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Putative intestinal permeability markers do not correlate with cardiometabolic health and gut microbiota in humans, except for peptides recognized by a widely used zonulin ELISA kit

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Abstract *Background and aims:* Cardiometabolic diseases refer to a group of interrelated conditions, sharing metabolic dysfunctions like insulin resistance, obesity, dyslipidemia, and hypertension. The gut microbiota has been associated with CMD and related conditions. Alterations in the intestinal epithelium permeability triggered by chronic stress and diet could bridge gut microbiota with inflammation and CMD development. Here, we assessed the relationship between intestinal permeability and circulating SCFAs with cardiometabolic health status (CMHS) and gut microbiota in a sample of 116 Colombian adults.

Methods and results: Plasma levels of lipopolysaccharide-binding protein (LBP), intestinal fatty acid-binding protein (I-FABP), claudin-3, and purported zonulin peptides (PZP) were measured by ELISA, whereas plasmatic levels of acetate, propionate, butyrate, isobutyrate, and valerate were measured by gas chromatography/mass spectrometry. In addition, for further statistical analysis, we took data previously published by us on this cohort, including gut microbiota and multiple CMD risk factors that served to categorize subjects as cardiometabolically healthy or cardiometabolically abnormal. From univariate and multivariate statistical analyses, we found the levels of I-FABP, LBP, and PZP increased in the plasma of cardiometabolically abnormal individuals, although only PZP reached statistical significance.

Conclusions: Our results did not confirm the applicability of I-FABP, LBP, claudin-3, or SCFAs as biomarkers for associating intestinal permeability with the cardiometabolic health status in these subjects. On the other hand, the poorly characterized peptides detected with the ELISA kit branded as "zonulin" were inversely associated with cardiometabolic dysfunctions and gut microbiota. Further studies to confirm the true identity of these peptides are warranted.

Abbreviations: Blood pressure, BP; Cardiometabolic diseases, CMD; Cardiometabolic health status, CMHS; Co-abundant microorganisms, CAGs; Enzyme-linked immunosorbent assays, ELISA; Intestinal fatty acid-binding protein, I-FABP; Intestinal permeability, IP; Short-chain fatty acids, SCFAs; Lipopolysaccharide, LPS; Lipopolysaccharide-binding protein, LBP; Purported zonulin peptides, PZP.

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1. Introduction

Cardiometabolic diseases, including cardiovascular diseases and type 2 diabetes, are major causes of the global disease burden, affecting mainly middle-to low-income countries [1,2]. The transition of lifestyles towards more westernized habits, such as less physical activity and diets rich in fats and sugars and low in fiber, has increased the prevalence, over the past thirty years, of metabolic dysfunctions like insulin resistance, glucose intolerance, dyslipidemia, hypertension, and central obesity, which are independent risk factors associated with cardiometabolic diseases (CMD). Furthermore, in Latin America, the percentage of adults accumulating more than three of these metabolic dysfunctions is about 20%. As a result, one out of seven people is at risk of having a cardiovascular event [3].

Variations in the gut microbiota composition have been associated with the development of cardiometabolic diseases in North American, European, and Asian populations [4–7]. Recently, we demonstrated that the gut microbiota of Colombians (South America) is composed of five consortia of co-abundant microorganisms that are differentially associated with cardiometabolic health, lifestyle, and diet [8]. Although such associations have been commonly described, the underlying mechanisms remain unclear. Increased intestinal permeability has been proposed to trigger dysbiosis, inflammation, and CMD development [9]. Gut microbiota has been suggested to modulate energy metabolism by producing short-chain fatty acids (SCFAs) like acetate, propionate, butyrate, and valerate by the anaerobic fermentation of indigestible dietary carbohydrates. SCFAs taken up by the gut epithelium are used as metabolic precursors in lipogenesis (acetate), gluconeogenesis (propionate), and as a source of energy for the colonocytes (butyrate) [10]. Furthermore, SCFAs produced by the gut microbiota improve gut health through several local effects, including the production of mucus, the depletion of intracellular O_2, and maintenance of the intestinal barrier integrity [11,12].

The gut epithelium can be divided into three anatomical regions: (i) the mucus layer, followed by (ii) a semi-permeable monolayer of different specialized cells joined through protein complexes forming tight junctions that allow the paracellular transport of substances and the diffusion of ions in both directions (lumen-circulation), and (iii) the immunological component found in the lamina propria below the epithelial cells [13]. The mucus layer acts as the first line of defense, and it is made up of glycoproteins called mucins [14]. The intestinal epithelial cells also work as a line of defense to protect the different tissues from the translocation of potentially inflammatory antigens [15]. Some factors can negatively affect the integrity of the gut barrier, increasing intestinal permeability, like dysbiosis [16], chronic stress [17], and high-fat and high-sugar diets [18]. Impairment of the gut barrier is tightly linked to a disruption in intestinal anaerobiosis, which drives the expansion of *Proteobacteria*, a dysbiotic signature of epithelial dysfunction [16]. In addition, increased intestinal permeability facilitates bacterial antigens to translocate [19] and activate the immune system [9], which contributes to low-grade inflammation, and the development of cardiometabolic diseases [20].

Increased intestinal permeability has been reported in multiple human and animal studies of cardiometabolic diseases. It is generally identified using a combination of circulating markers and functional tests [21]. The oral administration of non-metabolizable probes is the method of choice to assess intestinal permeability. The percentage of urinary excretion of the probe, reflects the degree of intestinal permeability [22]. However, this method is challenging to use in large cohorts or simply unfeasible in cross-sectional studies. As an alternative, the analysis of blood-circulating membrane and tight-junction proteins, such as the intestinal fatty acid-binding protein (I-FABP) [23], claudin-3 [24], and zonulin (although some technical concerns have risen when using commercially available ELISA kit's) [25], as well as markers of translocated bacterial products, like lipopolysaccharide (LPS) binding protein (LBP) and D-lactate [26], have been proposed and used.

Here, we quantified plasmatic intestinal permeability biomarkers I-FABP, claudin-3, and PZP, as well as the SCFAs acetate, propionate, butyrate, isobutyrate and valerate, to assess the associations among intestinal permeability, gut microbiota, and cardiometabolic health status (CMHS), in a sample of 116 adult men and women (18–62 years) from a Colombian cohort previously characterized by us, with known gut microbiota that is differentially associated with the CMHS of the subjects.

2. Methods

2.1. Studied population

We selected a convenience sample of 116 male and female adult subjects (18–62 years) from our previous study on Colombians (South America) [27], whose gut microbiota was described by five consortia of co-abundant microorganisms (CAGs) (*i.e.,* CAG-Prevotella, CAG-Lachnospiraceae, CAG-Pathogen, CAG-Ruminococcaceae, and CAG-Akkermansia-Bacteroidales); differentially associated with CMD risk factors. Each of these subjects had a gut microbiota dominated by one of the CAGs mentioned above (but non-null abundances of the other CAGs; note that CAGs are not equivalent to enterotypes). The participants were categorized according to the strict criteria for the cardiometabolic profile assessment implemented by Tomiyama et al. [28], in which individuals accumulating at least two of the following metabolic dysfunctions were described as cardiometabolically abnormal: systolic/diastolic blood pressure >130/85 mm Hg or consumption of antihypertensive medication; fasting triglycerides >150 mg/dL; HDL cholesterol <40 mg/dL (men), <50 mg/dL (women) or consumption of lipid-lowering medications; fasting glucose >100 mg/dL or consumption of antidiabetic medication, homeostatic model assessment-insulin resistance (HOMA-IR) >3, and high-sensitivity C reactive protein (hs-CRP) >3 mg/L. Subjects with one or

none of the above dysfunctions were cardiometabolically healthy.

The original study excluded pregnant women, consumption of antibiotics and antiparasitics three months before enrollment, and subjects diagnosed with the following diseases: neurodegenerative, recent cancer (<1 year), or gastrointestinal (Crohn's disease, ulcerative colitis, short bowel syndrome, diverticulosis, or celiac disease). Participants had a BMI>18.5 kg/m^2 and were from the five most populated cities in Colombia (Bogota, Medellin, Cali, Barranquilla, and Bucaramanga). The study design was stratified by city, sex at birth, age range (18–40, 41–62), and BMI category (lean, overweight, obese). Anthropometric and biochemical parameters were retrieved from the original database [8,27]. The samples used in the present study consisted of those for which a second informed consent was obtained (n = 116; plasmas stored at −80 °C). The Ethics Institutional Committee for Research in Humans of CES University (Medellin, Colombia; approval acts 122–2018 and 149–2020) approved the described procedures.

2.2. Measurements of intestinal permeability biomarkers

Commercially available enzyme-linked immunosorbent assays (ELISA) were used to measure plasmatic levels of intestinal permeability biomarkers according to the manufacturer's instructions. The levels of I-FABP were measured in 1:10 diluted plasmas using a Duoset kit (R&D Systems, Minneapolis, USA). Claudin-3 levels were measured using the Human claudin-3 ELISA Kit (Novus Biologicals™, Colorado, USA). Plasma levels of purported zonulin were measured using the Human zonulin ELISA kit (Cusabio, Wuhan, China), a kit based on monoclonal antibodies against the 293 cell-derived full-length human recombinant prehaptoglobin-2, for which the supplier reports negligible cross reactivity with haptoglobin. After reactions, absorbance was measured using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) set at 450 nm wavelength and corrected at 570 nm. Plasma concentrations of each biomarker were interpolated from 4-parameter regression lines using Graphpad Prism Software version 9.

2.3. Measurement of plasmatic short-chain fatty acids (SCFAs)

To quantify the SCFAs, thawed plasma samples were mixed in a Thermomixer (Eppendorf, AG, Germany) for 1 min at 2000 rpm, and 200 μL were transferred into a 1.5 mL tube. Then, 50 μL of 5% trichloroacetic acid (Sigma, Darmstadt, Germany) was added and mixed using the same conditions. Next, 200 μL of chloroform were added and remixed for 4 min at 2000 rpm. The samples were then centrifuged at 4 °C for 10 min at 17,000×*g* in a Micro CL17R microcentrifuge (Thermo Scientific, Waltham, MA, USA). Afterward, 150 μL of supernatants were collected and transferred into chromatography vial inserts. Samples were injected in the splitless mode into an Agilent 7890A gas chromatography (GC) system (Wilmington, DE, USA) equipped with a 5975C mass spectrometer (MS) detector and an Agilent J&W DB-WAX column (30 m × 0.25 mm × 0.250 μm). The oven temperature was programmed to start at 70 °C (2 min), to increase to 230 °C at 20 °C/min, then to 240 °C, and finally at 270 °C. The MS was tuned during all experiments; the signal acquisition for quantification was made in the SCAN mode. The temperature of the ionization source and the quadrupole were 230 °C and 150 °C, respectively. The electron-impact ionization energy was 70 eV. For quantification, a mix of acetic, propionic, butyric, isobutyric, and valeric acids at 50 ppm prepared on the day of analysis was used as the external standard. The chromatographic peaks were checked for homogeneity using extracted ions of the characteristic fragments to optimize the resolution and peak symmetry. Concentrations of acetate, propionate, butyrate, isobutyrate, and valerate were expressed in μM. Total SCFAs were the sum of acetate, propionate, butyrate, isobutyrate, and valerate.

2.4. Statistical analysis

For the statistical analysis, we used different packages included in the R software version 4.1.1 (2021-08-10) "Kick Things" (Boston, MA). First, the readxl (V 1.3.1), tidyverse (V 1.3.1), mice (V 3.14.0), and lares (V 5.1.1) packages were used to retrieve, clean, impute missing values and structure the database of biochemical and anthropometrical measurements from the previous study [8]. Then, subjects were classified according to their gut microbiota composition and CMHS.

Quantitative variables were summarized as mean ± SD and 95% confidence intervals. Qualitative variables were organized in a contingency table and summarized as a percentage of the total; Chi-square tests were used to compare frequencies among groups. To determine the associations of intestinal permeability biomarker levels with gut microbiota composition and CMHS, we first analyzed the data using a generalized multivariate regression model assuming a quasi-Poisson distribution of the data, built to assess the differences in the levels of intestinal permeability biomarkers between subjects with different CMHS and gut microbiota. These models were adjusted for potential confounders, including sex, age range, and the participant's city of origin. Next, multiple testing was adjusted with a false recovery rate of 5% using the q-value package (V 2.24.0). A FDR-adjusted p-value (q) < 0.05 was considered statistically significant. Afterward, Spearman's correlations and principal component analysis (PCA) were obtained with the R packages Corrplot (V 0.92), Hmisc (V 4.6-0), FactomineR (V 2.4), and Factoextra (V 1.0.7). Finally, in the PCA, the difference between cardiometabolically healthy and cardiometabolically abnormal individuals was assessed using the Mahalanobis distance between the group's centroids with the package HDMD (V 1.1) applying the two-sample Hotelling's T^2 (V 1.0–8) test, according to Goodpaster and Kennedy [29]. Then, an F-test was used to determine whether the centroids' separation was statistically different.

3. Results

3.1. Cardiometabolic health status and gut microbiota

Of the 116 subjects included in this study, 72 (62%) were classified as cardiometabolically abnormal, while 44 (38%) were considered cardiometabolically healthy. As per the study design, there were no statistical differences between these two groups by age range, sex, or city of origin. In addition, there were no differences either in alcohol drinking or smoking habits (Table 1). As expected, statistically significant differences were found in anthropometric measurements, such as BMI and waist circumference, among individuals categorized as cardiometabolically healthy vs. cardiometabolically abnormal (Table 1). In the same line, plasmatic levels of triglycerides, blood pressure, insulin, fasting glucose, hs-CRP, as well as blood pressure, and HOMA-IR were significantly increased in cardiometabolically abnormal subjects ($p < 0.05$). On the other hand, HDL levels were significantly reduced in this group of individuals, while LDL and total cholesterol plasmatic concentration presented no statistical difference (Table 1). Sensitivity analyses, in which subjects were classified in healthy-abnormal categories according to lipid profile, blood pressure, BMI, and insulin resistance, showed similar results except for hs-CRP, which was no longer significant when individuals were categorized by lipid profile, blood pressure, or insulin resistance (Tables S1–S4).

Cardiometabolically healthy individuals tended to have a diverse gut microbiota enriched in bacteria classified within the CAG-Ruminococcaceae, although this was only borderline significant. In comparison, cardiometabolically abnormal subjects displayed a less diverse gut microbiota with a high representation of potentially pathogenic microbes (CAG-Pathogen) ($q = 0.01$). Abundances of gut microbiota belonging to the CAG-Prevotella, CAG-Lachnospiraceae, and CAG-Akkermansia-Bacteroidales remained similar between the two groups of subjects (Table 1).

3.2. Cardiometabolic health status and intestinal permeability

Significant Spearman correlation coefficients were observed between most variables informing about cardiometabolic dysfunctions, gut microbiota, and intestinal permeability (Fig. S1). PZP were positively and significantly correlated with BMI ($r = 0.21$) systolic blood pressure ($r = 0.19$), diastolic blood pressure ($r = 0.21$), HOMA-IR ($r = 0.21$), and the plasmatic levels of triglycerides ($r = 0.32$), and fasting insulin ($r = 0.20$), while inversely correlated with the plasmatic levels of HDL ($r = -0.26$), butyrate ($r = -0.24$) and isobutyrate ($r = -0.19$). LBP, on the other hand, positively and significantly correlated with plasmatic levels of hs-CRP ($r = 0.35$), claudin-3 ($r = 0.20$), and the gut microbiota (CAG-Pathogen; $r = 0.18$). Levels of I-FABP did not correlate with markers of cardiometabolic dysfunctions or the gut microbiota but did correlate negatively with levels of claudin-3 ($r = -0.18$). Additionally, besides LBP, plasmatic levels of claudin-3 also correlated positively with LDL ($r = 0.19$) and total cholesterol ($r = 0.20$). On the other hand, regarding plasmatic SCFA levels, we did not observe any correlations with CMD biomarkers. Finally, the gut microbiota alpha diversity was inversely correlated with the plasmatic levels of acetate ($r = -0.27$), whereas the fecal abundance of CAG-Lachnospiraceae correlated positively with the plasmatic levels of isobutyrate ($r = 0.21$) and valerate ($r = 0.24$), and inversely correlated with the plasmatic levels of acetate ($r = -0.20$). Intestinal permeability biomarkers, SCFAs, and gut microbiota are associated with CMD at different levels.

The multivariate-adjusted regression analyses indicated that, of the selected biomarkers of intestinal permeability, only the plasmatic levels of PZP were significantly higher in cardiometabolically abnormal compared to cardiometabolically healthy subjects ($q = 0.005$) (Table 2). Additionally, PZP levels were significantly increased in the subjects categorized by independent cardiometabolic dysfunctions, including hypertension ($q = 0.004$), dyslipidemia ($q = 0.02$), insulin resistance ($q = 0.05$), and overweight ($q = 0.01$) (Fig. 1A–D (Fig. 1A–D). The plasmatic levels of LBP tended to be higher in cardiometabolically abnormal ($q = 0.13$) (Table 2) and hypertensive ($q = 0.15$) subjects, but these differences did not reach statistical significance (Table S1). The plasmatic levels of I-FABP and claudin-3 and those of acetate, propionate, butyrate, isobutyrate, and valerate were similar between cardiometabolically abnormal and cardiometabolically healthy subjects (Table 2). The same trend was observed when subjects were categorized by blood pressure, lipid profile, and HOMA-IR (Tables S1–S3). However, dyslipidemic and insulin-resistant subjects tended to have lower plasmatic levels of the measured SCFAs than normolipidemic and insulin-sensitive individuals (Tables S2 and S3). Noteworthy, lean and overweight subjects had significantly higher plasmatic propionate and butyrate levels than obese subjects, but the statistical significance was lost after adjusting p-values (Table S4). In addition to cardiometabolic dysfunctions classification, there was no difference in the circulating levels of acetate, propionate, isobutyrate, butyrate, valerate or the total of the SCFA and intestinal permeability biomarkers, LBP, I-FABP, PZP, and claudin-3 among individuals categorized by CAGs. Nonetheless, CAG-Lachnospiraceae had the highest levels of propionate, isobutyrate, and valerate; while CAG-Ruminococcaceae had increased levels of butyrate (Table S5).

Next, we performed a PCA after excluding the variables used to categorize the subjects by CMHS to avoid multicollinearity. To visualize patterns in the data and identify variables that could differentiate between cardiometabolically healthy and cardiometabolically abnormal individuals, we generated a scatter biplot of the observed individuals and variables using the first two principal components (PC1 and PC2). These PCs accounted for 35.8% of the total variance (Fig. 2). PC1 explained 21.8% of the variance and was significantly associated

Table 1 Characteristics of the studied population according to CMHS. Data presented as the mean ± SD and 95% CI.

Parameter	Cardiometabolically healthy n = 44		Cardiometabolically abnormal n = 72		p-value	q-value
Sex (%)						
Female	22		34		0.58	–
Male	22		38			
City of origin (%)						
Medellin (%)	8.26		9.92		0.30	
Barranquilla (%)	4.96		17.36			
Cali (%)	12.40		14.05			
Bogota (%)	6.61		8.26			
Bucaramanga (%)	8.26		9.92			
Life style						
Smoking (%yes)	4.96		14.88		0.08	–
Drinking (%yes)	16.53		31.40		0.20	–
Anthropometry, blood pressure, and blood chemistry	**Mean ± SD**	**95% CI**	**Mean ± SD**	**95% CI**		
Age (years)	39 ± 12	(36–43)	41 ± 12	(39–44)	0.10	0.15
Waist circumference (cm)	86.0 ± 10.1	(82.9–89.1)	98.4 ± 12.5	(95.5–101.3)	**< 0.001**	**< 0.001**
BMI (kg/m^2)	25.1 ± 3.5	(24.1–26.2)	29.4 ± 4.6	(28.3–30.5)	**< 0.001**	**< 0.001**
Systolic blood pressure (mm Hg)	117 ± 15	(112–121)	131 ± 20	(126–136)	**< 0.001**	**< 0.001**
Diastolic blood pressure (mm Hg)	74 ± 10	(71–77)	83 ± 13	(80–86)	**< 0.001**	**< 0.001**
Triglycerides (mg/dL)	101.2 ± 41.6	(88.5–113.9)	179.0 ± 126.1	(149.4–208.7)	**< 0.001**	**< 0.001**
HDL (mg/dL)	50.7 ± 8.2	(48.2–53.2)	40.6 ± 10.8	(38.1–43.2)	**< 0.001**	**< 0.001**
LDL (mg/dL)	119.2 ± 33.6	(109.0–129.4)	116.8 ± 33.5	(109.0–124.7)	0.58	0.68
Total cholesterol (mg/dL)	187.40 ± 39.30	(175.50–199.40)	189.60 ± 39.10	(180.40–198.80)	0.94	0.94
Fasting glucose (mg/dL)	84.10 ± 6.00	(82.20–85.90)	91.20 ± 16.10	(87.40–95.00)	**0.03**	0.06
Fasting insulin (uU/ml)	8.40 ± 2.60	(7.60–9.20)	16.60 ± 9.70	(14.30–18.90)	**< 0.001**	**< 0.001**
hs-CRP (mg/L)	1.60 ± 1.00	(1.30–1.90)	3.90 ± 5.60	(2.60–5.30)	**0.004**	**0.01**
HOMA-IR	1.70 ± 0.60	(1.60–1.90)	3.80 ± 2.50	(3.20–4.40)	**< 0.001**	**< 0.001**
Gut microbiota (%)						
CAG-Prevotella	20.95 ± 25.27	(13.27–28.63)	13.71 ± 21.14	(8.75–18.68)	0.19	0.25
CAG-Pathogen	8.67 ± 14.98	(4.11–13.22)	23.10 ± 29.07	(16.27–29.93)	**0.004**	**0.01**
CAG-Lachnospiraceae	19.99 ± 22.14	(13.26–26.72)	25.22 ± 23.39	(19.72–30.71)	0.19	0.25
CAG-Akkermansia-Bacteroidales	20.62 ± 26.08	(12.69–28.55)	16.67 ± 23.33	(11.19–22.16)	0.53	0.66
CAG-Ruminococcaceae	14.08 ± 15.67	(9.32–18.85)	8.48 ± 12.02	(5.66–11.31)	0.06	0.10
α–Diversity (OTU richness)	132.34 ± 38.77	(120.55–144.13)	116.53 ± 34.33	(108.46–124.60)	0.06	0.10

BMI: Body mass index; HDL: High-density lipoprotein, LDL: Low-density lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; hs-CRP: high-sensitive C-reactive protein, CAG: co-abundant groups; OTU: operational taxonomic unit. P-values from multivariate models adjusted by sex, city, and age range; q-values <0.05 from multivariate-adjusted models were considered significant.

Table 2 Plasmatic levels of putative intestinal permeability biomarkers and SCFAs according to CMHS. Data presented as the mean ± SD and 95% CI.

	Cardiometabolically healthy n = 44		Cardiometabolically abnormal n = 72		p-value	q-value
	Mean ± SD	95% CI	Mean ± SD	95% CI		
Intestinal Permeability markers (ng/ml)						
Claudin-3	2.81 ± 1.77	(2.27–3.35)	3.04 ± 2.08	(2.55–3.53)	0.75	0.79
I-FABP	0.97 ± 0.68	(0.76–1.17)	0.99 ± 0.70	(0.83–1.16)	0.62	0.69
LBP	4.32 ± 1.48	(3.87–4.77)	5.07 ± 1.60	(4.70–5.45)	0.08	0.13
PZP	3.81 ± 5.43	(2.16–5.46)	6.67 ± 9.12	(4.53–8.81)	**0.002**	**0.005**
Plasma Short chain fatty acids (μM)						
Acetate	598.24 ± 543.85	(432.90–763.59)	626.24 ± 847.90	(426.99–825.48)	0.41	0.59
Propionate	130.10 ± 125.80	(91.85–168.34)	148.08 ± 141.79	(114.76–181.40)	0.61	0.72
Butyrate	59.60 ± 90.05	(32.22–86.98)	46.71 ± 55.32	(33.71–59.70)	0.72	0.77
Isobutyrate	73.85 ± 80.48	(49.39–98.32)	79.14 ± 75.82	(61.32–96.95)	0.17	0.29
Valerate	24.41 ± 24.18	(17.06–31.76)	23.97 ± 27.97	(17.40–30.55)	0.72	0.77
Total SCFAs	886.2 ± 641.33	(691.2–1081.19)	924.13 ± 888.12	(715.43–1132.83)	0.61	0.72

LBP: lipopolysaccharide-binding protein, I-FABP: intestinal fatty acid-binding protein, PZP: purported zonulin peptides. P-values from multivariate models adjusted for sex, age range, and the participant's city of origin, q-values < 0.05 were considered significant.

(correlation coefficient to the dimension; −log10 p-value) with BMI (0.8; 50.4), waist circumference (0.7; 49.7), PZP levels (0.3; 8.5), the abundances of the CAG-Pathogen (0.5; 20), and CAG-Prevotella (0.2; 3.6). PC2 accounted for 14% of the variance and was significantly associated with waist circumference (0.3; 8.1), PZP (0.3; 5.3), claudin-3 (−0.2; 3.9) and LBP levels (−0.5; 21.7), gut microbiota alpha diversity (OTU richness; 0.5; 17.4), and the abundances of the CAG-Prevotella (0.5; 12.6), CAG-Ruminococcaceae (0.5; 15.8), CAG-Pathogen (−0.2; 3.8), and the CAG-Akkermansia-Bacteroidales (−0.7; 33.5). Analysis using the Mahalanobis distance (MD) from the centroids of both groups of subjects and their comparison according to the Hotelling T^2 distribution indicated that there was a separation between subjects (MD = 1.67, T^2 = 76.8, $F_{(2,113)}$ = 37.1, $p < 0.05$). Cardiometabolically healthy subjects displayed higher gut microbiota diversity (i.e., OTU richness) and higher abundances of the CAG-Ruminococcaceae and the CAG-Akkermansia-Bacteroidales than cardiometabolically abnormal subjects. In contrast, cardiometabolically abnormal subjects were associated with higher waist circumference, BMI, plasmatic levels of PZP, and the abundances of the CAG-Prevotella and the CAG-Pathogen (Fig. 2).

4. Discussion

In recent years, gut microbiome and intestinal dysfunction have emerged as potential contributors to the development of cardiometabolic diseases [30]. Gut microbiota dysbiosis has been associated with the risk of cardiometabolic diseases. Individuals with insulin resistance, glucose intolerance, dyslipidemia, hypertension, and central obesity have a less diverse gut microbiota with an increased abundance of pathobionts, particularly from the phylum *Proteobacteria* [31]. In addition, an altered gut permeability has been suggested to bridge the extra-intestinal effects of gut microbiota on human health [32]. In this study, we selected a sample of a deeply phenotyped cohort, including 116 subjects previously characterized in gut microbiota, and analyzed with different risks of cardiometabolic diseases and previously characterized gut microbiota to understand the interplay among intestinal permeability, cardiometabolic dysfunctions, and gut dysbiosis. We found that, among the tested biomarkers of intestinal permeability, the plasmatic levels of protein/peptides detected by the antibodies set from the Cusabio zonulin ELISA kit were significantly augmented in cardiometabolically abnormal individuals. A similar trend was observed in LBP analysis but not in claudin-3 or I-FABP.

Zonulin is described as a family of structurally and functionally similar proteins related to haptoglobin that evolved from a complement-associated protein (mannose-binding lectin-associated serine protease, or MASP) that modulate intercellular tight junctions to reversibly control intestinal permeability [33]. The first member described of this family was the inactive precursor (pre-HP2) of haptoglobin-2 (HP2). Mature HP2 is known to generate complexes with hemoglobin to protect it from oxidative damage [34]. Pre-HP2 is secreted mainly from the liver but can also be secreted from enterocytes and adipose tissue [35]. It is released under different stimuli in which the bacterial exposure in the epithelium is intesified, leading to its activation. Once active, zonulin can open or close the tight-junction protein complex, leading to the regulation of intestinal permeability [25]. Furthermore, zonulin transgenic mouse (Ztm) studies have shown that zonulin can trigger gut barrier impairment and is an early step leading to the development of chronic inflammatory diseases [36].

In our Colombian cohort, we found that the poorly characterized protein/peptides detected by the Cusabio zonulin ELISA kit (that we referred to as PZP) were significantly increased in cardiometabolically abnormal, dyslipidemic, and insulin-resistant individuals. It correlated positively with plasmatic levels of triglycerides and insulin resistance (HOMA-IR) and negatively with HDL cholesterol. Interestingly, when comparing the PZP levels across BMI categories, they were significantly higher in individuals with excess weight, supporting the idea that

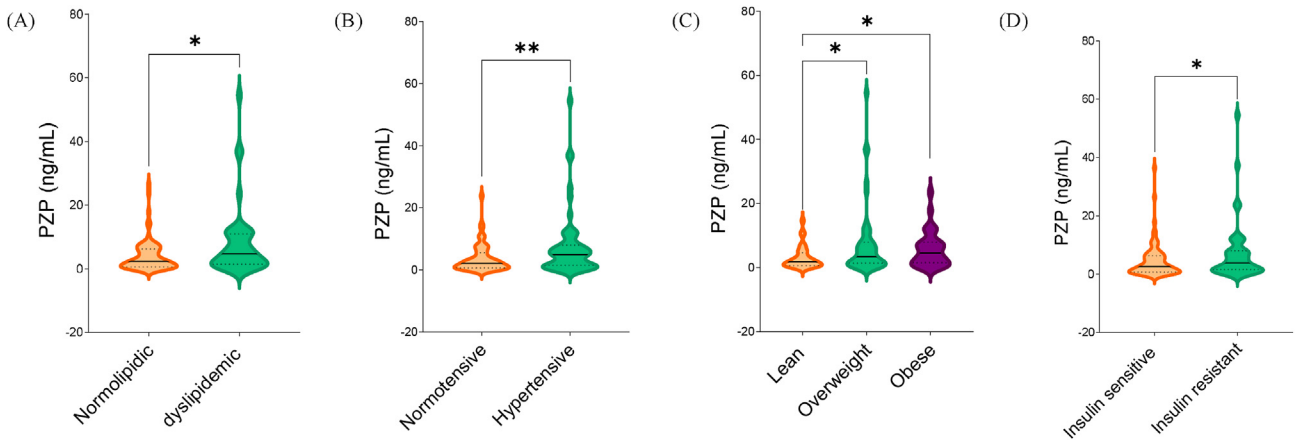

Figure 1 Plasmatic levels of purported zonulin peptides (PZP) among subjects categorized by cardiometabolic dysfunctions. Subjects with cardiometabolic dysfunction display higher levels of PZP. Violin plots of PZP with mean and error bars (95% confidence interval) A) Lipid profile, B) Blood pressure, C) BMI and D) Insulin resistance. * q-values < 0.05 from multivariate regression models adjusted by sex, age range, and the participant's city of origin were considered significant.

these peptides might be related to glucose and lipid metabolism. Furthermore, they were not only associated with metabolic risk factors in our study. We also found that their levels were positively associated with gut microbiota, displaying higher protein levels in the plasma of individuals with dysbiotic signatures, such as reduced microbial diversity and exacerbated abundance of potentially pathogenic microbes (CAG-Pathogen). A recent study

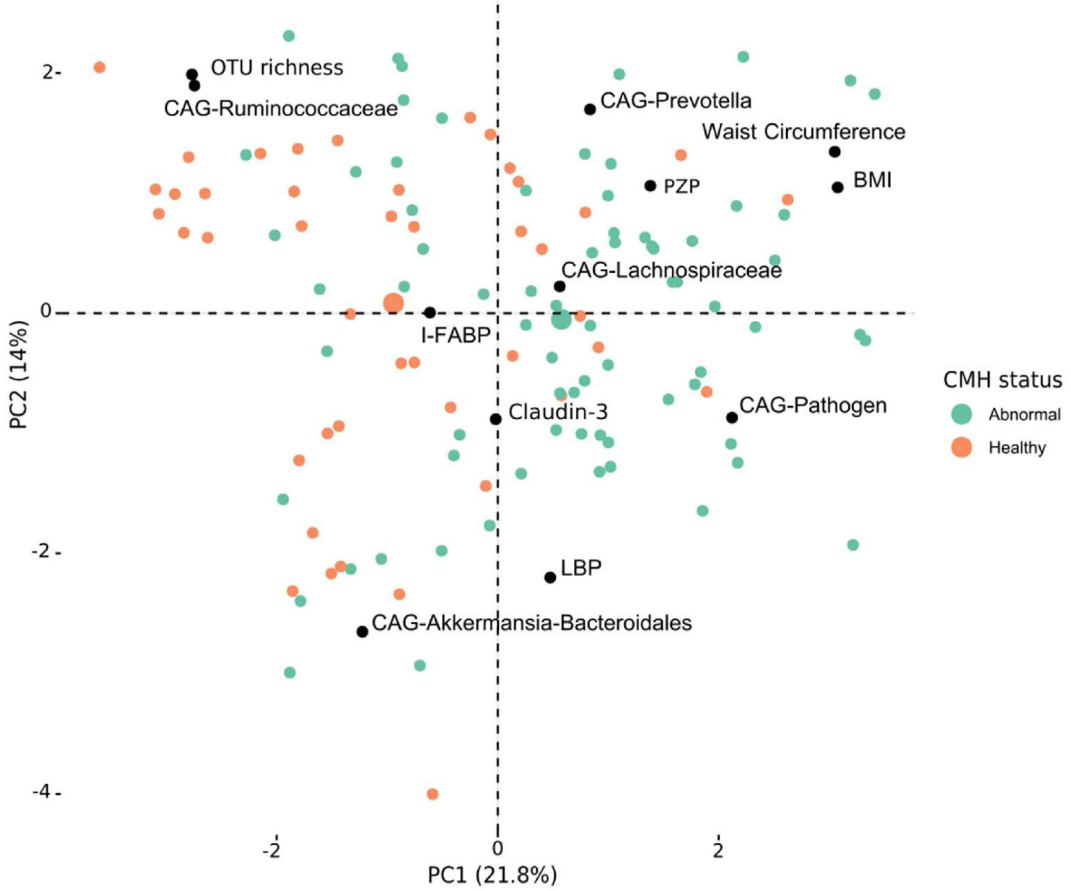

Figure 2 Scores and loadings PCA biplot grouping individuals by CMHS. (Cardiometabolically healthy = Orange dots, cardiometabolically abnormal = green dots). Cardiometabolically abnormal subjects are associated with higher levels of purported zonulin peptides (PZP), waist circumference, BMI, and gut microbiota (CAGs Prevotella, Pathogen, and Lachnospiraceae). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed that increased zonulin in patients with arthritis was associated with gut dysbiosis that could later affect the transition from asymptomatic autoimmunity to an inflammatory state [37]. Ztm mice have an altered gut microbiota in which there was a reduction in the abundance of the phylum Verrucomicrobia [36], especially the genus *Akkermansia*, which has been associated with cardiometabolic health and gut barrier integrity [38]. Additionally, the gut microbiota of Ztm mice was enriched in Betaproteobacteria, potentially pathogenic microorganisms associated with a higher risk of metabolic disease [20,36]. Our results are in line with these findings.

While our results on purported zonulin protein family are coherent with the association among cardiometabolic risk factors, gut microbiota, and intestinal permeability, they must be interpreted with caution. Recently the identity of the peptides quantified by different commercially available ELISA assays [39,40] and their correlation to functional intestinal permeability tests, such as the lactulose-mannitol [41–43], have been extensively debated. For instance, Meira de-Faria et al. [44] found that the plasma levels of zonulin (CUSABIO's) or pre-HP2 (Bio-Rad, specific monoclonal antibody, not yet available on the market) did not directly correlate with ^{51}Cr-EDTA permeability test in colonic biopsies of irritable bowel syndrome (IBS) patients. Also, there was no increase in the circulating levels of pre-HP2 in the IBS patients, opposite to PZP levels that increased in IBS and celiac disease patients (CeD) suggesting that purported zonulin peptides (PZP) could be more related to inflammation rather than intestinal permeability [44]. Even more, studies have shown that commercially available ELISA kits do not detect significant amounts of pre-HP2 but several unknown and possibly related proteins [39,40]. For example, Scheffler et al. [40] measured serum zonulin in subjects from a well-characterized cohort and found that the immundiagnostik set of antibodies capture complement C3 as shown in western blots but the ELISA kit failed to quantify pre-HP2 or complement C3 isolated from human plasma [40]. Later, after further characterization of the antigens captured by the kit's polyclonal antibody, a chymotrypsin-like serine protease was identified, most likely properdin, which is structurally and possibly functionally related to zonulin [40]. Then, Ajamian et al. [39] compared the CUSABIO's and Immundiagostik's, the most widely used commercial zonulin ELISA kits, and found that they do not detect significant levels of recombinant zonulin/pre-haptoglobin 2. Further, these authors found a poor correlation between the results for the same sample using two different kits, meaning that kits might not share the same capture antibody and do not detect the same antigens [39]. In contrast to Scheffler et al. [40], albumin and C3 complement were identified as the most relevant proteins detected by the Immunodiagnostik's set of antibodies, whereas complement C3, haptoglobin, and albumin might by the CUSABIO's [39], the one used in the present study but wether CUSABIOs ELISA kit could quantify complement C3 or haptoglobin has not been directly tested. Either way, recent studies have shown that acute microbial exposure leads to the production of complement proteins C3a and C5a, which can later form immune complexes that increase epithelial permeability [45]. Whether the complement C3 acts in synergy with or is independent of the proposed zonulin pathway in the regulation of the intestinal epithelial barrier remains unclear. Whether the protein is zonulin, properdin, complement C3, or a related protein, it is highly correlated with cardiometabolic health and associated diseases in our study. It seems that most of the protein/peptides quantified by the CUSABIOs kit could be better related to inflammatory molecules rather than to intestinal permeability markers. Thus, the true identity and role in intestinal inflammation, and the epithelium barrier function, of these peptides/proteins remain to be established.

Beyond the series of poorly characterized protein/peptides detected by the zonulin ELISA kit, we quantified additional biomarkers to get complementary information about intestinal permeability (I-FABP, LBP, and claudin-3). I-FABP is a low molecular weight intracellular protein (15 kDa) expressed in mature enterocytes, mainly in the small intestine and in the colon to a lesser extent [23]. Basal plasmatic levels of this protein reflect the normal turnover of the epithelium [23]. However, it has been shown that I-FABP can be released into circulation, increasing its plasmatic concentration when there is intestinal mucosal injury and cell damage [46], thereby being proposed as a potential biomarker of intestinal dysfunction. I-FABP has been most studied in chronic gastrointestinal diseases such as necrotizing enterocolitis [46], celiac disease [47], and diarrhea-predominant inflammatory bowel syndrome [42], conditions in which the epithelial cells are more compromised, and the intestinal damage is more severe due to the higher inflammation compared to the low-grade inflammation derived from cardiometabolic dysfunctions. Indeed, evidence about the association between I-FABP and cardiometabolic dysfunctions is scarce. However, it has been reported that alterations in glucose metabolism, such as hyperglycemia, can be associated with enterocyte damage and, therefore, with plasmatic levels of I-FABP [48]. In addition, the levels of I-FABP have been found in subjects with type 2 diabetes, especially in the presence of uncontrolled glycemia and lipid profile [49]. Here we found that I-FABP did not correlate with the other intestinal permeability biomarkers assessed, and there were no differences among subjects stratified by CMHS, blood lipid profile, insulin resistance, or blood pressure. These results agree with other studies where I-FABP levels were increased in the jejunum but decreased in the plasma and were not associated with metabolic and inflammatory parameters, such as LPS, glucagon-like peptide-2, HOMA-IR, and monocyte chemoattractant protein-1 in a high-fat diet-induced obesity rat model [23]. Similar results were found in a healthy-obese cohort, where I-FABP did not correlate with lactulose/mannitol, zonulin, or LBP [41]. The comparable levels of circulating I-FABP between cardiometabolically healthy and cardiometabolically abnormal individuals could be an adaptive response to dietary fat consumption

[23]. Thus, although I-FABP could work as a biomarker of severe gut barrier dysfunction in gastrointestinal diseases, it was not sensitive to the type of mild damage induced by cardiometabolic dysfunctions.

In addition to I-FABP, we measured plasmatic levels of LBP as a surrogate marker of intestinal permeability. LBP mediates the innate immune responses triggered by LPS [26], a component of the cell wall of gram-negative bacteria that translocate from the intestinal lumen into circulation [50]. Previous studies have found that LBP correlates with different risk factors associated with metabolic conditions like type 2 diabetes [51], obesity [26], and atherosclerosis [52]. A dysbiotic state could increase intestinal permeability and the translocation of bacterial products from the lumen into the circulation leading to low-grade inflammation and the development of cardiometabolic diseases. Furthermore, the levels of LBP display a good correlation with the lactulose/mannitol test in different cohort studies [41]. First, we found that LBP levels tended to be increased in cardiometabolically abnormal and hypertensive subjects but did not change across BMI categories. Similar results were found in a Dutch cohort, where LBP levels did not correlate with BMI, central obesity, or lipid profile. Still, the authors found that plasmatic LBP levels were slightly increased in the metabolically compromised subjects, as we did [53]. Also, after six years of following an adult Chinese population (50—70 years old), BMI was not correlated with LBP levels at the baseline or the follow-up [54]. Next, LBP levels positively correlated with hs-CRP, a biomarker of systemic inflammation and a risk factor used to metabolically classify our cohort. Both LBP and hs-CRP have been used as predictors of metabolic diseases [55] still Kim et al. [56] found that compared to hs-CRP, tumor necrosis factor-α and interleukin-6, LBP had the best diagnostic accuracy for detecting insulin resistance in young adolescents [56]. Finally, low-grade inflammation has been related to gut microbiota composition [10]. We found that LBP correlated positively with the CAG-Pathogen; previously this CAG has been reported to be involved in LPS biosynthesis, which triggers metabolic endotoxemia and promotes metabolic diseases [8]. One of the reasons of the marginal statistical significance of this difference could be our sample size, which constrains our statistical power.

Claudin-3 is another protein used to indicate the loss of integrity of the intestinal epithelium. It was found that there is an association between the disassembly of tight junctions with the increase of this protein in both plasma and urine in different models of gastrointestinal diseases, such as necrotizing enterocolitis, inflammatory bowel disease, or during low-grade inflammation processes, such as metabolic endotoxemia, which may reflect bacterial LPS translocation [24]. Here, we found that claudin-3 correlated positively with LBP, in line with the idea of increased translocation of LPS into circulation due to the tight junction dysfunction and cardiometabolic abnormalities. However, in our cohort, the plasmatic levels of claudin-3 were similar among cardiometabolically healthy and cardiometabolically abnormal subjects.

Finally, SCFAs produced by the gut microbiota have been shown to improve gut health through several local effects, including the production of mucus and maintenance of anaerobiosis and intestinal barrier integrity [11]. Results from animal models of obesity have described that plasmatic levels of SCFAs tend to be lower in obese animals [57,58]. We found that plasmatic levels of the measured SCFAs tended to be increased in metabolically abnormal and hypertensive subjects and decreased in hyperlipidemic, obese, or insulin resistant subjects, although no statistical differences were detected after adjusting our statistical models for potential confounders. Human studies about the actual effect of SCFAs are scarce and inconclusive. For example, the plasma levels of SCFAs did not change in a German cohort of obese subjects that underwent a 12 weeks dietary weight loss program [59]. In contrast, in a Chinese cohort including overweight and obese individuals, BMI and waist circumference correlated positively with plasmatic SCFAs [59]. On the other hand, we found that plasma levels of butyrate and isobutyrate inversely correlated with circulating levels of PZP. This result is in line with a study showing that butyrate-producing bacteria from the genera *Roseburia*, *Butyricicoccus,* and *Faecalibacterium* correlate inversely with the PZP levels in older adults under a polyphenol-rich diet [60], associating these peptides and butyrate indirectly.

The strengths of this work include the comprehensive assessment of surrogates of gut permeability and cardiometabolic dysfunctions in the studied cohort. Also, we selected a convenience sample of an understudied population based on gut microbiota composition that is differentially associated with cardiometabolic health. However, we also acknowledge limitations. Our findings are based on observational data from a small sample that cannot provide causal inference and might have low statistical power to detect differences in variables like LBP. We recognize some limitations, we used a commercial kit that has been proved to be unreliable for detecting zonulin and that the true identity of the peptides recognized by the set of antibodies included in the kit is still matter of debate. However, these unknown proteins/peptides have been clearly associated with human disease as also shown by Refs. [39—42,44,53]. In the same line, our results also reflect significant physiological changes associated with metabolic dysfunctions, which is crucial in the context of chronic non-communicable diseases, making it necessary to determine the precise nature of these peptides.

5. Conclusion

The protein/peptides detected by the CUSABIO zonulin ELISA kit could be potential markers for the association of cardiometabolic dysfunctions and gut microbiota, however it is mandatory a deep characterization of what is being detected by this “zonulin” ELISA kit. In addition, although LBP could be another promising biomarker bridging gut permeability with bacterial translocation, inflammation, and cardiometabolic dysfunctions, additional cross-sectional and case-control studies with bigger sample

sizes and controlled clinical trials are warranted to gain further insights into this complex association.

Author contributions

OJL-G, JSE, and JAS contributed to the conception and design of the project. AA-G, OJL-G, DAR, DS-S, RM-A, and JAS contributed to the laboratory experiments and analysis. AA-G, OJL-G, JSE, RMA, K-MD, and JAS contributed to the interpretation of data, preparation of the manuscript, and approval of the final version. All authors contributed to the revision and editing of the manuscript.

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Declaration of competing interest

AA-G, OJL-G, JSE, DA-R, K-MD, and JAS are employees of a research center that belongs to a food company (Grupo Nutresa). Grupo Nutresa has not had any role in designing or conducting the study, in the collection, analysis, or interpretation of the data, in the preparation, review, or approval of the manuscript, or in the decision to submit the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2022.09.026.

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