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Serum zonulin and colorectal cancer risk

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Intestinal permeability has been related to colorectal cancer (CRC) development. Zonulin, a protein able to regulate tight junction function and intestinal permeability, emerges as a promising marker to elucidate the contribution of bacterial translocation in CRC. An Italian case-control study included 77 CRC cases, 72 intestinal adenoma and 76 healthy controls (for a total of 148 tumor-free subjects), aged 20–85. Serum zonulin levels were quantified by ELISA kit and blood 16S rRNA gene copies by DNA extraction and polymerase chain reaction. We applied logistic regression models adjusted for center, sex, age and education. There was a positive association between zonulin and CRC risk. The odds ratio (OR) of CRC for the highest *versus* lowest tertile of zonulin as compared to tumor-free subjects was 2.36 (95% confidence interval, 1.14–4.86). The ORs were similar in colon and rectal cancers. The OR of colon cancer for the highest *versus* lowest levels of both zonulin and 16S rRNA gene copies was 4.55.

Circulating levels of zonulin were higher in CRC patients compared to tumor-free controls supporting the hypothesis of an interplay of gut barrier dysfunction and bacterial translocation in colorectal carcinogenesis. Zonulin may interact with 16S rRNA gene copies and serve as a further biomarker in the evaluation of CRC diagnosis.

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide, with about 1.8 million new cases diagnosed and about 1 million deaths each year[1].

Intestinal microbiome is associated with the development of CRC[2,3], and microbial translocation from the gastrointestinal tract to the bloodstream has been suggested to be implicated, too[2,4–7]. Intestinal permeability, also known as "leaky gut syndrome," is a measure of the function of the intestinal barrier, which is responsible for selecting which elements enter the bloodstream from the intestine[8]. Intestinal dysbiosis, an alteration in the composition and function of intestinal microbiota, has been associated with an increase in intestinal permeability and an increased risk of CRC[9,10]. Moreover, intestinal permeability can exacerbate gastrointestinal chronic inflammation and inflammatory diseases, such as Crohn's disease and ulcerative colitis, which are known risk factors for CRC[11–13].

Zonulin is the only human protein able to (reversibly) regulate intestinal permeability by modulating intercellular tight junctions and is responsible for their opening and closing, allowing for the transit of molecules and fluids in and out of the gut[14]. In physiological conditions, zonulin plays an important role in regulating paracellular exchanges (e.g. dietary factors), and in protecting the gut barrier from the translocation of microbial cells[15,16]. In pathological conditions, zonulin has also been implicated in the development of several metabolic diseases, such as type 2 diabetes and obesity, which are also recognised risk factors for CRC[12,17,18].

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Increased serum levels of zonulin are an indicator of increased intestinal permeability, which can promote higher microbial translocation leading to pro-inflammatory signalling cascades and immune responses in the colorectal carcinogenesis. Serum levels of zonulin have been found to be related with a higher microbial translocation[19], measured by bacterial 16S rRNA gene copies in blood, and our previous investigation on 100 CRC cases and 200 controls from Italy reported an over-representation of blood bacterial 16S rRNA gene copies in colon cancer patients compared to tumor-free controls[20].

Despite the potential of serum zonulin to elucidate CRC pathways and to serve as a basis to develop new CRC diagnostic strategies, the association between intestinal permeability and CRC has been scarcely investigated[21].

The aim of our study is to investigate the relationship between serum zonulin, as a marker of intestinal permeability, and CRC risk using data from a case-control study conducted in Italy[20]. This analysis will be also evaluated according to bacterial DNA load and profiling in blood.

Materials and methods

Setting and subjects' recruiting

This case-control study was carried out between 2017 and 2019 in two university hospitals in the metropolitan area of Milan, Italy[20]. Eligible subjects were 20–85 years old patients, scheduled for a colonoscopy. Subjects with immunodeficiency, selected inflammatory diseases, liver/kidney/heart failure, reported previous cancer, recent hospitalization or colonoscopy, as well as any dietary modifications in the previous month, were excluded. Based on colonoscopies and histological examinations, reviewed by two pathologists, the participants were assigned to different groups. Cases were incident and histologically confirmed CRC. For each CRC case, subject with intestinal adenoma (IA) and a subject free from IA/CRC (hereafter referred to as a healthy control) were frequency-matched (1:1:1) by study center, sex and age (±5 years).

The study was conducted according to the guidelines of the Declaration of Helsinki and the protocol was approved by the ethical committees of the involved hospitals: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (No. 742–2017; December 14, 2017) and ASST Grande Ospedale Metropolitano Niguarda (No. 477–112016; November 25, 2016). Enrolled subjects signed a written informed consent. Less than 2% of contacted eligible patients declined participation.

Out of 300 recruited subjects, we analysed data on subjects with available serum samples: 77 CRC cases and 72 IA and 76 healthy controls (for a total of 148 tumor-free subjects).

Definition and set up of the interview

The questionnaire was managed by trained and blinded interviewers, and included socio–demographic, education, lifestyle habits, anthropometric data, medical history and daily use of selected drugs. Information on the habitual diet of each participant was collected before the colonoscopy through a reproducible[22] and validated[23] food frequency questionnaire (FFQ).

Blood and serum collection

Blood samples were collected in 7 mL EDTA tube and in 3 mL blank tube before the colonoscopy. Part of the EDTA sample was immediately stored at −80 °C for the microbiomic analysis. The sample without anticoagulant was processed and centrifuged (1400 g × 15 min, 4 °C), aliquoted in small vials, and then stored at −80 °C for the circulating zonulin analysis.

Evaluation of intestinal permeability and 16S rRNA gene copies and profiling

Serum zonulin levels were quantified using the ELISA kit (cat. # K5601) from Immunodiagnostik® (Bensheim, Germany). Aliquots of serum samples were thawed (for the first time) at room temperature. The 96-well plate was pre-coated with a polyclonal anti-zonulin antibody. The competitive ELISA method provided for the addition of a biotinylated zonulin tracer to each sample (including the standards). The peroxidase-labeled streptavidin was added in order to bind the biotinylated zonulin tracer. Finally, following the reaction time, the reading of the fluorescence at 450 nm was performed with a Tecan plate reader (TECAN Infinite F200, Tecan Group Ltd. Mannedorf, Switzerland). The quantification of serum zonulin levels was carried out using a standard curve calculated by a 4-parameter algorithm as reported by the manufacturer.

For 16S rRNA gene copies analyses, DNA extraction, quantitative polymerase chain reaction (qPCR) experiments and sequencing of 16S rRNA gene amplicons were performed by Vaiomer SAS (Labège, France) through an optimized blood-specific technique, described elsewhere[20]. All samples were analyzed in the same experiment and with the same reagent batches and manipulator.

For both estimates, operators were blinded to the group assignment.

Statistical analysis

We evaluated differences in terms of zonulin by cancer diagnosis using an Anova test. We computed zonulin tertiles among healthy controls. Using multiple logistic regression models adjusted for study center, sex, age and education, we estimated the odds ratios (ORs) of CRC and the corresponding 95% confidence intervals (CIs) as compared to healthy controls, IA and tumor-free subjects (IA and healthy controls combined). We also estimated the ORs for an increment of zonulin equal to one standard deviation (computed among healthy controls; 4.7 ng/ml). In addition, we estimated the ORs of colon and rectal cancer separately. We also considered models further adjusted for body mass index (BMI), diabetes, and drugs use.

We carried out stratified analyses by strata of sex, age (< and ≥ 70) and education (< and ≥ 12 years), using tumor-free subjects as comparison group. Tests for heterogeneity between strata were based on the likelihood ratio test between models with and without interaction terms.

To estimate the relationship between zonulin and 16S rRNA gene copies, we applied a robust linear regression of 16S rRNA gene copies on zonulin using the Huber function[24] in order to consider the outliers effect in the model. We then computed the OR of CRC for zonulin tertiles, as compared to tumor-free subjects, further adjusting for quintiles of 16S rRNA gene copies, according to our previous findings[20]. Using the same model, we estimated the OR of colon cancer for highest tertile of zonulin and the highest quintile of 16S rRNA gene copies compared to the lowest zonulin tertile and the lowest 16S three quintiles. Interaction of zonulin and 16S rRNA gene copies on colon cancer risk was tested comparing models with and without interaction terms.

We identified the genera significantly different according to zonulin tertiles (III vs. I-II) in terms of relative abundance using the Wilcoxon rank sum test and in terms of presence using a chi-square test.

All analyses were performed using SAS 9.4.

Results

Table 1 reports the number and percentages of CRC, IA and healthy controls by study center, sex, age, education, BMI, and diabetes. Their distributions were similar according to these characteristics; 36% of cases, 35% of IA and 33% of controls were women. Mean age was 65.7 (range: 31–85) for CRC, 66.6 (range:32–82) for IA and 65.3 (range: 26–85) for healthy controls.

Figure 1 shows the distribution of zonulin among CRC, IA and healthy controls. The mean and median of zonulin were 31.0 ng/ml (SD, 4.2) and 31.0 ng/ml (range IQ, 6.2) in CRC cases, 28.9 ng/ml (SD, 3.7) and 28.6 ng/ml (range IQ, 5.0) in IA and 28.9 ng/ml (SD, 4.6) and 29.2 ng/ml (range IQ, 4.9) in healthy controls. There was heterogeneity between the three groups ($p=0.002$), between CRC vs. healthy controls ($p=0.003$), and CRC vs. IA ($p=0.002$). No heterogeneity was found between IA and healthy controls ($p=0.86$).

Table 2 gives the number and the percentage of CRC (including colon and rectal cancer, separately), IA and healthy controls according to tertiles of zonulin. We reported the ORs and the corresponding 95% CIs of CRC for the second and third *versus* the first zonulin tertile and for an increment of one standard deviation, using as comparison groups: healthy controls, IA and tumor-free subjects (IA and healthy controls combined). There was a positive association between zonulin and CRC risk. The OR of CRC for the highest *versus* the lowest tertile as compared to healthy controls was 2.55 (95% CI, 1.12–5.82), with a p for trend of 0.018; the OR of CRC as compared to IA was 2.16 (95% CI, 0.91–5.14), with a p for trend of 0.047; the OR of CRC as compared to tumor-free subjects was 2.36 (95% CI, 1.14–4.86), with a p for trend of 0.011. The OR of colon cancer was 2.66 (95% CI, 1.03–6.87), with a p of trend of 0.025, and the OR of rectal cancer was 2.11 (95% CI, 0.83–5.39), with a p of trend of 0.087, considering tumor-free subjects as comparison group.

Characteristic	CRC	IA	Healthy controls	χ^2 *p*-value
Study center				0.88
Niguarda	44 (57.1%)	41 (56.9)	46 (60.5)	
Policlinico	33 (42.9%)	31 (43.1)	30 (39.5)	
Sex				0.90
Male	49 (63.6%)	47 (65.3)	51 (67.1)	
Female	28 (36.4%)	25 (34.7)	25 (32.9)	
Age group				0.60
<50	9 (11.7%)	3 (4.2)	6 (7.9)	
50–59	15 (19.5%)	13 (18.1)	19 (25.0)	
60–69	20 (26.0%)	25 (34.7)	16 (21.1)	
70–79	24 (31.2%)	24 (33.3)	26 (34.2)	
≥80	9 (11.7%)	7 (9.7)	9 (11.8)	
Mean (SD)	65.7 (12.2)	66.6 (10.4)	65.3 (12.5)	0.81[a]
Education (years)[b]				0.50
<7	17 (22.1%)	14 (19.4)	9 (11.8)	
7–11	18 (23.4%)	21 (29.2)	21 (29.2)	
≥12	41 (53.9%)	37 (51.4)	37 (51.4)	
Body mass index (kg/m^2)				0.91
<25	40 (51.9%)	35 (48.6%)	37 (49.3%)	
≥25	37 (48.1%)	37 (51.4%)	38 (50.7%)	
Mean (SD)	25.2 (4.2)	25.6 (3.7)	25.8 (3.6)	0.66[a]
Diabetes				0.87
Yes	9 (11.7)	10 (13.9)	11 (14.5)	
No	68 (88.3)	62 (86.1)	65 (85.5)	

Table 1. Distribution of 77 colorectal cancer (CRC), 72 intestinal adenoma (IA) and 76 healthy controls by selected characteristics; Italy 2017–2019. [a]p-value from Anova for heterogeneity. [b]The sum does not add up to the total because of one missing value.

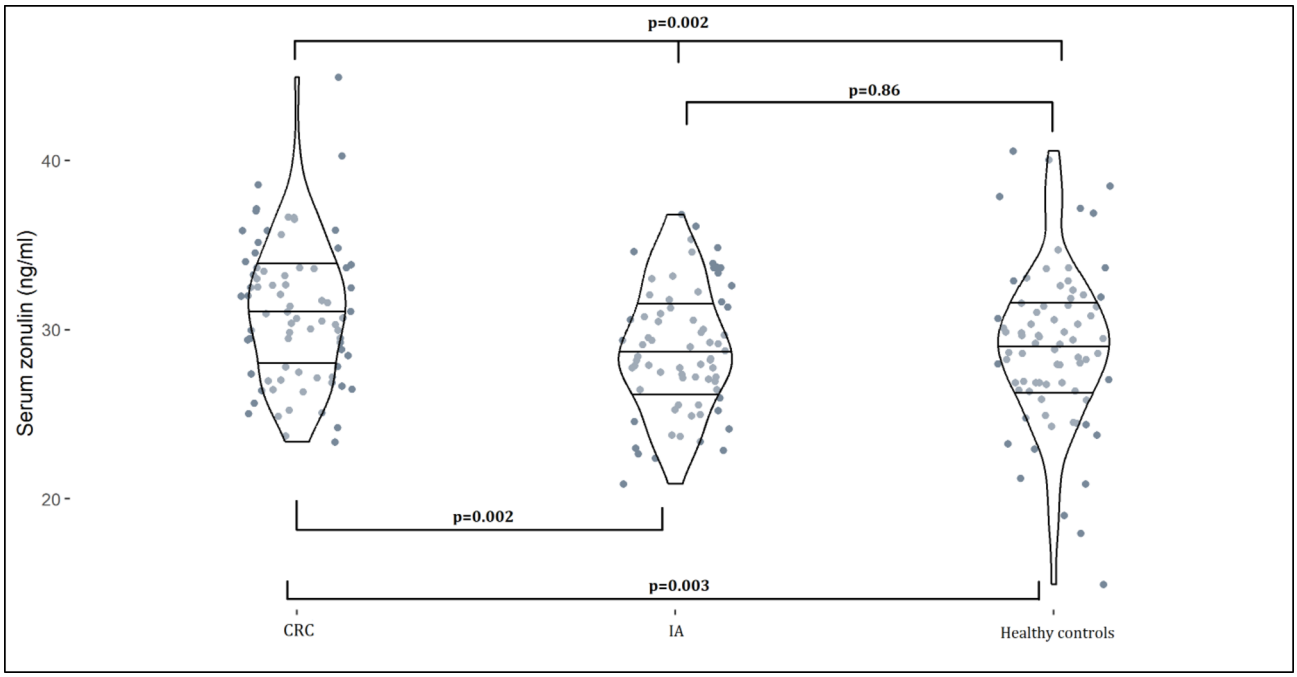

Fig. 1. Plot[a] of serum zonulin distribution among colorectal cancer (CRC) cases ($n=77$), intestinal adenoma (IA) ($n=72$) and healthy controls ($n=76$). [a]Lines indicate first, second and third quartiles.

When we further adjusted for BMI, diabetes and drug use, the OR of CRC was 2.71 (95% CI, 1.27–5.76). We also performed a sensitivity analysis by removing diabetic subjects and found similar results (OR, 2.21; 95% CI, 1.02–4.79).

Figure 2 shows the ORs of CRC for the highest *versus* the lowest tertile of zonulin in strata of sex, age and education. The associations were apparently stronger in women (OR, 4.86), young individuals (OR, 3.51) and subjects more educated (OR, 3.43), although there was no significant heterogeneity ($p=0.21$, 0.20, 0.96, respectively).

When we investigated the relationship between 16S rRNA gene copies and zonulin trough robust regression models, we found that 16S rRNA gene copies tended to increase with higher zonulin levels, with a slope of 136 ($p=0.04$) for an increment of a zonulin unit, among healthy subjects (**Supplementary Fig. 1**). Overall, the slope was 58, not significantly different from 0 ($p=0.18$).

Further adjusting for 16S rRNA gene copies, the ORs of CRC for zonulin tertiles did not appreciably change: the ORs were 2.40 for CRC, 2.82 for colon and 2.41 for rectal cancer.

Supplementary Fig. 2 shows the combined effect of zonulin and 16S rRNA gene copies on colon cancer risk. For subjects in the lowest tertile of zonulin and the highest quintile of 16S, the OR of colon cancer was 2.34 (95% CI = 0.76–7.19) as compared to subjects in both the lowest zonulin tertile and the lowest three 16S quintiles, whereas for subjects in the highest tertile of zonulin and the lowest three quintiles of 16S, the OR was 3.33 (95% CI = 1.12–9.87). For subjects in the highest tertile of zonulin and the highest quintile of 16S rRNA, the OR of colon cancer was 4.55 (95% CI = 1.17–17.66). There was no significant interaction ($p=0.51$).

When we evaluated the differences of bacteria genera in terms of zonulin, the relative abundances or prevalences of the genera *Alkanindiges*, *Daeguia*, *Methylobacterium*, *Limnohabitans*, and *Sphingobacterium* were higher in the highest as compared to the lowest two zonulin tertiles. Conversely, the genera *Sphingobium*, *Corynebacterium 1*, and *Staphylococcus* were lower in the highest zonulin tertile.

Discussion

Our study shows an association between circulating levels of zonulin and CRC risk, supporting the hypothesis that an increased intestinal permeability (or leaky gut syndrome) could play a role in CRC development. This novel result, together with the additive effect of serum zonulin and circulating bacterial 16S rRNA gene copies found on colon cancer risk, supports the evidence regarding the interplay between gut barrier dysfunction, microbial translocation, and colon cancer risk.

These results align with the findings from a Russian study, including 152 CRC patients and 134 controls[21], which reported that CRC patients had significantly higher levels of serum zonulin compared to healthy subjects and patients with non-tumor inflammatory bowel disease (IBD). That study found that the level of zonulin increased with the progression of the disease. In addition, a nested case-control study from the Health Professionals Follow-Up Study, including 261 incident CRC cases and 261 controls, supported the involvement of microbial translocation, as indicated by plasma sCD14 levels and potentially EndoCAb IgM, in the development of CRC[6]. In another case-control study, including 26 CRC cases and 41 healthy controls from South Africa, circulating bacterial toxins, such as lipopolysaccharides (LPS), were significantly elevated in the CRC patients

Zonulin	Mean (SD)	Tertiles[b]			P_{trend}[c]	Continuous OR[d]
		I	II	III		
Upper cutpoint (ng/ml)		27.05	30.58	–		
Healthy controls (%)	29.0 (4.7)	25 (32.9)	25 (32.9)	26 (34.2)		
CRC (%)	31.0 (4.1)	16 (20.8)	20 (26.0)	41 (53.3)		
IA (%)	28.9 (3.7)	20 (27.8)	28 (38.9)	24 (33.3)		
OR (CRC vs. healthy controls)		1	1.25	2.55	0.018	1.72
(95% CI)		–	(0.50–3.10)	(1.12–5.82)		(1.18–2.52)
OR (CRC vs. IA)		1	0.90	2.16	0.047	2.009
(95% CI)		–	(0.37–2.19)	(0.91–5.14)		(1.31–3.09)
OR (CRC vs. IA/ healthy controls)		1	1.07	2.36	0.011	1.82
(95% CI)		–	(0.49–3.35)	(1.14–4.86)		(1.30–2.55)
COLON cancer	31.5 (4.8)					
Cases (%)		8(20.5)	9 (23.1)	22 (56.4)		
OR (Cancer vs. healthy controls)		1	1.27	2.89	0.030	1.82
(95% CI)		–	(0.39–4.13)	(1.03–8.10)		(1.18–2.80)
OR (CRC vs. IA/ healthy controls)		1	1.05	2.66		1.96
(95% CI)		–	(0.36–3.04)	(1.03–6.87)	0.025	(1.30–2.95)
RECTAL cancer	30.6 (3.4)					
Cases (%)		8 (21.5)	11 (29.0)	19 (50.0)		
OR (Cancer vs. healthy controls)		1	1.16	2.32	0.085	1.57
(95% CI)		–	(0.37–3.65)	(0.83–6.46)		(0.98–2.54)
OR (CRC vs. IA/ healthy controls)		1	1.06	2.11	0.087	1.66
(95% CI)		–	(0.38.2.95)	(0.83–5.39)		(1.06–2.59)

Table 2. Odds ratios (ORs)[a] and 95% confidence intervals (CIs) of colorectal cancer (CRC) for zonulin tertiles as compared to healthy controls, intestinal adenoma (IA) and tumor-free controls among 77 CRC cases, 72 IA and 76 controls. Italy 2017–2019. [a]Estimated from logistic regression models adjusted for study center, sex, age and education. [b]Computed among healthy controls. [c]Across tertiles. [d]For an increment equal to one standard deviation (among healthy controls).

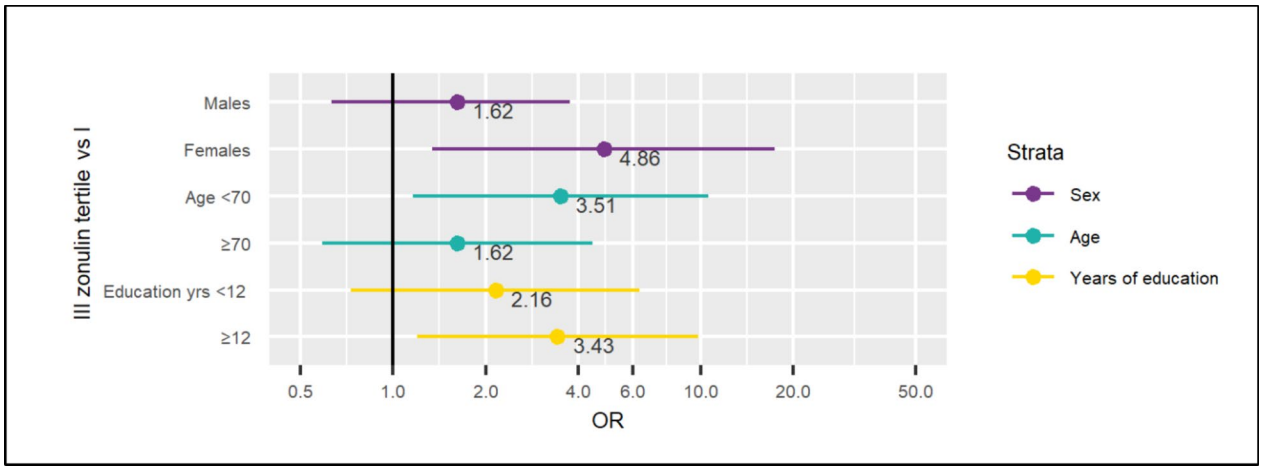

Fig. 2. Odds ratios[a] (ORs) of colorectal cancer (CRC) and 95% confidence intervals (CIs) for the highest versus the lowest zonulin tertile by strata of sex, age and education among 77 CRC and 148 tumor-free subjects. Italy 2017–2019. [a] Estimated from logistic regression model adjusted for study center, sex, age, education.

compared to healthy controls[4]. Moreover, a Greek study, including 397 adjuvant or metastatic CRC cases and 32 healthy controls, reported a positive association between microbial translocation, measured by blood bacterial 16S rRNA gene copies, and CRC[5], similarly to our previous investigation on these data[20].

Increased serum levels of zonulin have been associated with increased blood 16S rRNA gene copies in an elderly Italian population comprising 43 healthy subjects[19]. Our data were consistent with those findings in healthy controls, but did not show any significant correlation between serum zonulin and 16S rRNA gene copies overall and among CRC cases, suggesting that higher intestinal permeability and higher bacterial DNA translocation independently act on the risk of colon cancer. Zonulin is one of the different markers associated with intestinal permeability, and 16S rRNA gene copies, as a marker for microbial translocation, is grounded in the well-established concept that bacterial DNA in the bloodstream reflects increased permeability of the gut barrier[25,26]. In CRC patients, compromised intercellular integrity can facilitate bacterial entry into the bloodstream, potentially influencing our observed elevation in 16S rRNA gene copies. Our study also includes information on bacteria in blood in relation to zonulin levels, providing further insights on gut barrier functions.

Zonulin is part of the haptoglobin family proteins[27] and the structural and functional diversity within the zonulin family underscores the complexity of its role in regulating intestinal barrier function[17,28]. It has been reported that the Immunodiagnostik kit does not recognize prohaptoglobin[27]. However, Fasano and colleagues[29] highlighted the broader impact of the zonulin family on intestinal health. Measuring the entire zonulin family could offer a more comprehensive assessment of the dynamic interactions within the intestinal barrier. This integrative approach aligns with emerging research that emphasizes the interconnectedness of various proteins in maintaining gut homeostasis[30]. In our study, measuring the complete zonulin family allows capturing the collective influence of these proteins on intestinal permeability.

An increasing evidence suggests that the disruption of the protective mucosal barrier in the gastrointestinal tract contributes to the development of CRC[2,10,16]. The impairment of the colonic barrier integrity leads to heightened exposure of colonocytes and other components of the mucosal immune system to toxins present in the colonic environment, thereby amplifying inflammatory processes and the release of reactive oxygen species[16]. Zonulin has been implicated in many chronic inflammatory diseases[17]. Serum zonulin is upregulated in irritable bowel syndrome (IBS) and the levels are comparable to those in celiac disease[31]. Levels of faecal and serum zonulin were also elevated in patients diagnosed with Crohn's disease, while no association was observed with ulcerative colitis in a study on 40 IBD patients and 40 healthy subjects[32]. However, another study based on 118 IBD patients and 23 healthy controls found higher serum zonulin concentrations in patients with Crohn's disease and ulcerative colitis and an inverse correlation between serum zonulin concentration and disease duration[33]. Those findings suggest that zonulin dysregulation may be a common feature of gastrointestinal disorders, including CRC.

In addition to the overall association, we explored potential effect modifiers in the zonulin-CRC relationship. The associations between zonulin levels and CRC risk appeared to be stronger in women and young individuals, without however significant heterogeneity. These findings are consistent with a study by Maget et al.[34], which reported that females exhibited significantly higher levels of serum zonulin than males, suggesting a gender-related difference in zonulin regulation.

Furthermore, we computed the ORs by BMI categories and found that the OR of CRC was 1.48 among subjects with BMI < 25 and 4.06 among subjects with BMI ≥ 25 kg/m^2, without a significant heterogeneity ($p = 0.43$). In other studies using zonulin as intestinal permeability marker, a positive association was found between intestinal permeability alteration and obesity[35,36]. Additionally, a study on obese children patients found that zonulin levels were associated with HDL-C and leptin levels, even after adjusting for age and BMI[36]. These results indicate a potential interaction between zonulin, obesity, and CRC risk. The well-known association between obesity and increased CRC risk[37] can at least in part be mediated by a gut barrier dysfunction and an altered gut microbiota. Dietary habits can influence that interaction, but intervention studies are not univocal to date in supporting the influence of dietary modifications on circulating zonulin levels[38–40]. Investigating (dietary) interventions targeting zonulin and bacterial translocation may offer promising avenues for personalized preventative strategies in individuals at high risk of CRC.

In our study, the data on serum zonulin aim to shed a light on the underlying mechanisms of microbial translocation in CRC, indicating the importance of considering both host and microbial factors in CRC development. When we considered the relationship between zonulin and CRC risk by stage, we found an OR of 1.25 for I-II stage and of 9.18 for III-IV stage. Our findings could also offer innovative data useful to conceive new non-invasive strategy for the detection of CRC diagnosis and severity. Circulating levels of biological markers have been widely studied in relation to CRC, with the aim of identifying potential biomarkers for early detection applicable to screening and early diagnosis of the disease[41,42]. In line with our study, most of the proposed diagnostic tests for CRC are increasingly based on microbial signatures in faecal or blood samples[3,5,43].

Given the lack of data on faecal microbiota, we were not able to evaluate as a whole the pathway linking gut microbiota, intestinal permeability, and inflammation in CRC carcinogenesis. However, we identified blood bacterial DNA that were different according to zonulin levels and assessed the ORs of colon cancer according to combined levels of zonulin and 16S rRNA gene copies. Another limitation is that our results cannot distinguish whether the elevated zonulin is a consequence or a predictor of the disease, and we cannot infer on a direct causal relationship between intestinal permeability and CRC. However, most cases were detected at the first CRC-diagnosing colonoscopy, allowing us to recruit incident cases from the very beginning of the diagnostic process.

With reference to selection bias, CRC cases, healthy and IA subjects were recruited in the same hospital among patients admitted for colonoscopy. An *ad hoc* data collection was applied to recruit all patients according to the same procedures (e.g. similar setting, catchment areas). We took care to exclude subjects with history of

cancer or with selected chronic and inflammatory diseases. However, we cannot rule out residual selection bias due to the characteristics of the population undergoing colonoscopy. The participation rate was satisfactory.

Our questionnaire was administered by trained interviewers, reducing information bias. Interviewers and investigators were blinded to the group assignment, as data collection was performed before endoscopy. In addition, our cases were unaware of the cancer diagnosis at the time of the interview, ideally removing the over-reporting bias of cases. With reference to confounding, we were able to adjust for study center, sex, age, education, BMI, diabetes, and use of selected drugs. Further controlling for 16S rRNA gene copies in the models did not modify the associations with zonulin. In addition, the use of healthy controls, IA or tumor-free subjects as comparison group did not change the results. A major limitation of this study remains the relatively small sample size. The fact that results from this case-control study are in line with previous findings involving different modifiable factors associated with CRC risk is partially reassuring[44–47]. Additional biological experiments could provide further evidence to the current findings.

In conclusion, circulating levels of zonulin, which is considered a measure of intestinal permeability, were higher in CRC patients compared to tumor-free controls supporting the hypothesis that intestinal permeability may contribute to the development of this neoplasm. Future prospective studies are needed to elucidate the underlying mechanisms and validate the clinical utility of zonulin as a potential biomarker for CRC risk stratification.

Data availability

All the reads are publicly available in the European Nucleotide Archive (ENA) with the accession number: PRJEB46474.

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Author contributions

MR and PR conceived the work. MR and CLV designed the work. MC, RP, GG, CC, PL, AA, MV, RB, BO, PC, MVa, MMu and MR acquired data. MM, SM, KM, MF, LP, CDB, SG, PR, CLV and MR interpreted the results. MM, GG, CDB and RB contributed to data elaboration. SM and MR conducted statistical analysis. MM, SM and MR drafted the manuscript. All authors critically revised and approved the final version of the manuscript.

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Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and ap-proved by the Ethics Committees of ASST Grande Ospedale Metropolitano Niguarda (No. 477–112016; November 25, 2016)

and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (No. 742–2017; December 14, 2017). Written informed consent was obtained from all subjects involved in the study.

Consent for publication

All authors consent to the publication of the present version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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