Interpersonal Variability in Gut Microbial Calprotectin Metabolism

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. 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 UC3), 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 = 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...
Measured calprotectin as % of A. muciniphila control

A. muciniphila
S. variabile
fecal microbiota

*p = 0.05

Participant ID (same order as in panel C)

Subdoligranulum
Akkermansia

Bacterial growth (OD600)

0 6 12 18 24

Time (hrs)

calprotectin concentration (AU)

0 50 100 150 200

Differential calprotectin ELISA

Sterile fecal microbiota
Fecal microbiota + LYHBHI media + calprotectin
Sterile LYHBHI media + calprotectin
Sterile LYHBHI lowAA media + calprotectin
Sterile LYHBHI lowAA media

AAs in media:
std low low low

Fecal calprotectin (μg per g stool)

Fecal calprotectin ELISA

Harvey-Bradshaw Index

Simple Clinical Colitis Activity Index

Ulcerative colitis

Crohn’s disease

Relative abundance

Participant ID (same order as in panel C)

Subdoligranulum
Akkermansia

Measured calprotectin as % of A. muciniphila control

* p = 0.05

Participant ID

Crohn’s disease
Ulcerative colitis
Ileal disease
Colonic disease
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:

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\text{calprotectin}_{\text{normalized}} = \frac{\text{calprotectin}_{\text{measured}} - \text{control}_{\text{sterile media only}}}{\text{control}_{\text{sterile media plus calprotectin}} - \text{control}_{\text{sterile media only}}}
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Negative controls used for normalization corresponded to the same media as the experimental samples being normalized (e.g., 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. We observed variability in microbiome composition in our IBD population and a predominance of Firmicutes, also consistent with prior reports. 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 OD600 measurements. However, given calprotectin’s antibacterial effect (which curiously is reported to be media dependent), 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.

**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 LYHBHI_lowAA growth media in an anaerobic chamber. Calprotectin is then quantified via ELISA. (C) Normalized calprotectin levels in LYHBHI and in LYHBHI_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 LYHBHI_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.
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.

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Supplementary Materials
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