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RESEARCH ARTICLE

Biomarkers for assessment of intestinal permeability in clinical practice

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Abstract

Intestinal permeability is an important diagnostic marker, yet its determination by established tests, which measure the urinary excretion of orally administered tracer molecules, is time consuming and can only be performed prospectively. Here, we aim to validate proposed surrogate biomarkers, which allow measuring intestinal permeability more easily. In this cross-sectional study, we included two independent cohorts comprising nonobese (Healthy cohort, n = 51) and individuals with obesity (Obesity cohort, n = 27). The lactulose/mannitol (lac/man) ratio was determined in all individuals as an established marker of intestinal permeability. Furthermore, we measured six potential surrogate biomarkers, being albumin, calprotectin, and zonulin, measured in feces, as well as intestinal fatty acid binding protein (I-FABP), lipopolysaccharide binding protein (LBP) and zonulin, measured in plasma. Correlation analyses and multiple linear regression models were conducted to assess possible associations between the established lac/man ratio and the proposed biomarkers by also evaluating a potential effect of age, body mass index (BMI), and sex. The lac/man ratio correlated with plasma LBP levels in all cohorts consistently and with the amount of fecal zonulin in overweight and obese individuals. Multiple linear regression models showed that the association between the lac/man ratio and plasma LBP was independent of age, BMI, and sex. Fecal zonulin levels were associated with the lac/man ratio as well as BMI, but not age and sex. Our data suggest plasma LBP as a promising biomarker for intestinal permeability in adults and fecal zonulin as a potential biomarker in overweight and obese individuals.

NEW & NOTEWORTHY This study shows that biomarkers from blood and fecal samples are associated with the cumbersome established tests of intestinal permeability throughout different cohorts. Therefore, such biomarkers could be used to assess gut barrier function in prospective cohort studies and large-scale clinical trials for which tracer-based tests may not be feasible.

gut barrier; gut health; gut permeability; LBP; zonulin

INTRODUCTION

Intestinal permeability is a diagnostic measure of intestinal barrier integrity and is frequently used to assess intestinal mucosal injury in several disorders, including Crohn's disease and celiac disease (1, 2). Besides gastrointestinal disorders, a broad range of conditions, including aging (3) and overweight (4), can cause increased intestinal permeability, a condition which is associated with several different diseases such as cancer, diabetes, and cardiovascular disease (1). Furthermore, sex-associated differences in intestinal permeability have been observed, with women showing lower intestinal permeability than men (5).

How to measure intestinal permeability has been a matter of debate for many years. There are well-established methods which determine the urinary excretion of orally administered tracer molecules, such as labeled chromium-ethylenediaminetetraacetic acid (^{51}Cr-labelled EDTA), or nondigestible sugars like lactulose (lac) and mannitol (man). These tests are highly respected and are considered as accurate; however, they are time-consuming, lack standardization, cannot be performed retrospectively and still have limited validity based on uncertainty of the proposed normal values (2).

Besides these tests, numerous endogenous proteins have been proposed as intestinal permeability biomarkers, yet so far there is no consensus as to which such parameters are reliable for clinical practice (6, 7). Some proposed biomarkers directly or indirectly measure the translocation of molecules via the intestinal barrier. When the intestinal barrier is damaged, albumin can pass from the blood vessels into the interstitial space and finally into the gut lumen. Therefore, fecal

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albumin has been suggested as a biomarker of intestinal permeability (8), which is commonly used in animal models. Lipopolysaccharide binding protein (LBP) is an acute-phase protein produced by hepatocytes that circulates in the bloodstream. LBP binds to bacterial lipopolysaccharides which in part derive from translocation from the intestine and is therefore considered as a marker of endotoxemia as well as a biomarker of intestinal permeability (9). Zonulin comprises a family of structurally and functionally related proteins, also being described as the zonulin family peptides (10). Zonulin is an acute-phase reaction protein that controls intestinal permeability by decreasing the stability of tight junctions (11) and has been suggested as a biomarker of intestinal permeability (11, 12), which can be measured in both blood and fecal samples. Intestinal fatty acid binding protein (I-FABP; also known as fatty acid binding protein 2) is a cytosolic protein which plays a key role in the cellular uptake and metabolism of fatty acids in enterocytes and is released into the gut lumen in cases of injury (13). Under physiological conditions, only little amounts of I-FABP are found in the bloodstream (14). Therefore, circulating I-FABP may reflect translocation from the gut into the bloodstream and has been suggested as a biomarker of intestinal permeability (15). Calprotecin is released by neutrophils as part of the inflammatory response. Because intestinal inflammation is accompanied both by an increased intestinal permeability and an increase in calprotectin secretion, fecal calprotectin levels have been proposed as an indirect marker of intestinal permeability (1, 16).

In the present study, we aim to validate six potential intestinal permeability biomarkers against the established lac/man test in two independent cohorts comprising normal weight, overweight, and obese individuals.

METHODS

Clinical Trials

Written informed consent was obtained from all individuals involved in the present study. All studies were approved by the respective ethics committee and were registered on clinicaltrials.gov. Healthy cohort (Ethik-Kommission der Landesärztekammer Baden-Württemberg; clinicaltrials.gov, NCT04667208); Obesity cohort (Ethik-Kommission des Universitätsklinikums Tübingen; clinicaltrials.gov, NCT01344525).

Study Cohorts

Healthy cohort.

Fifty-one healthy adults were recruited and examined at the University of Hohenheim for a two-day cross-sectional pilot study, aiming to determine possible associations between potential intestinal permeability biomarkers and the lac/man ratio. On the first study day, the participants provided fecal and blood samples and subsequently drank the test solution, which is described in *Lactulose/Mannitol Test*. Henceforth, urine was collected for 6 h and kept refrigerated at 6–8°C until the samples were returned the next day. The sample size was set to $n = 50$, as a typical sample size for pilot studies. Inclusion criteria comprised written informed consent, age between 18 and 70 yr, and a body mass index (BMI) between 19 and 35 kg/m^2. Exclusion criteria comprised intolerance of artificial sweeteners; acute or chronic disease, including acute or chronic gastrointestinal disorders; serum c-reactive protein levels $>$ 5.0 mg/L; smoking; medication which might interact with gut health, including the intake of e.g., antibiotics, nonsteroidal anti-inflammatory drugs, and immunosuppressants 3 mo before the study; pregnancy and nursing; and withdrawal of consent.

Our group has previously shown that intestinal permeability is associated with body weight (4). To tests such differences, we divided the Healthy cohort according to the BMI into a normal weight subgroup (BMI $<$ 25 kg/m^2; $n = 31$) and an overweight subgroup (BMI $\geq$ 25 kg/m^2; $n = 20$). Details of the Healthy cohort are shown in Table 1.

Obesity cohort.

Individuals who underwent a weight-reduction program at our institute (Optifast^{52}; Nestlé Nutrition) were asked if they would like to participate in a study called "Obesity and the gastrointestinal tract," which was designed to run in parallel to the weight-reduction program. Participants who agreed to volunteer were examined to see if they satisfy the inclusion criteria, being age between 18 and 65 yr, a BMI $\geq$ 30 kg/m^2, and no acute or chronic disorders, including acute or chronic gastrointestinal disorders. Before the start of the study, written informed consent was provided by all participants.

For the present study, we included all participants of the "Obesity and the gastrointestinal tract" study in the Obesity cohort who fulfilled the following criteria. Both plasma and fecal samples were available; no reported intake of medication which might interact with gut health, including e.g., antibiotics, nonsteroidal anti-inflammatory drugs, immunosuppressants 3 mo before the samples were taken; and no diabetes mellitus. This approach led to 27 individuals with obesity which were included in the present study. All plasma and fecal samples were taken at baseline, before start of the weight-reduction program. Details of the Obesity cohort are shown in Table 1.

Lactulose/Mannitol Test

The %lactulose-to-%mannitol excreted ratio (lac/man) was determined in both cohorts after the participants received 5 g of lactulose (Ratiopharm, Ulm, Germany) and 2 g of mannitol (Sigma-Aldrich, St. Louis, MO), whose urinary excretion was analyzed in 6-h collective urine samples. The methodology for the Obesity cohort has been described previously (4). In the Healthy cohort, sample pretreatment was as follows. To remove proteins, 100 μL of fresh 20% sulfosalicylic acid was added to 1,000 μL urine, incubated for 10 min and frozen overnight at −20°C. On the next day, the thawed samples were centrifuged for 10 min at 20,000 *g*. Afterward, ion exchange resin (No. m8157, Sigma-Aldrich, St. Louis, MO) was added and after 20-min incubation the samples were centrifuged for 10 min at 20,000 *g*. High-performance liquid chromatography was performed similarly for both cohorts on a DIONEX Ultimate 3000 device (Thermo Scientific, Waltham, MA) using a Rezex column (no. 00H-0138-K0, Phenomenex, Aschaffenburg, Germany; 70°C) and a Shodex RI-101 Detector (Sho°wa Denko° K.K., Tokyo, Japan; 50°C). As eluent, 0.003 N

Table 1. *Characteristics of the cohorts included in the present study*

	Healthy Cohort		Obesity Cohort
	Normal weight	Overweight	Obese
n	31	20	27[a]
Female/male, *n*	22/9	14/6	14/13
Age, yr	31.0 (28–36)	41.5 (29–54.8)	45.0 (38–49)
BMI, kg/m^2	21.7 (20–24)	28.9 (25–32)	42.2 (41–47)
CRP, mg/L	0.7 (0.3–0.9)	2.1 (1.0–4.2)	7.6 (5.5–11)
Lactulose/mannitol test			
Lactulose, % excreted	0.04 (0.01–0.05)	0.04 (0.02–0.07)	1.1 (0.5–1.1)
Mannitol, % excreted	6.2 (4.4–8.6)	6.7 (4.6–10)	21.7 (20–26)
lac/man ratio	0.006 (0.004–0.007)	0.008 (0.005–0.010)	0.045 (0.028–0.053)
Increased IP, %[b]	3%	15%	93%
IP biomarkers			
Fecal albumin, ng/mg	3.0 (0.9–6.4)	1.9 (1.1–4.7)	0.6 (0.3–1.1)
Fecal calprotectin, ng/mg	17 (9–37)	25.2 (15–44)	36.1 (25–79)
Fecal zonulin, ng/mg	109 (84–155)	130 (94–206)	171 (87–360)
Plasma I-FABP, pg/mL	477 (368–804)	502 (267–829)	840 (495–1072)
Plasma LBP, μg/mL	6.5 (5.6–7.5)	8.0 (6.5–10)	13.7 (8.5–17.1)
Plasma zonulin, ng/mL	42.5 (38–51)	38.5 (30–48)	52.0 (35–75)

The Healthy cohort was stratified according to the body mass index (BMI) into a normal weight subgroup (BMI 19–24.9 kg/m^2) and an overweight subgroup (BMI ≥ 25 kg/m^2). Median and interquartile range (25th–75th percentile) are shown except for female/male which is expressed in absolute numbers and increased IP which is expressed in percent. CRP, c-reactive protein; I-FABP, intestinal fatty acid binding protein; IP, intestinal permeability; lac, lactulose; LBP, lipopolysaccharide binding protein; man, mannitol. [a]For plasma samples n = 20; [b]Percentage of individuals with a lac/man ratio exceeding normal range, defined as means + SD of the normal weight subgroup in the Healthy cohort.

H_2SO_4 with an isocratic rate of 0.4 mL/min was used. Sample time was 40 min.

Intestinal Permeability Biomarkers

We assessed all biomarkers using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocols. Fecal biomarkers comprised albumin, calprotectin, and zonulin (Refs: K6330, K6927, K5600; Immundiagnostik, Bensheim, Germany). Plasma biomarkers comprised I-FABP, LBP, and zonulin (Refs: KR6809, KR6813, K5601; Immundiagnostik).

Statistical Analyses

Spearman's rank correlation coefficients (expressed as r) were used to assess associations between two variables. Multiple linear regression models were used to assess associations between multiple variables after nonmulticollinearity for the independent variables was confirmed. Non-normally distributed variables, tested by Shapiro–Wilk tests, were log-transformed to assure normal distribution. Multiple testing was adjusted with a false discovery rate of 10% according to Benjamini et al. (17). An adjusted P value (q) < 0.1 was considered as statistically significant. Statistical analyses were conducted using Prism version 9.0.2 (GraphPad Software).

RESULTS

According to the lac/man test, 93% in the Obesity cohort, 15% of the overweight participants in the Healthy cohort, and 3% of the normal weight participants from the Healthy cohort had an increased intestinal permeability (Table 1), indicating that in our cohorts intestinal permeability may be associated with body weight. Furthermore, the amounts of fecal calprotectin, fecal zonulin, as well as plasma I-FABP and plasma LBP seemed to increase in parallel to the BMI, being lowest in normal weight individuals and highest in individuals with obesity (Table 1). In contrast, fecal album levels seemed to decrease in parallel to BMI. Plasma levels of zonulin did not seem to be associated with BMI (Table 1).

For the first step in statistically testing a possible relationship between the established lac/man ratio and the six potential surrogate biomarkers, correlation analyses were performed. We conducted these tests separately for the normal weight participants from the Healthy cohort, the overweight participants from the Healthy cohort, and the individuals from the Obesity cohort, resulting in 18 correlations, as shown in Supplemental Table S1 (all Supplemental material is available at https://doi.org/10.6084/m9.figshare.14510622.v3). The amount of fecal albumin correlated with the lac/man ratio solely in the Obesity cohort. Fecal levels of zonulin correlated with the lac/man ratio in the overweight subgroup of the Healthy cohort and the lac/man ratio in the Obesity cohort but not in the normal weight subgroup of the Healthy cohort (Fig. 1, *A–C*), suggesting that the association between the lac/man ratio and fecal zonulin levels might be weight-dependent. Plasma LBP levels correlated with the lac/man ratio in all three cohorts consistently (Fig. 1, *C–F*). There was no correlation between the lac/man ratio and the amount of fecal calprotectin, and also no correlation between the lac/man ratio and plasma levels of zonulin or I-FABP in any of the cohorts (Supplemental Table S1).

To investigate the associations between the lac/man ratio and the potential surrogate biomarkers in more detail, we performed multiple linear regression analyses with the biomarkers as dependent variables and the lac/man ratio, age, BMI, and sex as independent variables. We used this approach for all six biomarkers in the Healthy cohort and the Obesity cohort individually, resulting in 12 multiple regression analyses. Here, all 51 participants from the

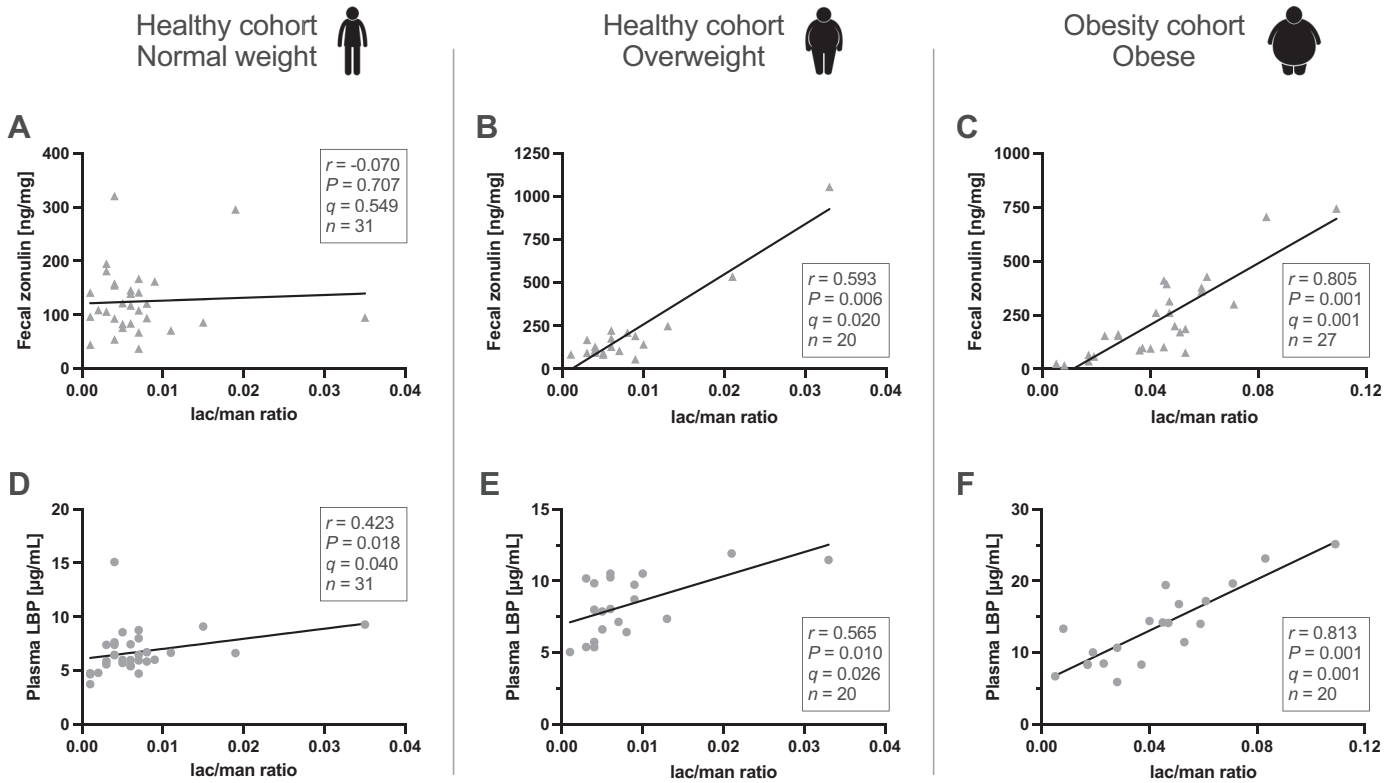

Figure 1. Correlation analyses between the lactulose-to-mannitol (lac/man) ratio and the amounts of the potential intestinal permeability biomarkers zonulin, measured in feces, and lipopolysaccharide binding protein (LBP), measured in plasma. The amount of fecal zonulin correlated with the lac/man ratio in the overweight subgroup of the Healthy cohort and the Obesity cohort, with no such correlation in the normal weight subgroup of the Healthy cohort (*A*–*C*). Plasma LBP levels correlated with the lac/man ratio in all cohorts (*D*–*F*). Spearman's rank correlation coefficient (*r*) with a false discovery rate (FDR) of 10% was conducted. *n* = 20–31 subjects. The *q* value represents the FDR-adjusted *P* value.

Healthy cohort were grouped together because potential weight-dependent effects will be determined by including the BMI as an independent variable into the model.

The multiple linear regression models showed that the amount of fecal albumin was by trend associated with the lac/man ratio in the Obesity cohort, but not in the Healthy cohort ($q = 0.101$; Table 2). This relationship was independent of age, BMI, and sex. Fecal calprotectin levels as well as BMI were associated with the lac/man ratio in the Healthy cohort, but not in the Obesity cohort, with no effect of age and sex on the amount of fecal calprotectin. Fecal zonulin levels were associated with the lac/man ratio as well as BMI in both cohorts consistently, confirming our findings from the initial correlation analyses, that fecal zonulin levels are related to body weight. There was no effect of age and sex on the amount of fecal zonulin. Plasma LBP levels were associated with the lac/man ratio in both cohorts consistently, with no significant effect of age, BMI, or sex. Plasma zonulin levels were inversely associated with the BMI in the Healthy cohort, but not in the Obesity cohort. There was no association between plasma zonulin levels and the lac/man ratio, age, or sex in either of the cohorts and no association between plasma levels of I-FABP and any of the independent variables in either of the cohorts (Table 2).

Finally, we pooled the data derived from the Healthy cohort and the Obesity cohort ($n = 78$) and performed multiple linear regression analyses with the biomarkers as dependent variables and the lac/man ratio, age, BMI, and sex as independent variables. We used this approach for all biomarkers individually, resulting in six multiple linear regression models. In this pooled collective, LBP levels were associated with the lac/man ratio, with no significant effect of age, BMI, or sex (Supplemental Table S2). Fecal zonulin levels were associated with the lac/man ratio ($P = 0.017$), yet this association was not statistically significant after the correction for multiple testing ($q = 0.213$). There was no association between fecal zonulin levels and age, BMI, and sex in the pooled data. Fecal albumin, fecal calprotectin, plasma I-FABP, and plasma zonulin levels showed no association to the lac/man ratio, age, BMI, or sex (Supplemental Table S2).

DISCUSSION

Gut barrier function is of importance in the pathophysiology of numerous diseases of the intestine and beyond. However, implementation of this knowledge into clinical practice was hampered by limitations in the diagnostics regarding both the established tests, like the lac/man ratio, but also regarding potential surrogate biomarkers (1, 2). Here, we show strong associations between the lac/man ratio and plasma LBP in our cohorts of normal weight, overweight, and obese individuals, as well as strong associations between the lac/man ratio and fecal zonulin in overweight and obese individuals. Fecal albumin, fecal calprotectin,

Table 2. *Multiple linear regression models showing the associations between potential permeability biomarkers and the lactulose/mannitol ratio as well as potential confounders, being age, body mass index (BMI) and sex*

		Healthy Cohort ($n = 51$)			Obesity Cohort ($n = 27$)[a]		
Dependent Variable	**Independent Variable/R^2**	**β**	***P* (*q*)**	**R^2 (Adj. R^2)**	**β**	***P* (*q*)**	**R^2 (Adj. R^2)**
Fecal albumin, ng/mg	lac/man ratio	−0.30	0.214 (0.449)		11.5	0.023 (0.101)	
	Age, yr	−0.01	0.408 (0.606)		−0.01	0.414 (0.606)	
	BMI, kg/m^2	−0.01	0.714 (0.794)		0.03	0.116 (0.283)	
	Sex (f/m)	−0.08	0.627 (0.745)		−0.01	0.944 (0.868)	
	R^2			0.06 (−0.02)			0.67 (0.61)
Fecal calprotectin, ng/mg	lac/man ratio	**0.42**	**0.013 (0.069)**		9.41	0.032 (0.129)	
	Age, yr	0.01	0.469 (0.606)		−0.01	0.236 (0.471)	
	BMI, kg/m^2	**0.03**	**0.005 (0.069)**		−0.03	0.156 (0.343)	
	Sex (f/m)	0.19	0.105 (0.283)		0.23	0.040 (0.135)	
	R^2			0.30 (0.24)			0.29 (0.16)
Fecal zonulin, ng/mg	lac/man ratio	**0.28**	**0.005 (0.069)**		**8.07**	**0.012 (0.069)**	
	Age, yr	0.01	0.250 (0.479)		0.01	0.456 (0.606)	
	BMI, kg/m^2	**0.02**	**0.009 (0.069)**		**0.04**	**0.010 (0.069)**	
	Sex (f/m)	0.07	0.290 (0.518)		0.05	0.549 (0.671)	
	R^2			0.29 (0.23)			0.78 (0.74)
Plasma I-FABP, pg/mL	lac/man ratio	0.09	0.694 (0.794)		−7.7	0.109 (0.283)	
	Age, yr	−0.1	0.462 (0.606)		−0.01	0.722 (0.794)	
	BMI, kg/m^2	0.01	0.378 (0.594)		0.03	0.111 (0.283)	
	Sex (f/m)	−0.3	0.841 (0.841)		0.02	0.892 (0.853)	
	R^2			0.03 (0.02)			0.06 (0.03)
Plasma LBP, µg/mL	lac/man ratio	**0.21**	**0.001 (0.004)**		**169**	**0.008 (0.069)**	
	Age, yr	0.00	0.035 (0.129)		−0.02	0.769 (0.826)	
	BMI, kg/m^2	0.01	0.102 (0.283)		0.02	0.947 (0.868)	
	Sex (f/m)	−0.03	0.295 (0.518)		−2.10	0.147 (0.341)	
	R^2			0.43 (0.38)			0.78 (0.72)
Plasma zonulin, ng/mL	lac/man ratio	0.04	0.455 (0.606)		−444	0.376 (0.594)	
	Age, yr	0.01	0.871 (0.852)		−0.15	0.829 (0.841)	
	BMI, kg/m^2	**−0.01**	**0.019 (0.090)**		1.49	0.490 (0.616)	
	Sex (f/m)	0.03	0.350 (0.593)		3.31	0.788 (0.826)	
	R^2			0.14 (0.06)			0.09 (−0.15)

The results of 12 multiple linear regressions adjusted with a false discovery rate (FDR) of 10% are summarized in this table. Results with an FDR-adjusted *P* value (*q* value) < 0.1 are shown in bold. β, β-coefficient, has the unit of the dependent variable divided by the unit of the independent variable; CRP, c-reactive protein; f, female; I-FABP, intestinal fatty acid binding protein; lac, lactulose; LBP, lipopolysaccharide binding protein; m, male; man, mannitol; R^2, coefficient of determination, shows the goodness of fit of the models. [a]For plasma samples n = 20.

plasma I-FABP, and plasma zonulin showed no or inconsistent associations with the lac/man ratio. One reason could be that fecal albumin and fecal calprotectin are rather inflammatory than barrier markers. I-FABP has been successfully used as a barrier marker mostly in severely ill patients (14, 15), but seems less suitable for healthy or obese subjects according to our data. The failure of plasma zonulin as a barrier marker according to our study might be related to the limited specificity of the human ELISA kits, as described below in more detail, whereas mouse data seem to be more consistent (18).

According to our data, LBP might be a valuable biomarker for the assessment of intestinal permeability in adults, independent of age, BMI, and sex. Furthermore, Kuzma et al. (19) recently presented that LBP showed very low intraindividual variation over time in normal weight to obese adults, complementing our findings that LBP might be a robust surrogate marker of intestinal permeability. It should be considered, however, that not only LPS translocation from the intestine but also other factors like bacterial infection can increase circulating LBP (9). Therefore, LBP is possibly not a suitable marker for intestinal permeability in patients with bacterial infections. So far, associations between LBP and established intestinal permeability tests have shown inconsistent results. Some studies reported no correlation between LBP and the established intestinal permeability tests (20–22), whereas in others LBP was markedly higher in patients with an increased lac/man ratio (23). The reason for such differences between the studies is unclear at present.

Fecal zonulin levels were strongly associated with the lac/man ratio and were also affected by BMI, with no significant effect of age and sex. By longitudinal evaluation of another obesity cohort, we showed that BMI, waist circumference as well as fecal zonulin levels and the lac/man ratio decrease in the course of weight-loss therapy, being most pronounced after 3 mo of intervention (4). The lack of correlation in healthy normal weight individuals might be due to the fact that these individuals just have no impairment of their barrier function which is related to the tight junction modulator zonulin. Absence of correlations between fecal zonulin and the lac/man ratio in healthy normal weight individuals have also been reported by others (24, 25). To our knowledge, associations between fecal zonulin levels and established intestinal permeability tests have not been assessed for overweight and/or obese individuals.

Using commercially available kits to assess zonulin levels is a matter of debate since it was shown that these kits do not detect zonulin, previously thought to be a single molecule, but rather several structurally resembling proteins (26–28). However, from a clinical point of view, this question may not be of major

relevance for using these ELISA kits to assess zonulin as a marker of intestinal permeability, if the results correlate well with the established tests (29). Furthermore, these concerns based on ELISA kits which measured circulating zonulin and, to our knowledge, it is unknown whether this aspect also affects measurement of fecal zonulin. Further studies are needed, both on a methodological side to strengthen the reliability of commercially available zonulin kits, and on a clinical side, to test the reliability of zonulin as a marker of intestinal permeability.

Some studies indicate that intestinal permeability may also be altered in a proportion of first-degree relatives (30, 31) whereas others did not find such association (32, 33). In patient history assessment, we did not evaluate severe gastrointestinal disease in first-degree relatives of our study participants. Another limitation of our study might be that the cohorts were not matched in terms of age and sex, with the Healthy cohort comprising younger participants and a higher ratio of women than the Obesity cohort. The potential role of age and sex in this context needs to be further analyzed in future studies. Despite these limitations, our independent study cohorts uniformly show strong statistical associations between the lac/man ratio and the amount of fecal zonulin and plasma LBP levels.

To sum up, our data suggest LBP as a promising biomarker of intestinal permeability in adults, which according to our data is independent of age, BMI, and sex. Furthermore, fecal zonulin may be a biomarker of intestinal permeability for overweight and obese adults.

SUPPLEMENTAL DATA

Supplemental Tables S1 and S2: https://doi.org/10.6084/m9.figshare.14510622.v3.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.S., M.B., A.M.N., J-A.N., J.W., N.M.D., and S.C.B. conceived and designed research; B.S. and M.B. performed experiments; B.S. analyzed data; B.S. and S.C.B. interpreted results of experiments; B.S. prepared figures; B.S. and S.C.B. drafted manuscript; B.S., M.B., A.M.N., J.-A.N., J.W., N.M.D., and S.C.B. approved final version of manuscript.

REFERENCES

1. **Bischoff SC**, **Barbara G**, **Buurman W**, **Ockhuizen T**, **Schulzke J-D**, **Serino M**, **Tilg H**, **Watson A**, **Wells JM.** Intestinal permeability—a new target for disease prevention and therapy. *BMC Gastroenterol* 14: 189, 2014. doi:10.1186/s12876-014-0189-7.
2. **Camilleri M.** Leaky gut: mechanisms, measurement and clinical implications in humans. *Gut* 68: 1516–1526, 2019. doi:10.1136/gutjnl-2019-318427.
3. **Man AL**, **Bertelli E**, **Rentini S**, **Regoli M**, **Briars G**, **Marini M**, **Watson AJM**, **Nicoletti C.** Age-associated modifications of intestinal permeability and innate immunity in human small intestine. *Clin Sci* 129: 515–527, 2015. doi:10.1042/CS20150046.
4. **Damms-Machado A**, **Louis S**, **Schnitzer A**, **Volynets V**, **Rings A**, **Basrai M**, **Bischoff SC.** Gut permeability is related to body weight, fatty liver disease, and insulin resistance in obese individuals undergoing weight reduction. *Am J Clin Nutr* 105: 127–135, 2017. doi:10.3945/ajcn.116.131110.
5. **Edogawa S**, **Peters SA**, **Jenkins GD**, **Gurunathan SV**, **Sundt WJ**, **Johnson S**, **Lennon RJ**, **Dyer RB**, **Camilleri M**, **Kashyap PC**, **Farrugia G**, **Chen J**, **Singh RJ**, **Grover M.** Sex differences in NSAID-induced perturbation of human intestinal barrier function and microbiota. *FASEB J* 32: 6615–6625, 2018. doi:10.1096/fj.201800560R.
6. **Schoultz I**, **Keita ÅV.** The intestinal barrier and current techniques for the assessment of gut permeability. *Cells* 9: 1909, 2020. doi:10.3390/cells9081909.
7. **Bischoff SC**, **Kaden-Volynets V**, **Filipe Rosa L**, **Guseva D**, **Seethaler B.** Regulation of the gut barrier by carbohydrates from diet—underlying mechanisms and possible clinical implications. *Int J Med Microbiol* 311: 151499, 2021. doi:10.1016/j.ijmm.2021.151499.
8. **Wang L**, **Llorente C**, **Hartmann P**, **Yang A-M**, **Chen P**, **Schnabl B.** Methods to determine intestinal permeability and bacterial translocation during liver disease. *J Immunol Methods* 421: 44–53, 2015. doi:10.1016/j.jim.2014.12.015.
9. **Schumann R**, **Leong SR**, **Flaggs G**, **Gray P**, **Wright S**, **Mathison J**, **Tobias P**, **Ulevitch R.** Structure and function of lipopolysaccharide binding protein. *Science* 249: 1429–1431, 1990. doi:10.1126/science.2402637.
10. **Wang W**, **Uzzau S**, **Goldblum SE**, **Fasano A.** Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci* 113: 4435–4440, 2000. doi:10.1242/jcs.113.24.4435.
11. **Fasano A**, **Not T**, **Wang W**, **Uzzau S**, **Berti I**, **Tommasini A**, **Goldblum SE.** Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 355: 1518–1519, 2000. doi:10.1016/S0140-6736(00)02169-3.
12. **Sapone A**, **de Magistris L**, **Pietzak M**, **Clemente MG**, **Tripathi A**, **Cucca F**, **Lampis R**, **Kryszak D**, **Cartenì M**, **Generoso M**, **Iafusco D**, **Prisco F**, **Laghi F**, **Riegler G**, **Carratu R**, **Counts D**, **Fasano A.** Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 55: 1443–1449, 2006. doi:10.2337/db05-1593.
13. **Ockner RK**, **Manning JA.** Fatty acid-binding protein in small intestine. Identification, isolation, and evidence for its role in cellular fatty acid transport. *J Clin Invest* 54: 326–338, 1974. doi:10.1172/JCI107768.
14. **Thuijls G**, **van Wijck K**, **Grootjans J**, **Derikx JPM**, **van Bijnen AA**, **Heineman E**, **Dejong CHC**, **Buurman WA**, **Poeze M.** Early diagnosis of intestinal ischemia using urinary and plasma fatty acid binding proteins. *Ann Surg* 253: 303–308, 2011. doi:10.1097/SLA.0b013e318207a767.
15. **Wells JM**, **Brummer RJ**, **Derrien M**, **MacDonald TT**, **Troost F**, **Cani PD**, **Theodorou V**, **Dekker J**, **Méheust A**, **de Vos WM**, **Mercenier A**, **Nauta A**, **Garcia-Rodenas CL.** Homeostasis of the gut barrier and potential biomarkers. *Am J Physiol Gastrointest Liver Physiol* 312: G171–G193, 2017. doi:10.1152/ajpgi.00048.2015.
16. **von Martels JZH**, **Bourgonje AR**, **Harmsen HJM**, **Faber KN**, **Dijkstra G.** Assessing intestinal permeability in Crohn’s disease patients using orally administered 52Cr-EDTA. *PLoS One* 14: e0211973, 2019. doi:10.1371/journal.pone.0211973.
17. **Benjamini Y**, **Krieger AM**, **Yekutieli D.** Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* 93: 491–507, 2006. doi:10.1093/biomet/93.3.491.
18. **Tajik N**, **Frech M**, **Schulz O**, **Schälter F**, **Lucas S**, **Azizov V**, **Dürholz K**, **Steffen F**, **Omata Y**, **Rings A**, **Bertog M**, **Rizzo A**, **Iljazovic A**, **Basic M**, **Kleyer A**, **Culemann S**, **Krönke G**, **Luo Y**, **Ûberla K**, **Gaipl US**, **Frey B**, **Strowig T**, **Sarter K**, **Bischoff SC**, **Wirtz S**, **Cañete JD**, **Ciccia F**, **Schett G**, **Zaiss MM.** Targeting zonulin and intestinal epithelial barrier function to prevent onset of arthritis. *Nat Commun* 11: 1995, 2020. doi:10.1038/s41467-020-15831-7.
19. **Kuzma JN**, **Hagman DK**, **Cromer G**, **Breymeyer KL**, **Roth CL**, **Foster-Schubert KE**, **Holte SE**, **Weigle DS**, **Kratz M.** Intra-individual variation in markers of intestinal permeability and adipose tissue inflammation in healthy normal weight to obese adults. *Cancer Epidemiol Biomarkers Prev* 28: 610–615, 2019. doi:10.1158/1055-9965.EPI-18-0641.

20. **Kuzma JN**, **Cromer G**, **Hagman DK**, **Breymeyer KL**, **Roth CL**, **Foster-Schubert KE**, **Holte SE**, **Weigle DS**, **Kratz M.** No differential effect of beverages sweetened with fructose, high-fructose corn syrup, or glucose on systemic or adipose tissue inflammation in normal-weight to obese adults: a randomized controlled trial. *Am J Clin Nutr* 104: 306–314, 2016. doi:10.3945/ajcn.115.129650.
21. **Nylund CM**, **D'Mello S**, **Kim M-O**, **Bonkowski E**, **Däbritz J**, **Foell D**, **Meddings J**, **Trapnell BC**, **Denson LA.** Granulocyte macrophage-colony-stimulating factor autoantibodies and increased intestinal permeability in Crohn disease. *J Pediatr Gastroenterol Nutr* 52: 542–548, 2011. doi:10.1097/MPG.0b013e3181fe2d93.
22. **Pedersen C**, **Ijaz UZ**, **Gallagher E**, **Horton F**, **Ellis RJ**, **Jaiyeola E**, **Duparc T**, **Russell-Jones D**, **Hinton P**, **Cani PD**, **Ragione RML**, **Robertson MD.** Fecal Enterobacteriales enrichment is associated with increased in vivo intestinal permeability in humans. *Physiol Rep* 6: e13649, 2018. doi:10.14814/phy2.13649.
23. **Vogt A**, **Reuken PA**, **Stengel S**, **Stallmach A**, **Bruns T.** Dual-sugar tests of small intestinal permeability are poor predictors of bacterial infections and mortality in cirrhosis: a prospective study. *World J Gastroenterol* 22: 3275–3284, 2016. doi:10.3748/wjg.v22.i11.3275.
24. **de Roos NM**, **van Hemert S**, **Rovers JMP**, **Smits MG**, **Witteman BJM.** The effects of a multispecies probiotic on migraine and markers of intestinal permeability-results of a randomized placebo-controlled study. *Eur J Clin Nutr* 71: 1455–1462, 2017. doi:10.1038/ejcn.2017.57.
25. **Russo F**, **Linsalata M**, **Clemente C**, **Chiloiro M**, **Orlando A**, **Marconi E**, **Chimienti G**, **Riezzo G.** Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers. *Nutr Res* 32: 940–946, 2012. doi:10.1016/j.nutres.2012.09.010.
26. **Ajamian M**, **Steer D**, **Rosella G**, **Gibson PR.** Serum zonulin as a marker of intestinal mucosal barrier function: may not be what it seems. *PLoS One* 14: e0210728, 2019. doi:10.1371/journal.pone.0210728.
27. **Massier L**, **Chakaroun R**, **Kovacs P**, **Heiker JT.** Blurring the picture in leaky gut research: how shortcomings of zonulin as a biomarker mislead the field of intestinal permeability. *Gut*, 2020. In press. doi:10.1136/gutjnl-2020-323026.
28. **Scheffler L**, **Crane A**, **Heyne H**, **Tönjes A**, **Schleinitz D**, **Ihling CH**, **Stumvoll M**, **Freire R**, **Fiorentino M**, **Fasano A**, **Kovacs P**, **Heiker JT.** Widely used commercial ELISA does not detect precursor of haptoglobin2, but recognizes properdin as a potential second member of the zonulin family. *Front Endocrinol (Lausanne)* 9: 22, 2018. doi:10.3389/fendo.2018.00022.
29. **Fasano A.** Zonulin measurement conundrum: add confusion to confusion does not lead to clarity. *Gut*, 2020. In press. doi:10.1136/gutjnl-2020-323367.
30. **Teshima CW**, **Goodman KJ**, **El-Kalla M**, **Turk S**, **El-Matary W**, **Valcheva R**, **Danchak R**, **Gordon M**, **Ho P**, **Mullins A**, **Wong D**, **Kao D**, **Meddings J**, **Huynh H**, **Dieleman LA.** Increased intestinal permeability in relatives of patients with Crohn's disease is not associated with small bowel ulcerations. *Clin Gastroenterol Hepatol* 15: 1413–1418.e1, 2017. doi:10.1016/j.cgh.2017.02.028.
31. **Secondulfo M**, **de Magistris L**, **Fiandra R**, **Caserta L**, **Belletta M**, **Tartaglione MT**, **Riegler G**, **Biagi F**, **Corazza GR**, **Carratù R.** Intestinal permeability in Crohn's disease patients and their first degree relatives. *Dig Liver Dis* 33: 680–685, 2001. doi:10.1016/S1590-8658(01)80045-1.
32. **Kevans D**, **Turpin W**, **Madsen K**, **Meddings J**, **Shestopaloff K**, **Xu W**, **Moreno-Hagelsieb G**, **Griffiths A**, **Silverberg MS**, **Paterson A**, **Croitoru K**; **GEM Project.** Determinants of intestinal permeability in healthy first-degree relatives of individuals with Crohn's disease. *Inflamm Bowel Dis* 21: 879–887, 2015 [Erratum in *Inflamm Bowel Dis* 21: 1673, 2015]. doi:10.1097/MIB.0000000000000323.
33. **Turpin W**, **Espin-Garcia O**, **Bedrani L**, **Madsen K**, **Meddings JB**, **Raygoza Garay JA**, **Silverberg MS**, **Smith MI**, **Griffiths AM**, **Moayyedi P**, **Marshall JK**, **Mack D**, **Seidman EG**, **Ropeleski M**, **Feagan BG**, **Jacobson K**, **Turner D**, **Walters T**, **Paterson AD**, **Project Research Consortium CCG**, **Xu W**, **Croitoru K**; **CCC GEM Project Research Consortium.** Analysis of genetic association of intestinal permeability in healthy first-degree relatives of patients with Crohn's disease. *Inflamm Bowel Dis* 25: 1796–1804, 2019. doi:10.1093/ibd/izz116.