a positive correlation with markers of systemic (CRP rho=0.44; P=0.010) and intestinal inflammation, (fecal calprotectin rho=0.53; p=0.007) (Figure 4A and supplementary material, Table S3). We used a generalized linear model to explain diabetic status taking into account age, sex, BMI, permeability to FITC-Dextran 4 kDa at baseline and after lipid challenge. We found that age and jejunal permeability after lipid challenge were the only independent variables explaining diabetic status (P=0.008 and P=0.013 for age and permeability, respectively). When corrected for age, BMI, sex and baseline permeability, permeability after micelles was significantly higher in diabetic obese than in non-diabetic-obese patients (Figure 6D; P=0.012). Thus an increased permeability to large molecules after a lipid challenge was linked to the diabetic status.

DISCUSSION

Combining *ex vivo* and *in vivo* approaches, we demonstrate an alteration of the intestinal barrier function in human obesity evidenced by impaired tight junctions and increased levels of seric zonulin and LBP. Increased jejunal permeability was not detected in fasted obese patients but unveiled after a challenge with dietary lipids and linked with inflammation and metabolic alteration.

Intestinal permeability is controlled by tight junctions, which are impaired in inflammatory bowel disease (IBD) [32, 33]. Among the large repertoire of tight junction proteins, our study focused on tricellulin and occludin, two proteins involved in the barrier to macromolecules [34, 35], the latter being found decreased in rodent models of genetic- or diet-induced obesity [11, 36, 37]. In our obese patients, total jejunal occludin and tricellulin were unchanged. However, their levels were decreased in tight junctions, demonstrating for the first time a mislocalization of both proteins in human obesity, as described in other inflammatory conditions [38, 39]. This suggests that human obesity is associated with altered intestinal barrier properties. Contrary to IBD [40], we did not observe any epithelial lesions (data not shown) and we assessed whether the modification of tight junctions in obese patients were sufficient to lead to increased permeability. Considered as the gold standard, the Ussing chamber method allows for direct assessment of permeability in specific intestinal segments, regardless of confounding factors (i.e. intestinal transit time and surface area, tracer degradation, renal function, etc) and has been used to characterize barrier alterations in several animal models [41]. Measured during the fasting state in obese individuals being operated for bariatric surgery, jejunal permeability to small and large molecules was not significantly increased compared to non-obese patients. This result contrasts with obese mice fed a HFD, in which 2 to 10-fold increase in intestinal permeability to macromolecules was reported [11, 20, 21, 42]. However, in our cohort, accurate analysis of phenotypes linked to

obesity revealed associations between jejunal permeability to small molecules and inflammation status. Thus, despite much more modest barrier defects compared to rodent models of obesity, our results demontraste in humans the links between jejunal permeability and obesity-associated low-grade inflammation.

When evaluated in the fasting state, alterations of the intestinal barrier of obese patients were probably too subtle to be clearly evidenced in comparison to non-obese patients. The major finding of our study is that an acute *ex vivo* exposure to dietary lipids mimicking a meal is sufficient to unmask jejunal hyperpermeability to large molecules in severe obesity. Our results in Caco-2/TC7 cells indicate that lipids *per se* are able to increase paracellular permeability through direct effects on tight junctions, and especially tricellular tight junctions, which were recently identified as a pathway for macromolecules [34]. As we previously demonstrated, complex lipid micelles used in the current study preserved cell viability and epithelial integrity [31]. Therefore, increased permeability to macromolecules cannot be attributed to cellular damage. Among lipid micelle components, some bile acids have been shown to alter intestinal barrier function [37, 43]. However, in our conditions, taurocholic acid, the most abundant conjugated primary bile acid, alone or mixed with the other main bile components (cholesterol and L-α-lysophosphatidylcholine), did not change epithelial permeability. Altogether, our results suggest that tight junctions could be modified through the rapid activation of signalling pathways triggered specifically by micelles containing triglyceride hydrolysis products, as previously shown for dietary lipid sensing through the scavenger receptor SR-B1/CLA-1 [44]. Such acute effects of lipid micelles on permeability were replicated in human jejunal samples, with more prominent effects in obese patients, supporting the hypothesis that acute lipid consumption may impair intestinal barrier function with an impact on host response. By promoting the trans-epithelial passage of macromolecules, lipid-mediated alterations of tight junctions could exacerbate local immune

responses with systemic consequences [30]. This is in accordance with our results showing a correlation between gut permeability upon lipid exposure and systemic inflammation assessed by CRP. Furthermore, fecal calprotectin, a surrogate marker of intestinal inflammation in inflammatory bowel disease [45], was correlated with permeability after lipid challenge and thereby could constitute a reliable marker of postprandial-induced barrier alteration in obesity. Our results support the hypothesis that deleterious effects of acute lipid loads on intestinal barrier properties add up to those due to the chronic lipid consumption already described in several previous studies [12]. In obese patients, a weakened barrier during fasting would be more sensitive to nutrient/luminal challenges. To go further in this way, the links between daily food intake and gut permeability remains to be explored in detail. While lipids are a representative nutrient challenge, other molecules known to alter the intestinal barrier including alcohol or non-steroidal anti-inflammatory drugs [2, 46], would be of interest to test in future studies.

Another important result is the observation of an independent link between lipid-induced jejunal hyperpermeability and metabolic conditions of obese patients. Indeed, lipid-induced permeability was a significant explicative variable of type 2 diabetes, independently of age, sex, BMI or baseline jejunal permeability, whereas intestinal permeability measured at the fasting state, regardless of the method, was comparable between diabetic and non-diabetic obese patients. Various tissue and systemic alterations associated with diabetes may increase jejunal sensitivity to lipid-mediated barrier impairment. This includes proinflammatory cytokines, which have been shown to trigger intestinal barrier dysfunction through mecanisms initiated at the basal side of epithelial cells [3]. Importantly, we previously showed immune cell accumulation and local cytokine expression in the jejunum of obese patients according to their metabolic status [10]. Recently, Thaiss et al demonstrated a hyperglycemia-mediated barrier disruption through a mechanism initiated at the basal membrane [47]. Interestingly,

hyperglycemia is associated with decreased bacterial-epithelial distance in the colon, which may contribute to barrier dysfunction [48]. Therefore, the integration of the different signals originating from both luminal and basolateral environments and the interaction between the microbiota and the mucus layer are important factors to investigate in future studies.

In order to establish a reliable clinical measure easily applicable to obese patients, we examined *in vivo* markers of intestinal barrier dysfunction. Urinary L/M ratio has been commonly used to investigate permeability in intestinal and extra-intestinal diseases [49]. Despite this, few studies have investigated *in vivo* permeability in obese patients and the current studies have shown conflicting results [14-16, 50]. Herein, we report no statistical difference in L/M ratios between non-obese patients and patients with severe obesity and found no link with clinical and biological phenotypes**.** However, zonulin, a marker of intestinal tight junction impairment in celiac disease and type 1 diabetes [51] and LBP, a LPS transporter, were increased in obese patients, in accordance with previous observations in overweight or moderately obese patients [18, 52-54]. Notably, none of these *in vivo* biomarkers of altered barrier function correlated with permeability measured in Ussing chambers. Collectively, these results show that a reliable tool measuring permeability for clinical practice remains to be identified in the context of human metabolic disease. The search for intestinal permeability and epithelial integrity biomarkers is also a major issue in other pathological conditions such as IBD. Moreover, the establishment of physiological values for permeability or other parameters reflecting healthy barrier function are a prerequisite for future prospective studies assessing the benefit of surgical or nutritional interventions [55].

In conclusion, the “leaky gut” paradigm can be extended to human severe obesity, although the intestinal barrier dysfunction differs significantly from the rodent models. In human obesity, whereas only subtle barrier alterations are evidenced in the fasting state,

increased jejunal permeability is unequivocally revealed by a lipid load and associated with inflammatory and metabolic status. These results emphasize the need to consider if and how repeated lipid challenges can alter intestinal permeability and play a role in the pathophysiology of intestinal barrier function and downstream metabolic impairment in obesity. Further research on this topic would pave the way for new nutrition strategies in preventive medicine.

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Author contribution statement. C.P, S.T and A.L designed the study. CP coordinated clinical investigation. J.M.O, C.P and KC contributed to obese subject recruitment, patient phenotyping, and sample collection. LG and J.E.S contributed to non-obese subject recruitment and phenotyping. J.C.V, L.G and P.W operated on the patients. L.G, L.D, S.T, D.A., M.R and E.B.L performed Ussing chamber experiments, tight junction analysis and Caco-2/TC7 cells experiments. M.C.M. and F.L performed zonulin, sCD14, and LBP measurements. L.T performed lactitol/mannitol tests. L.G, H.S and K.A performed statistical analyses. C.P, S.T and L.G analyzed data and wrote the manuscript. A.L, K.C, M.R and E.B.L contributed to data presentation and to the manuscript writing. All authors reviewed the manuscript. C.P. and S.T are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Figure 1. Decreased occludin and tricellulin at tight junctions in jejunum of fasted obese patients.

A: Occludin and tricellulin were analyzed by immunofluorescence and confocal microscopy in jejunum sections. Photos show their expected distributions at bicellular and tricellular tight junctions respectively. The schemes display the labeling profile obtained with either top views (as shown on the photos) or side views (shown in B and C and used for quantifications as described below).

B, C: Representative fields of side views of occludin **(B)** and tricellulin **(C)** labeling (white), with Na K ATPase basolateral co-labeling (green) (Scale bar: 10 µm; BB: Brush Border). Fluorescence intensity at the level of tight junction was quantified by Image J® (AU: arbitrary unit) as described in Supplementary Figure 1. For each subject, the value is the median fluorescence obtained by the measure of ~200 tight junctions of the mid-villus from at least 9 fields in tile images with correct orientation according to the Na K ATPase basolateral labeling. **B**: Occludin, non-obese C1 (n=9), obese (n=41) *** $P<0.001$; **C**: Tricellulin, non-obese C1 (n=8), obese (n=39) ***$P<0.001$. Obese patients include non-diabetic obese (black circles) and diabetic obese patients (black triangles). No significant statistical difference was observed according to the presence of type 2 diabetes ($P=0.34$ and $P=0.18$ for occludin and tricellulin respectively). Results are provided as dot plots with mean ± SEM; Generalized linear model (GLM) corrected for age and sex.

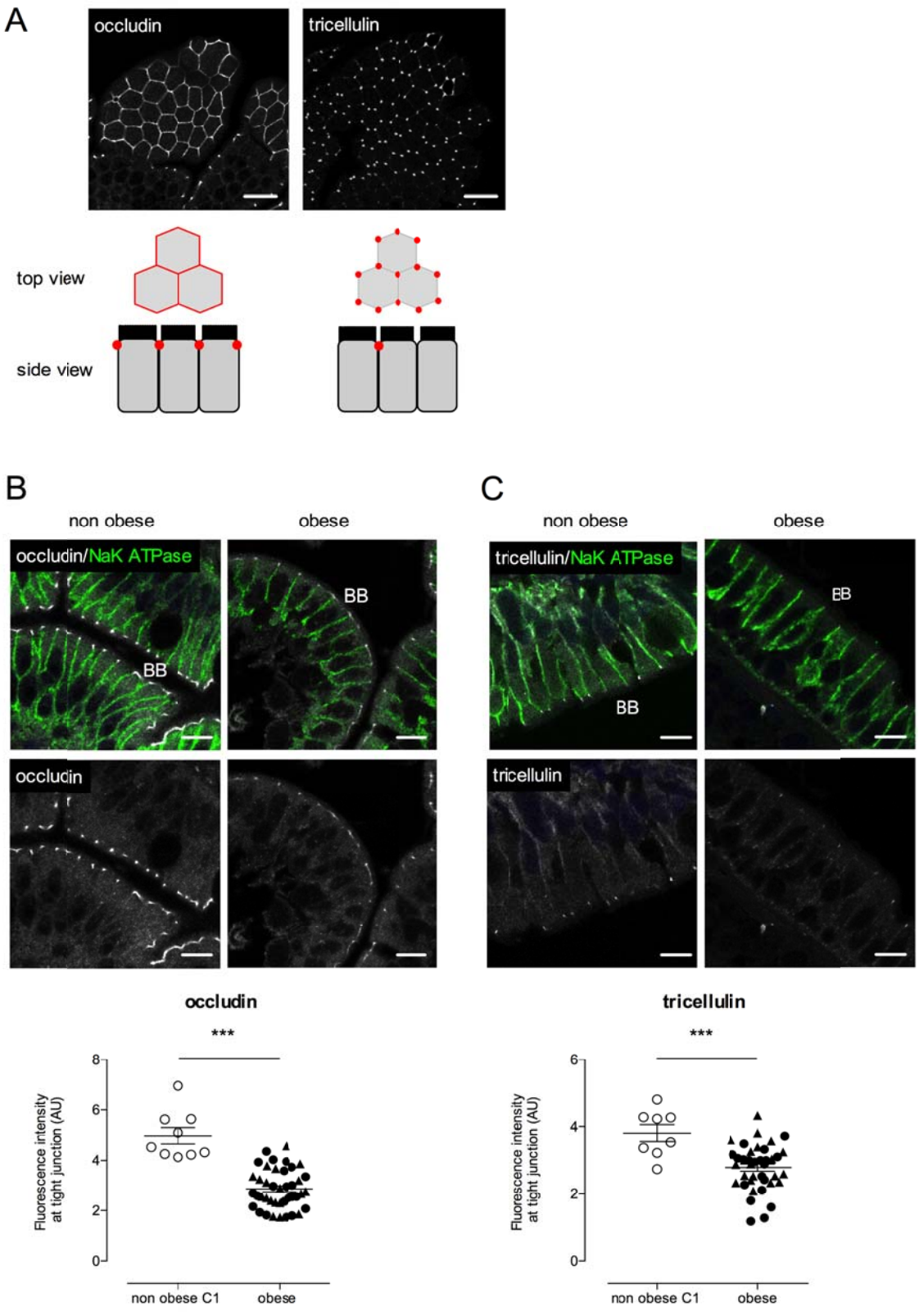
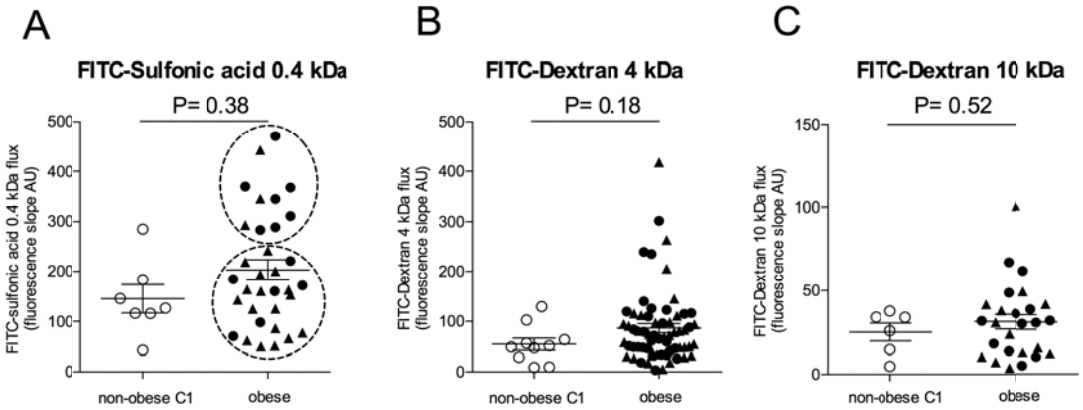


Figure 2: Jejunal permeability measured in Ussing chambers in obese and non-obese patients at fasting state.



Jejunum samples were mounted in Ussing chambers to measure permeability for **A**: FITC-Sulfonic Acid 0.4 kDa, non-obese C1 (n=7), obese (n=33). Dotted circles delineate two subgroups according to the nadir in the distribution of permeability values (Low versus High). **B**: FITC-Dextran 4 kDa, non-obese C1 (n=10), obese (n=65). **C**: FITC-Dextran 10 kDa, non-obese C1 (n=6), obese (n=26). Obese patients include non-diabetic obese (black circles) and diabetic obese patients (black triangles). *Ex vivo* permeability was lower in diabetic obese (ObD) than in obese (Ob) patients for FITC-SA 0.4 kDa (168.3±23 AU in ObD (n=20) versus 257.1±32.6 AU in Ob (n=13), p=0.01) but was comparable when considering FITC-Dextran 4 kDa (P=0.85) and 10 kDa (P=0.84). Data, expressed as fluorescence slope in Arbitrary Units (AU), are provided as dot plots with mean ± SEM; GLM corrected for age and sex.

Figure 3: Lactitol/Mannitol (L/M) excretion ratio, zonulin serum levels, endotoxemia-related parameters and fecal calprotectin in obese and non-obese patients at fasting state.

A: L/M urinary excretion ratio in non-obese C2 (n=29) and obese patients (n=27). **B:** Zonulin serum level (ng/ml) in non-obese C2 (n=30) and obese patients (n=26). * P<0.05. **C and D:** Serum levels of LBP (µg/ml) and sCD14 (µg/ml) in non-obese C2 (n=30) and obese patients (n=27). *** P<0.001. **E**: Fecal calprotectin concentration (µg/g stool) in non-obese C2 (n=29) and obese patients (n=41). * P<0.05. Obese patients include non-diabetic obese (black circles) and diabetic obese patients (black triangles). No differences between Ob and ObD patients were observed for any of these parameters (L/M ratio, P=0.46; zonulin, P=0.39; LBP, P=0.08; sCD14, P= 0.34; fecal calprotectin P= 0.6). Results are provided as dot plots with mean ± SEM; GLM corrected for age and sex.

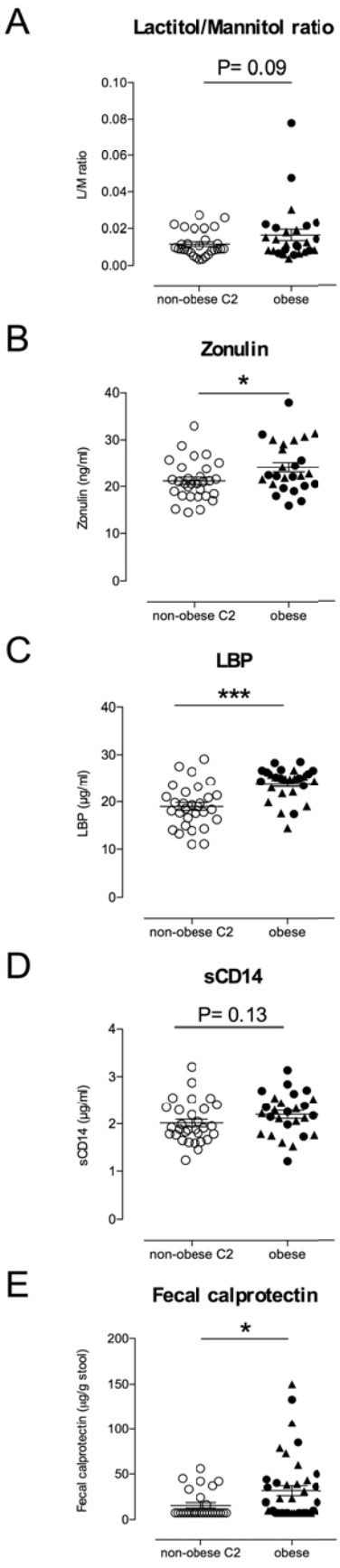


Figure 4: Obesity-related phenotype and jejunal permeability relationships

A: Heat map of correlations between parameters of intestinal barrier function (evaluated either by the L/M ratio, serum levels of zonulin, LBP or sCD14 or permeability measured directly in Ussing chambers) with baseline obesity-related clinical parameters adjusted for age, sex and BMI (partial Pearson correlations). Each colored square denotes a correlation at the intersection of columns and lines. Color intensity (color key) indicates the correlation coefficient magnitude using the red color for positive correlation and blue for negative correlation. Significant P values are indicated for each intersection: * P<0.05 ** P<0.01 *** P<0.001. Detailed Rho, P and n values are presented in Supplementary Table 3. PPM: post-prandial micelles. **B**: Biplot from the PCA of the inflammation markers. Vectors corresponding to the 7 inflammation markers are displayed and subgroups with low (black) and high permeability (green) to the 0.4 kDa tracer, determined according to Figure 2A, are delineated using the convex hull. **C:** Serum levels of haptoglobin, CRP and fibrinogen according to permeability to the 0.4 kDa tracer (low or high, see Figure 2A). ** P<0.01 *** P<0.001. Obese patients include non-diabetic obese (black circles) and diabetic obese patients (black triangles). Results are provided as dot plots with mean ± SEM; GLM corrected for age sex and BMI.

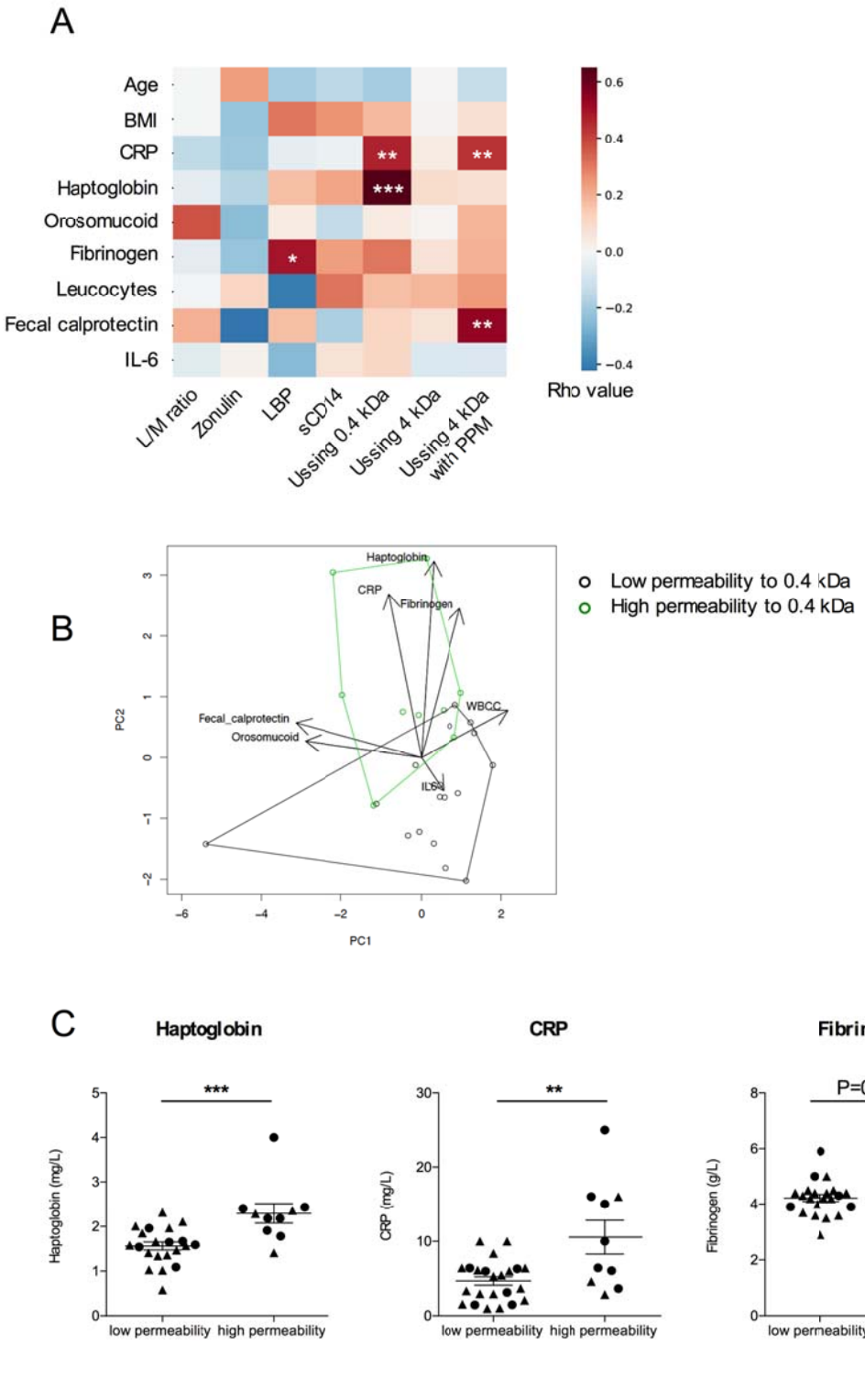
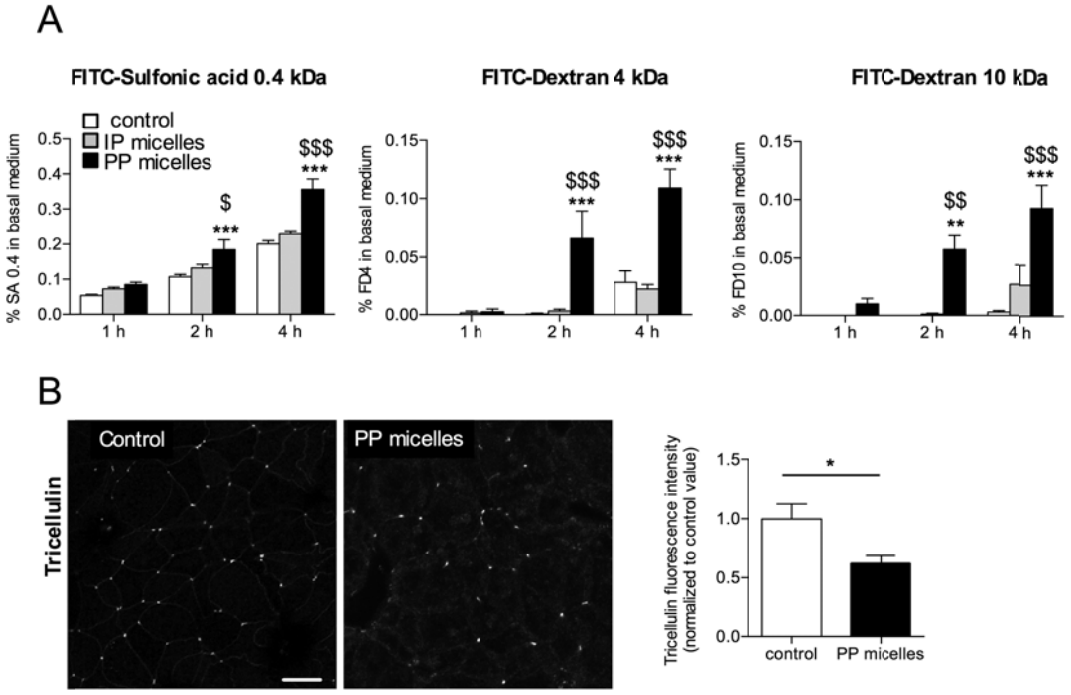
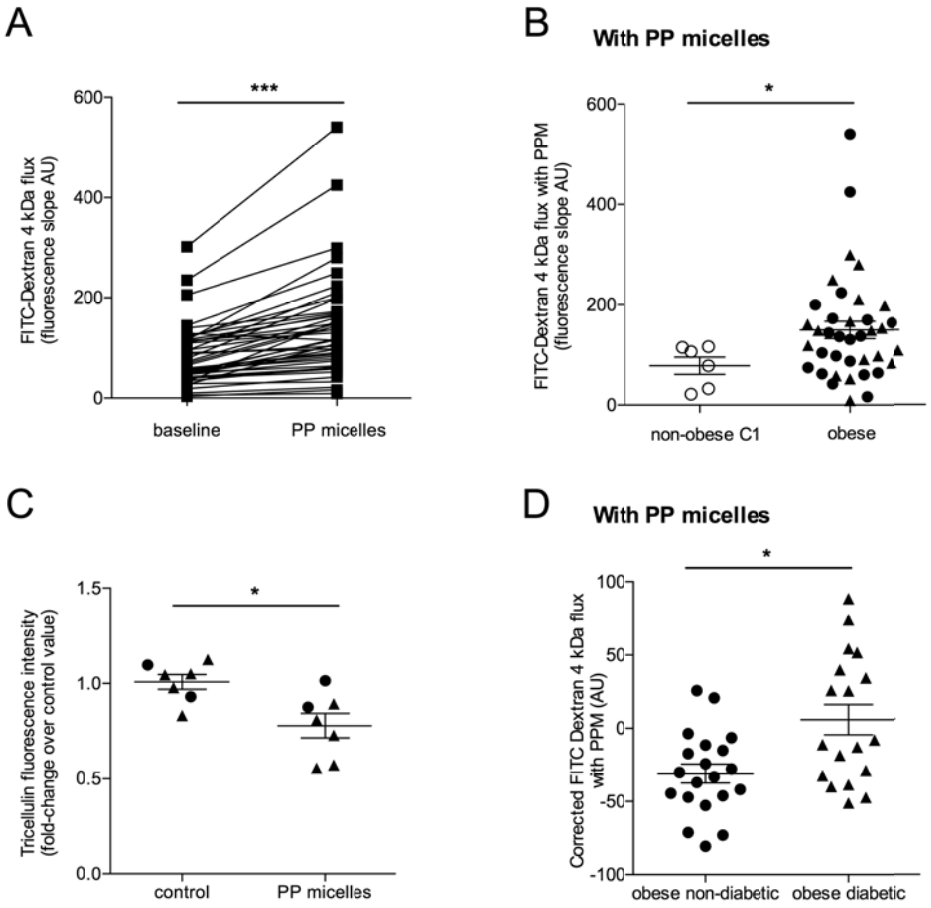


Figure 5: Lipid micelles increase permeability to macromolecules in Caco-2/TC7 enterocytes.



Caco-2/TC7 were cultured on Transwell filters and exposed on their apical side to post-prandial (PP) micelles or interprandial (IP) micelles. **A**: Paracellular permeability was evaluated by the passage of FITC-sulfonic acid (0.4 kDa) or FITC-Dextran (4 or 10 kDa) from the apical to the basal side of the monolayer. Results are expressed as percentage of the initial quantity of tracer in the apical compartment and are mean ± SEM of 3 to 6 replicates. ** P<0.01 *** P<0.001 versus control, $ P<0.05 $$P<0.01 $$$ P<0.001 versus IP micelles (2 way ANOVA; Tukey's post-test). One representative of 2 to 4 independent experiments is presented for each tracer. **B**: Immunofluorescence analysis of tricellulin after exposure to PP micelles; scale bar 10 µm. Fluorescence intensity was quantified using Fiji on 30 different fields from 4 independent experiments.

Figure 6: Lipid micelles reveal increased permeability to macromolecules in human jejunum from obese patients.



A: Jejunum samples mounted in Ussing chambers were analyzed for permeability to FITC-Dextran 4 kDa at baseline and after supply of PP micelles. Fluorescence slope values (AU) are presented for 6 non-obese C1 and 38 obese patients. GLM corrected for age and sex; *** P<0.001. **B**: Permeability to FITC-Dextran 4 kDa after exposure to PP micelles in non-obese C1 (n=6) and obese patients (n=38). Obese patients include non-diabetic obese (black circles) and diabetic obese patients (black triangles). GLM corrected for age, sex and BMI; *P<0.05. **C:** Modification of tricellulin fluorescence intensity at tight junction in presence of PP micelles in 7 obese patients. For each patient, 2 equivalent jejunum samples were taken close

to the mucosa sample used for jejunal permeability assessment in Ussing chambers and incubated in presence or not of PP micelles before fixation and immunolabelling. Non-parametric unpaired Mann-Whitney test. *P<0.05**.** **D**: Variation of FITC-Dextran 4 kDa flux after exposure to PP micelles according to type 2 diabetes status (GLM model: FITC-Dextran 4 kDa flux with PP micelles ~ Age + BMI + Sex + FITC-Dextran 4 kDa flux baseline + type 2 diabetes) in obese patients without (Ob, n=20) or with type 2 diabetes (ObD, n=18); * P<0.05.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods YES

Supplementary figure legends YES

Figure S1. Immunofluorescence analysis of occludin and tricellulin in human jejunum samples

Figure S2. WES analysis of occludin and tricellulin levels in human jejunum samples

Figure S3. Effect of different combinations of lipids on TEER and on permeability to FITC-Dextran 4 kDa

Table S1. Detailed P and n values corresponding to generalized linear model including different intestinal permeability hallmarks and anti-diabetic treatments as categorical variables

Table S2. Detailed Rho, P and n values corresponding to analyses of partial correlation between different hallmarks of altered intestinal barrier function

Table S3. Analyses of partial correlation between obesity-related phenotype and hallmarks of altered intestinal barrier function

		C1 non obese (n=14)	C2 non obese (n=30)	Obese (n=78)	P value*	
					C1 versus obese	C2 versus obese
Demographic data	Sex ratio M/F n (%)	7 (50)/7 (50)	2 (7)/28 (93)	18 (23)/60 (77)	0.037	0.049
	Age (Y)	60.1±3 [30.8-71]	43±2.2 [21.3-63.8]	42.6±1.5 [21-67]	0.0003	0.18
Corpulence and adipokines	Weight (kg)	68.5±3.5 [47-88]	60.7±1.3 [48.5-85]	125.5±2.3 [87.8-194]	<0.0001	<0.0001
	BMI (kg/m²)	23.5±0.9 [18.4-27.6]	22.5±0.3 [18.8-24.9]	45.5±0.6 [37-56.9]	<0.0001	<0.0001
	Fat mass (%)¥	-	27.8±1 [17-37]	47.3±0.6 [27.4-56.9]	-	-
Comorbidities	Type 2 diabetes n (%)	0	0	42 (53.8)	<0.0001	<0.0001
	Hypercholesterolemia n (%)	2 (14.3)	0	36 (46.1)	0.026	<0.0001
	Hypertriglyceridemia n (%)	0	0	41 (52.6)	0.0003	0.0003
	OSA n (%)	0	0	56 (71.8)	<0.0001	<0.0001
	Hypertension n (%)	2 (14.3)	0	36 (46.1)	0.0258	<0.0001
	Systolic blood pressure (mmHg)	127.4±1.7 [123-136]	110.1±1.8 [93-130]	120.8±1.5 [100-157]	0.02	<0.0001
	Diastolic blood pressure (mmHg)	56.2±3.5 [27-67]	71.2±1.3 [61-88]	68±1.3 [50-96]	0.004	0.025
Glucose metabolism	Glycemia (mmol/L)	-	4.6±0.1 [3.8-5.1]	6.6±2.2 [3.5-17.1]	-	<0.0001
	Insulinemia (mU/L)*	-	6.3±0.6 [2.2-18]	22.4±2.2 [0.5-85.6]	-	<0.0001
	HOMA-IR*	-	1.2±0.1 [0.4-3.7]	5.8±0.6 [0.1-25.1]	-	<0.0001
Lipid metabolism	Total Cholesterol (mmol/L)	-	4.9±0.2 [3.3-6.7]	4.7±0.1 [3.1-7.2]	-	0.23
	Triglycerides (mmol/L)	-	0.9±0.3 [0.3-1.8]	1.7±0.1 [0.5-6.2]	-	<0.0001
	HDL (mmol/L)	-	1.6±0.1 [0.8-2.4]	1±0.1 [0.6-1.8]	-	<0.0001
	LDL (mmol/L)	-	2.9±0.1 [1.3-4.3]	2.9±0.1 [1.3-5.3]	-	0.88

Table 1. Clinical and biological baseline characteristics of obese and non-obese (C1 and C2) patients enrolled in the present study

Mean±SEM [min–max]; non-parametric unpaired Mann-Whitney test (quantitative variables); Chi-square test (categorial variables); DXA: Dual-energy X ray absorptiometry; OSA: obstructive sleep apnea syndrome.

¥% of fat mass was measured by impedance for non-obese and DEXA for obese patients

* data not considered in 29 diabetic obese patients treated with sulphonylureas and/or insulin and/or GLP-1 agonists.

		Obese (n=78)
Low-grade inflammation	CRP (mg/L)	8±1 [0.8-58]
	Orosomucoïd (mg/l)	0.9±0.1 [0.2-2.2]
	Haptoglobin (mg/l)	1.8±0.1 [0.6-4]
	IL6 plasma (pg/ml)	6.9±1.9 [1.4-130.8]
	Fibrinogen (g/l)	4.5±0.1 [2.9-6.2]
	Leucocytes ($X10^9/mm^3$)	7.3±0.2 [4-11.7]
Adipokines	Adiponectin (µg/ml)	4.1±0.2 [1.7-11.3]
	Leptin (ng/ml)	61±3.5 [15-164.1]
Metabolic parameters	Visceral fat mass (%)	30.9±2.7 [9.2-75.6]
	HbA1c (%)	6.8±0.1 [5.1-10.6]

Table 2: Inflammation markers, adipokines and metabolic parameters in obese patients

Mean±SEM [min–max]