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Interpersonal Variability in Gut Microbial Calprotectin Metabolism

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Ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated chronic inflammatory bowel diseases (IBDs) of the gastrointestinal tract. The gold standard for assessing mucosal inflammation is through endoscopy with biopsies, which permits providers to define the severity and distribution of intestinal inflammation visually and histologically. However, endoscopy is impractical as a tool for monitoring patients on a daily or weekly basis. There is a clinical need for reliable noninvasive testing to monitor IBD patients. Fecal calprotectin is a promising but imperfect biomarker for inflammation. A meta-analysis found a pooled sensitivity of 85% (95% confidence interval: 82%, 87%) and specificity of 75% (95% confidence interval: 71%, 79%) for diagnosing endoscopically active disease among individuals with IBD.[1] Here we ask whether the gut microbiome can metabolize calprotectin and thereby potentially alter measured fecal calprotectin levels.

We recruited 22 individuals with IBD (64% female; 73% with colonic disease; Table), who provided stool samples and completed a symptom questionnaire 1–3 days before outpatient colonoscopy. Sixty-four percent ($n = 14$) had endoscopically inactive disease (Simple Endoscopic Score for Crohn's Disease 3 for ileal CD, Simple Endoscopic Score for Crohn's Disease 5 for colonic CD,[2] or Mayo Endoscopic Score of 0 for UC[3]), whereas 82% ($n = 18$) of participants had clinically inactive disease (Harvey-Bradshaw Index <5 or Simple Clinical Colitis Activity Index 2). Based on a cutoff of 50 μg/g, 9 had normal fecal calprotectin levels, whereas 13 had elevated fecal calprotectin levels. Calprotectin levels were higher in individuals with clinically or endoscopically active disease ($P < 10^{-4}$, $F =$

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Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

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27; one-way analysis of variance). Clinical disease activity metrics were significantly (but poorly) correlated with fecal calprotectin levels for CD ($r = 0.62$, $P = .008$) but not for UC ($r = -0.29$; $P = .6$; Figure A). Similarly, endoscopic disease activity was correlated with fecal calprotectin in CD ($r = 0.83$, $P < .001$) but not UC ($r = 0.50$, $P = .4$).

To characterize calprotectin metabolism by the microbiome, we designed a novel ex vivo functional assay in which we anaerobically cultured fecal samples in media containing calprotectin and quantified calprotectin 24 hours later (Figure B). Control samples lacked microbes, calprotectin, or both. Reasoning that gut microbes may not preferentially harvest amino acids from calprotectin, we tested both a standard bacterial growth media (LYHBHI) and a modified version of that growth medium containing low levels of amino acids ($LYHBHI_{lowAA}$) in which cultured bacteria would be "starved" for amino acids and therefore more likely to catabolize calprotectin. We normalized the measured calprotectin levels using the equation:

$$calprotectin_{normalized} = \frac{calprotectin_{measured} - \text{control}_{sterile\,media\,only}}{\text{control}_{sterile\,media\,plus\,calprotectin} - \text{control}_{sterile\,media\,only}}$$

Negative controls used for normalization corresponded to the same media as the experimental samples being normalized (eg, $LYHBHI_{lowAA}$ cultures were normalized to sterile $LYHBHI_{lowAA}$ media controls).

Microbiome-mediated calprotectin degradation varied between individuals (Figure C). We observed significantly lower normalized calprotectin levels in $LYHBHI_{lowAA}$ cultures than in LYHBHI cultures ($P < .0007$, paired Student's 2-tailed t-test). The difference in gut microbial calprotectin degradation between LYHBHI and $LYHBHI_{lowAA}$ media contexts was greater in UC microbiomes compared with CD microbiomes ($P < .02$, Student's 2-tailed t-test). This gut microbial trait is not specific to IBD: when we performed this assay using a fecal sample from an adult without IBD, we similarly observed depletion of calprotectin after a 24-hour incubation in $LYHBHI_{lowAA}$ only ($P = .005$, Student's 2-tailed t-test comparing $LYHBHI_{lowAA}$ vs LYHBHI media; Figure D).

We assessed the effect of calprotectin on bacterial growth using fecal microbiota suspensions from 7 individuals representing diverse IBD subtypes whose fecal microbiota exhibited variable calprotectin metabolism phenotypes (participant IDs 1–7). In all samples, bacterial community growth was hampered in $LYHBHI_{lowAA}$ compared with LYHBHI media (representative growth curves shown in Figure E). The presence of calprotectin in $LYHBHI_{lowAA}$ media was associated with more robust growth compared with $LYHBHI_{lowAA}$ media alone, although this effect was highly variable and not statistically significant.

To identify bacterial species correlated with calprotectin degradation, we generated shotgun metagenomic sequencing data from fecal samples. The relative abundances of reads mapping to bacterial proteases and peptidases were within a tight range (0.3%–0.6% of all reads), consistent with prior studies.[4] We observed variability in microbiome composition in our IBD population and a predominance of *Firmicutes*, also consistent with prior reports.[5,6]

The relative abundance of *Subdoligranulum* was significantly correlated with $LYHBHI_{lowAA}$ growth media-dependent calprotectin degradation ($P = .04$, $r(20) = 0.38$, Pearson; Figure F).

To determine whether *Subdoligranulum* species can degrade calprotectin, we anaerobically cultured a representative type strain, *Subdoligranulum variabile*, in $LYHBHI_{lowAA}$ growth media spiked with calprotectin for 5 days. Cultures of *Akkermansia muciniphila* (which was not correlated with $LYHBHI_{lowAA}$ growth media-dependent calprotectin degradation; Figure F) and a fecal microbiota suspension from study participant #2 served as negative and positive controls, respectively. As predicted, calprotectin levels were significantly lower in *S. variabile* cultures than in *A. muciniphila* cultures ($P = .03$, Student's 1-tailed *t*-test; Figure F). A direct growth benefit to *S. variabile* was not appreciated, as estimated by OD_{600} measurements. However, given calprotectin's antibacterial effect (which curiously is reported to be media dependent[7]), bacterial degradation of calprotectin could nonetheless serve an ecologically beneficial role. *Subdoligranulum* species were not detectable in 5 of the 22 fecal microbiomes; therefore, *Subdoligranulum* species are unlikely to be the lone mediators of this metabolic phenotype.

In our IBD cohort, just a single study participant (#9) had endoscopically active colitis with a relatively low fecal calprotectin level (Table). Consistent with our findings, this individual's microbiome harbored *Subdoligranulum* and was able to metabolize calprotectin in our ex vivo functional assay.

In summary, through the use of a novel ex vivo functional assay, we report interpersonal microbiome-dependent variation in calprotectin metabolism that is sensitive to amino acid levels. Consistent with our findings, a longitudinal study of pregnant women reported that *Subdoligranulum* (among other bacteria) negatively correlated with maternal fecal calprotectin in the third trimester.[8] Microbiome-based calibration could improve sensitivity and specificity of fecal calprotectin readouts, thereby facilitating more reliable real-time monitoring and ultimately enabling more timely interventions. A limitation of our study is small sample size, which precluded generation of such a model for calibrating readouts. Follow-up studies examining individuals with active colitis are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of Interest:

These authors disclose the following: S.D.L. is an advisory board member of Cornerstones Health, Janssen, Eli Lilly, Bristol-Myers Squibb, and Pfizer; and receives research funding from AbbVie, Janssen, Takeda, Celgene, and Pfizer. K.C.-S. is an advisory board member of Takeda, Bristol-Myers Squibb, Pfizer, and AbbVie. The remaining authors disclose no conflicts.

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Data Transparency Statement:

The sequencing datasets generated and analyzed during the current study are available in the ENA repository PRJEB51208.

Abbreviations used in this paper:

CD	Crohn's disease
IBD	inflammatory bowel disease
LYHBHI	brain heart infusion with L-cysteine and yeast extract
UC	ulcerative colitis

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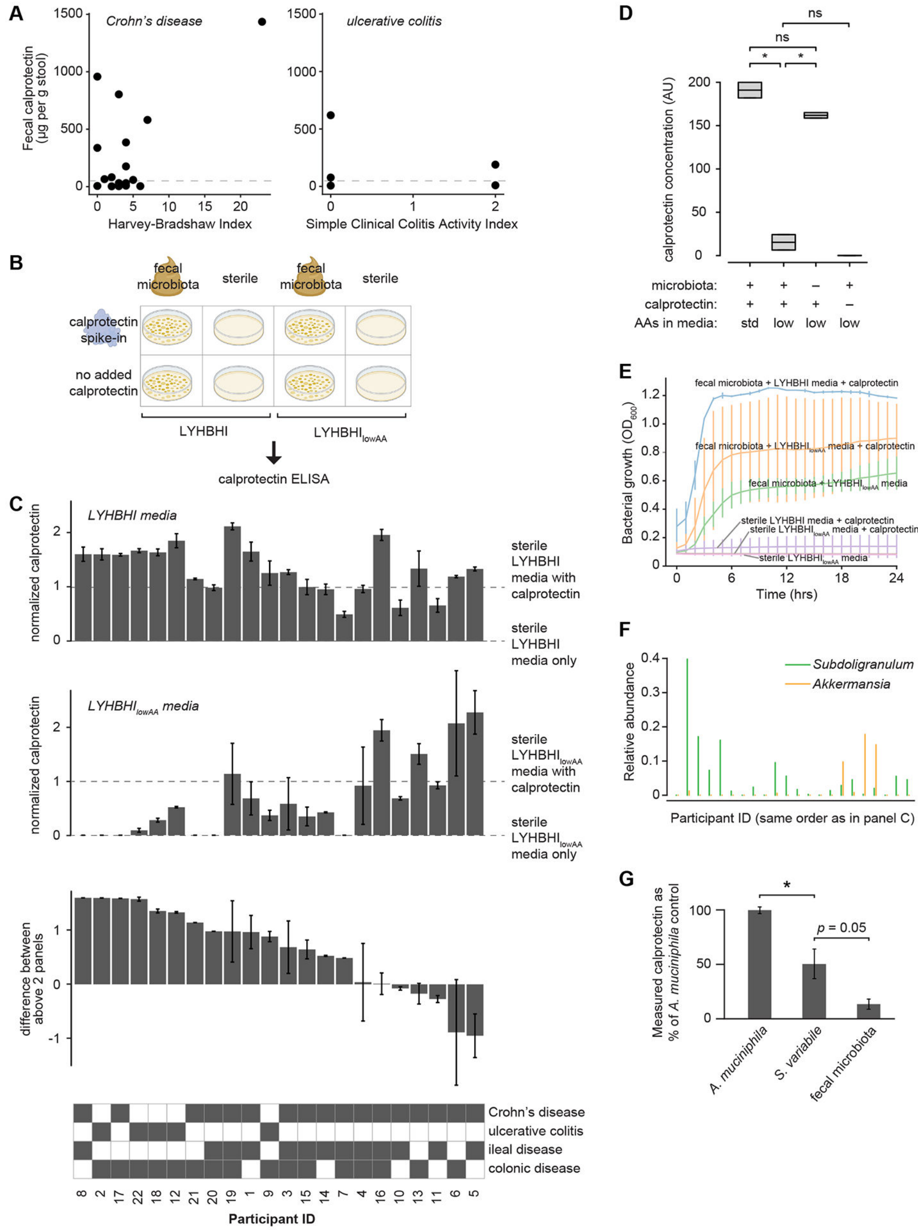

Figure.

(A) Fecal calprotectin vs clinical disease activity in CD and UC. The horizontal dashed line indicates the upper limit of the normal range for fecal calprotectin (50 μg per gram of stool). (B) Schematic of ex vivo gut microbial calprotectin degradation assay. Fecal microbiota samples (and sterile controls) are cultured with/without calprotectin for 24 hours in LYHBHI or in $\text{LYHBHI}_{\text{lowAA}}$ growth media in an anaerobic chamber. Calprotectin is then quantified via ELISA. (C) Normalized calprotectin levels in LYHBHI and in $\text{LYHBHI}_{\text{lowAA}}$ growth media. Key clinical characteristics of study participants are represented in the heatmap. (D) Media-dependent calprotectin degradation by a non-IBD fecal microbiome. (E) In vitro growth curves of a representative fecal microbiota suspension in $\text{LYHBHI}_{\text{lowAA}}$ growth media with or without calprotectin measured hourly over 24 hours, compared

with standard LYHBHI media control. (F) Relative abundances of *Subdoligranulum* and *Akkermansia* genera in fecal microbiomes, with samples ordered identically as in panel (C). (G) Detectable calprotectin in cultures of *S. variabile, A. muciniphila* (negative control), and a fecal microbiota suspension (positive control) after 5 days.

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Table.

Individual Calprotectin Levels and Clinical Metadata Collected for Each Study Participant

ID	IBD type	IBD distribution	Calprotectin level	Probiotic use	HBI	SCCAI	Clinical disease activity	SES-CD	Mayo	Endoscopic disease activity
1	Crohn's disease	Ileal	31	Yes	3		Inactive	3		Inactive
2	Ulcerative colitis	Colonic	10	No		2	Inactive		0	Inactive
3	Crohn's disease	Ileocolonic	4	Yes	6		Active	0		Inactive
4	Crohn's disease	Ileocolonic	581	Yes	7		Active	8		Active
5	Crohn's disease	Ileal	65	No	1		Inactive	11		Active
6	Crohn's disease	Colonic	385	No	4		Inactive	7		Active
7	Crohn's disease	Ileocolonic	6	No	0		Inactive	0		Inactive
8	Crohn's disease	Ileal	59	No	5		Active	3		Inactive
9	Ulcerative colitis	Colonic	189	No		2	Inactive		2	Active
10	Crohn's disease	Ileal	82	No	2		Inactive	0		Inactive
11	Crohn's disease	Ileal	176	NA	4		Inactive	0		Inactive
12	Ulcerative colitis	Colonic	620	Yes		0	Inactive		1	Active
13	Crohn's disease	Colonic	958	No	0		Inactive	9		Active
14	Crohn's disease	Ileal	4	No	3		Inactive	0		Inactive
15	Crohn's disease	Ileocolonic	803	Yes	3		Inactive	30		Active
16	Crohn's disease	Ileocolonic	337	No	0		Inactive	0		Inactive
17	Crohn's disease	Colonic	8	Yes	4		Inactive	0		Inactive
18	Ulcerative colitis	Colonic	77	No		0	Inactive		0	Inactive
19	Crohn's disease	Ileocolonic	1435	No	23		Active	33		Active
20	Crohn's disease	Ileocolonic	31	No	4		Inactive	3		Inactive
21	Crohn's disease	Colonic	3	No	2		Inactive	4		Inactive
22	Ulcerative colitis	Colonic	7	No		0	Inactive		0	Inactive

IBD, inflammatory bowel disease; HBI, Harvey-Bradshaw Index; SCCAI, Simple Clinical Colitis Activity Index; SES-CD, Simple Endoscopic Score for Crohn's Disease.