Bacterial Quorum-Sensing Molecules in Serum: A Potential Tool for Crohn's Disease Management

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Crohn's disease (CD) is an idiopathic inflammatory condition of the gastrointestinal tract with the primary method of diagnosis and follow-up being colonoscopy. A disturbed host-microbiome interaction, including the presence of pathobionts, is implicated in initiation and perpetuation of inflammation. As such, we hypothesized that bacterial quorum-sensing (QS) molecules (QSMs), small molecules bacteria generate to regulate gene expression, would be elevated in patients with CD. We collected serum at the time of colonoscopy from patients with CD and healthy controls, determining through biosensors for QSMs that patients with CD had significantly elevated levels of QSMs in serum. Expansion of these studies may allow for QSM levels in serum to serve as a biomarker for intestinal inflammation in patients with CD.

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A889

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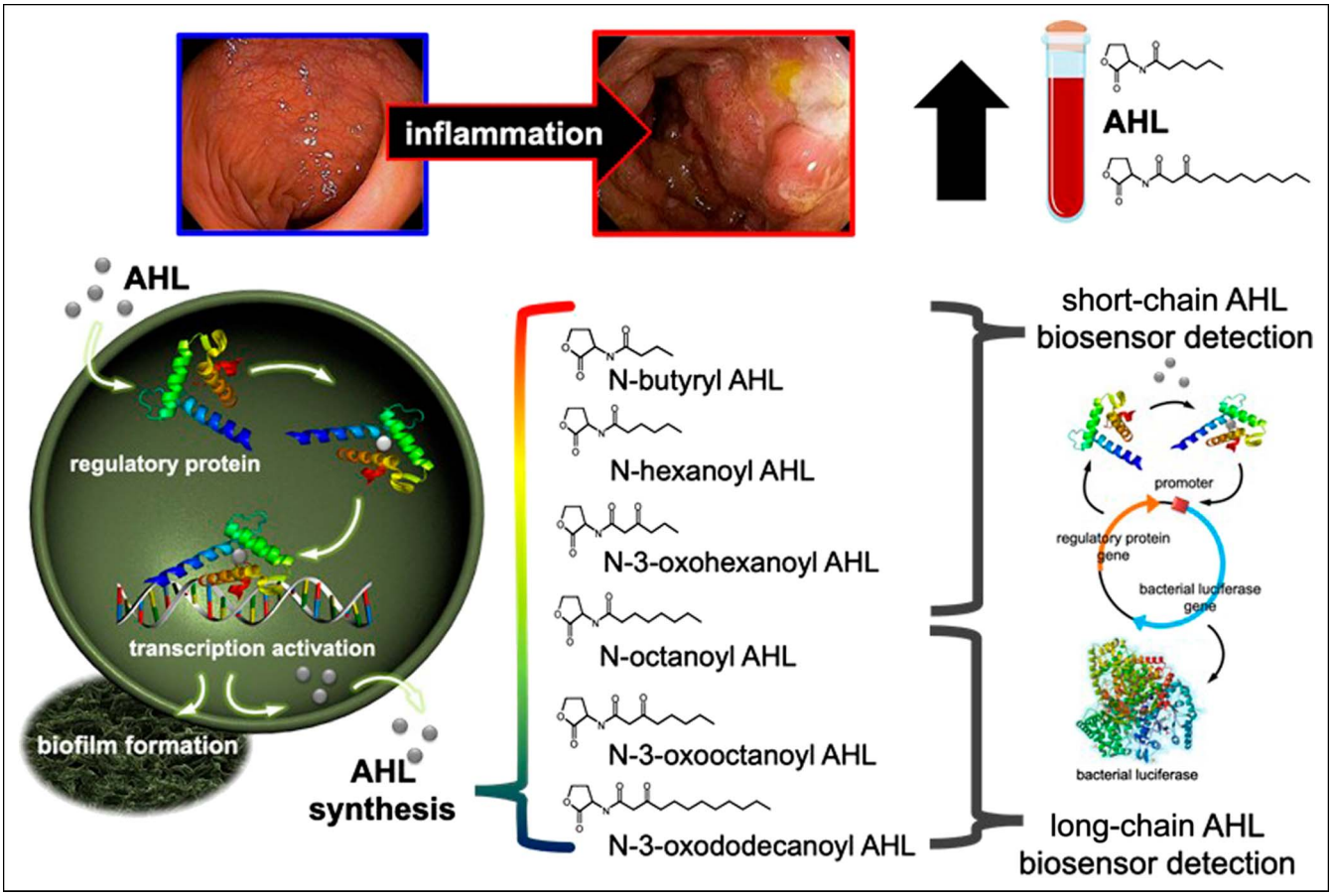

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INTRODUCTION

Crohn's disease (CD), an immune-mediated, chronic inflammatory disease that affects approximately 565,000 individuals in the United States. Although considered a disease of the Western world, CD incidence is on the rise in the developing world and in younger populations (1); thus, there is an increasing need for better diagnosis and management of CD. The field continues to rely on colonoscopy for a diagnosis of CD and to assess mucosal healing in response to therapy (2). The microbiome plays a critical role in the initiation and perpetuation of intestinal inflammation in patients with CD (3). Studies of the microbiota in patients with CD have revealed a decrease in overall diversity, specifically a reduction of distinct ribotypes of the phylum Firmicutes (4). Studies in children with a new diagnosis of CD before treatment has shown that decreased abundance in Erysipelotrichales, Bacteroidales, and Clostridiales correlates strongly with disease status (5). Continued work in this field has led to identification of various species of bacteria that are downregulated in patients with CD compared with healthy relatives, such as *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis* (6). The cause of the initial alteration to microbial homeostasis is unclear, but intestinal inflammation seems to play a role in the observed lack of bacterial diversity in patients with CD (7).

Bacteria interact in biofilms, such as occuring in ulcer beds and wounds, which can be impervious to antibiotics. Biofilms are extremely complex ecosystems that allow for many different species of bacteria to interact. Members of this biofilm community are characterized by distinct gene expression profiles, growth rate, interacting behavior, and/or structural appearance as compared with single isolated cells. *Helicobacter pylori*, *Pseudomonas aeruginosa*, and adherent invasive *Escherichia coli* strains have been shown to play a role in the formation of biofilms within patients with CD (8,9). These bacteria coordinate behavior and communicate using quorum sensing (QS); QS is a system of communication in which small quorum-sensing molecules (QSMs) are secreted and regulate gene expression in neighboring bacteria (10). QSMs are typically constitutively synthesized by bacteria at low levels, which then either passively diffuse or are actively transported into the environment. These QSMs accumulate in the environment as the bacterial population density increases and can then re-enter bacteria where they are recognized by receptor proteins and subsequently induce biofilm formation and expression of virulence factors. QSM production may also be induced by bacteria as a response to stress and other external stimuli (11). QS has been shown to mediate interkingdom communication between the gut microbiome and its human host (12). Many different classes of QSMs have been determined, including n-acyl homoserine lactones (AHLs), which are then further subdivided into short-chain AHLs and long-chain AHLs based on the length of the carbon tail on the molecule. AHLs have been of growing research interest as, in addition to their functions as a bacterial QSM and initiators of biofilm formation, AHLs have bene shown to cause inflammation in mammalian cells and initiate oncogenic pathways by binding to pancreatic cell receptors (13). *P. aeruginosa* and *E. coli*, mentioned above playing a role in biofilm formation in CD, are known to use the AHL QS circuits allowing them to produce and respond to AHLs (12,14). QSM detection is predominantly achieved through liquid or gas chromatography, mass spectrometry, or, more recently, with whole cell–based biosensors (WCBs) (15). These WCBs offer many advantages, including their ability to detect bioavailable levels of QSMs, adaptability to point-of-care settings, and low sample volumes required for assays.

We hypothesized that QSMs will be differentially generated by the microbiota in response to the inflamed mucosa of CD and that these differences can be detected in serum through biosensors; for individuals with CD during periods of intestinal inflammation, the integrity of the intestinal epithelial barrier is compromised (16), which may allow for bacterial QSMs to enter the blood stream. In the current study, we have developed and validated a biosensor method for detection of short-chain and long-chain AHLs, categories of QSMs used predominantly by Gram-negative bacteria (17), in human blood serum and determined the levels of AHLs in blood of patients with CD under conditions of confirmed intestinal inflammation and during uninflamed periods. As mentioned above, AHLs are synthesized by bacteria implicated in playing a role in CD, as well as by native Gram-negative bacteria of the gut microbiome, and can cause inflammation in mammalian cells. Thus, AHLs were selected for detection as a potential marker of intestinal inflammation in individuals with CD.

MATERIALS AND METHODS

Study population and sample collection

Patient data and samples were collected prospectively with the approval of the Institutional Review Board at the University of Miami Miller School of Medicine (Miami, FL). Whole blood was collected at the time of screening colonoscopy in serum collection tubes from patients with CD and age-matched and sex-matched healthy controls (Table 1). For patients with CD, intestinal inflammation was quantified using the validated Simple Endoscopic Score for CD.

Table 1. Age, ethnicity, inflammation (SES-CD score), and CRP levels for healthy controls, patients with noninflamed CD, and patients with active inflammation CD

	Control n = 31	Noninflamed CD n = 35	Active inflammation CD n = 35
Age (yr)			
Mean	56 (±9)	47 (±11)	46 (±16)
Range	26–81	25–56	26–79
Ethnicity			
White	8	16	23
Hispanic	23	17	10
African	0	1	1
Mixed/other	0	1	1
Inflammation (SES-CD)			
Uninflamed (SES-CD score 0)	31	35	0
Moderate (SES-CD score 7–15)	0	0	30
Severe (SES-CD score 15–27)	0	0	5
CRP levels (mg/L)			
Mean	0.47 (±0.17)	0.39 (±0.35)	1.37 (±0.33)

CD, Crohn's disease; CRP, C-reactive protein; SES-CD, Simple Endoscopic Score for Crohn's Disease.

Pathology results were also evaluated to confirm presence of histologic inflammation. Healthy controls were determined as individuals who, in addition to absence of intestinal inflammation, have not been diagnosed with IBD or any other GI disorder.

Liquid chromatography coupled with tandem mass spectrometry detection of AHLs in serum

A method for detection of AHLs through high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) (18) was optimized for use in serum by addition of a protein precipitation step at the start of the procedure. Serum forms an emulsion if methylene chloride is used for AHL extraction; therefore, we first precipitated the proteins from serum by adding acetonitrile to ensure that AHLs were solubilized in acetonitrile. An equivalent volume of acetonitrile (12 mL) was added to healthy control pooled serum (12 mL total, 1 mL/individual n = 12) to precipitate the proteins out. The solution was then centrifuged and the supernatant separated. The supernatant was extracted using methylene chloride, evaporated to dryness, reconstituted using mobile phase to approximately 100 μL, and C-7 HSL was added as an internal standard before analysis using LC-MS-MS.

Detection of AHLs in serum with biosensors

Bacterial-based whole-cell biosensors for short-chain AHLs (pSB406) and long-chain AHLs (pSB1075) were used as previously described (19). Five-milliliter cultures of cells containing plasmids pSB406 or pSB1075 were prepared and grown overnight (37 °C, 250 rpm). The next day, these cultures were refreshed 1:15 in LB Miller broth and grown to OD_{600} 0.45 (37 °C, 250 rpm). Dose-response curves to determine response to analyte were generated from the cognitive analyte for each sensor through serial dilution from 10^{-4} M to 10^{-10} M (C6-AHL for pSB406 and C12-AHL for pSB1075 [Millipore Sigma]). Ten microliters of standard analyte or diluted serum was added to 90 μL of sensor in a black 96-well plate in triplicate and incubated (37 °C, 175 rpm) for 2 hours before determination of bioluminescent intensity (CLARIOstar, BMG Labtech). To overcome the matrix effect, human serum samples were diluted 1:10 in distilled, deionized water.

Levels of C-reactive protein (CRP) in blood serum were determined using ELISA (Thermo Fisher Scientific) as per the manufacturer's instructions.

Statistics

Power analysis was performed to predict the sample size: to detect at least a 50% difference in luminescence intensity at 90% power, a

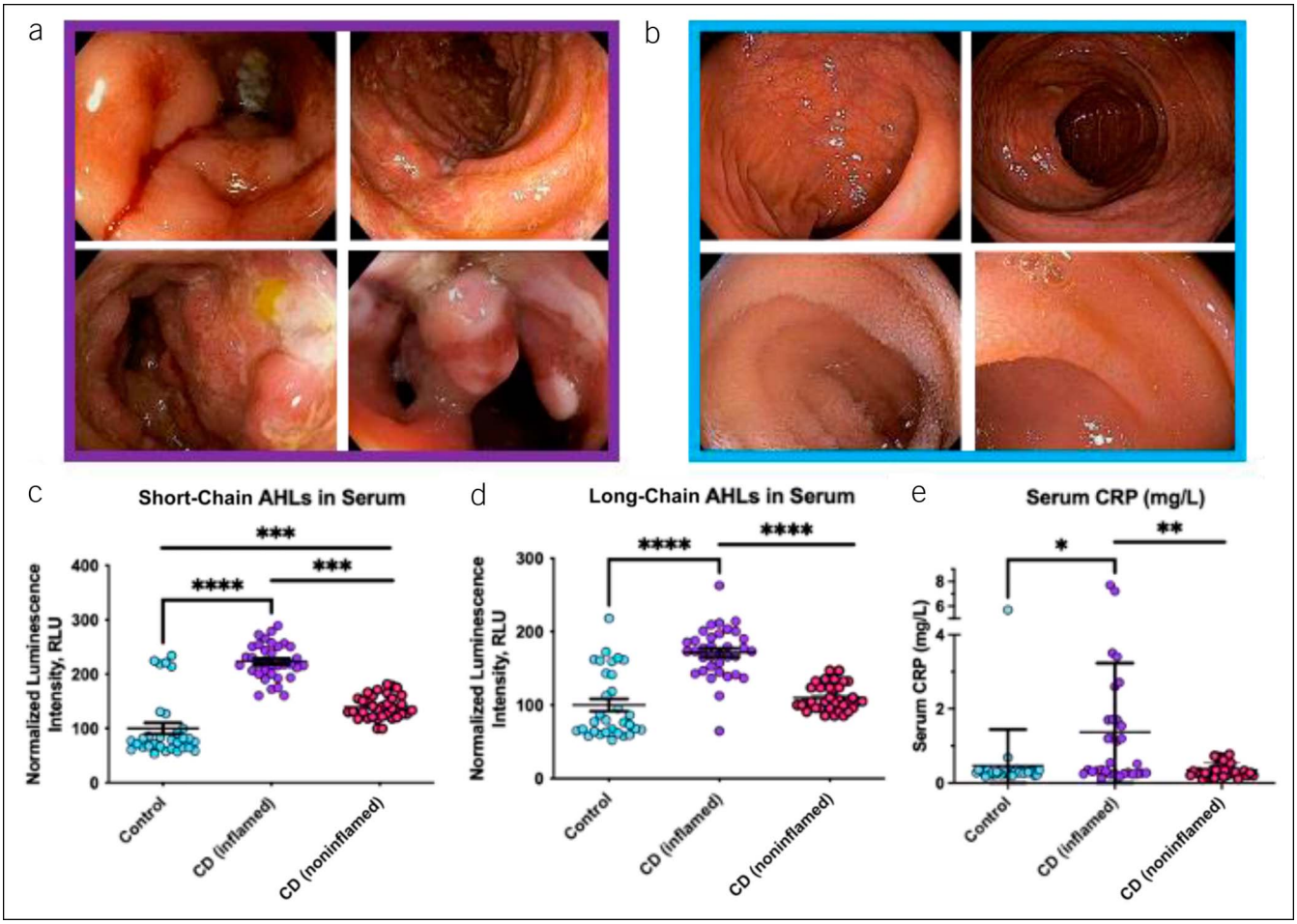

Figure 1. Colonoscopy images from (**a**) patients with active inflammation Crohn's disease (CD) and (**b**) healthy controls. Detection of short-chain AHLs (**c**), long-chain AHLs (**d**), and CRP (**e**) in serum from healthy controls (n = 31), patients with CD with active inflammation (n = 35), and patients with noninflamed CD (n = 35). (*t*-test, * = *P*value < 0.05, ** = *P*value < 0.01, *** = *P*value < 0.001, **** = *P*value < 0.0001). AHLs, n-acyl homoserine lactones; CRP, C-reactive protein.

minimum sample size of 28 individuals is necessary. Data were expressed as the mean ± the SD of mean and analyzed in GraphPad Prism 7.04. Statistical significance was assessed with the Student *t* test, unless otherwise stated. Power analysis was performed with GraphPad StatMate.

RESULTS

To confirm the presence of AHLs in serum of healthy individuals, we developed an LC-MS-MS analysis protocol. LC-MS-MS required large volumes of serum, and thus, we pooled serum from 12 healthy controls (1 mL serum per control person, 12 mL total volume) for analysis. C-8 AHL, C-10 AHL, C-12 AHL, and C-14 AHL, different types of AHLs, were detected in low nanomolar levels in extracted serum samples (see Supplementary Figure 1A, http://links.lww.com/CTG/A889). We then investigated AHLs in serum samples through WCBs because this greatly reduced the amount of serum necessary for analysis (~10 μL), allowing for AHL determination in individual samples at low nanomolar levels (see Supplementary Figure 1B–C, http://links.lww.com/CTG/A889) in agreement with LC-MS-MS.

We next investigated the levels of AHLs in serum from individual patients with CD with active intestinal inflammation and compared them with healthy controls; patients with CD had moderate to severe mucosal inflammation (Figure 1a,b, Table 1). Using biosensors, both short-chain and long-chain AHL levels were significantly elevated in the serum of patients with actively inflamed CD when compared to controls (Figure 1c–d). In addition to the patients with active inflammation CD, we investigated the levels of AHLs in serum from a separate group of patients with noninflamed CD compared with healthy controls. Short-chain AHL levels were found to be significantly upregulated in patients with noninflamed CD when compared to controls (Figure 1c–d), whereas levels of long-chain AHLs were similar for both groups.

We determined the levels of serum CRP, a biomarker of systemic inflammation (20), in patients with known active CD based on colonoscopy (Figure 1e, Table 1). CRP levels were found to be significantly elevated for the active inflammation CD group as compared to controls. By contrast, the patients with noninflamed CD had CRP levels similar to controls (Figure 1e, Table 1).

DISCUSSION

Although many studies of the microbiome have been performed in patients with CD implicating the microbiome and intestinal inflammation as potential biomarkers (21), there is not yet a hallmark measure of disease activity. As such, our laboratory is interested in QSMs as a surrogate for inflammation and disease pathogenesis in patients with CD due to bacterial QS being a dynamic system used by bacteria to respond to their environment and coordinate group behaviors (12). In this pilot study, we developed biosensor methods allowing us to demonstrate that patients with CD have increased serum levels of QSMs, specifically short-chain and long-chain AHLs. We show that high levels of both short-chain and long-chain AHLs are found in patients with documented active inflammation through colonoscopy and can distinguish these individuals from those with CD but no current inflammation, who have only increased levels of short-chain AHLs. AHL levels are lower in individuals with no intestinal inflammation or gastrointestinal symptoms. Our data suggest that serum measurements of QSMs may help identify patients with active intestinal inflammation. The presence of increased levels of short-chain AHLs may also be useful as a marker to stratify risk of CD in patients with GI problems that are not yet diagnosed; however, larger prospective studies would need to be conducted to examine the accuracy of QSMs vis-à-vis other noninvasive tests such as CRP and fecal calprotectin.

Production of AHLs is of great importance to bacteria as these molecules control virulence factor and biofilm production with AHL pathways inducing a positive feedback loop that synthesizes more AHLs (12). The integrity of the intestinal barrier is impaired in patients with CD due to inflammation, which allows bacteria to translocate to the adipose tissue and peripheral blood and increased tight junction permeability (22). This would allow AHLs produced by gut bacteria to diffuse into the blood stream. Interestingly, mammalian blood contains enzymes capable of degrading AHLs (23), known as paraoxonases (PONs). Thus, there exists a natural defense against bacterial QS in blood. In humans, 3 kinds of PONs have been identified (PON1, PON2, and PON3), with PON1 and PON3 being secreted into the blood after synthesis in the liver. One report has indicated reduced levels of PON1 enzymes in the serum of patients with CD and ulcerative colitis as compared to controls (24). Therefore, the increase in AHLs in serum of patients with CD may represent overproduction in the gut, increased leakage from the intestine and/or decreased destruction in blood.

CRP level detection was originally used to assess inflammation in cardiovascular disease, but it has been shown to correlate with disease activity in a large number of inflammatory conditions, including CD (20). In our studies to validate the potential use of the QSMs as a biomarker for intestinal inflammation, the levels of CRP were determined in our patients with active inflammation and in control healthy individuals (Figure 1e). The average level of CRP for the active inflammation CD group was significantly higher than the control group; however, this difference seems to be due to a subset of patients with greatly altered CRP levels. Many of the active inflammation CD group (19 of 35) had levels equal to that of the control, indicating that serum CRP was not a strong marker of intestinal inflammation for these patients despite the intestinal inflammation that was confirmed through colonoscopy. By contrast, patients with most active inflammation CD (33 of 35) had elevation of both short-chain and long-chain AHLs.

The link between AHL levels and intestinal inflammatory state, specifically elevation of both short-chain and long-chain AHLs during active inflammation CD, may prove useful in disease management and discovering potential therapeutic pathways. In the potential physiological impacts of elevated AHLs in blood serum, while more investigation is required, recent studies have demonstrated that AHLs can directly activate mammalian oncogenic pathways (25). Although the results of this study are promising, this preliminary investigation has several limitations that need to be expanded on with the future direction toward establishing AHL levels in serum as a potential biomarker of intestinal inflammation and CD status. Although n = 35 was sufficient to power this initial study, a larger number of individuals should be investigated to confirm the findings of elevated AHL levels in blood at the time of intestinal inflammation. Thus, a larger study that includes a separate validation cohort is required for these findings to become clinically relevant. Investigating a greater number of individuals will also allow for additional

parameters, such as race and ethnicity, to be factored into the evaluation. Furthermore, in the current study, we collected serum from individuals only once; moving forward it would be interesting and relevant to collect serum from the same individuals over time and across differing states of intestinal inflammation and disease to determine a temporal AHL profile. Although our study benefited from using colonoscopy to confirm intestinal inflammation, as we have shown that CRP levels in serum did not accurately reflect this state, patient colonoscopy preparations prevented us from collecting stool at the time when we confirmed intestinal inflammation. Collection of stools from individuals in addition to serum will allow for microbiome and metabolome analysis, which can be combined with QSM data to determine AHL-producing bacterial targets for potential therapeutic interventions. In addition, by determining the levels of QSMs in serum and stool through biosensors along with microbiome-sequencing data on QSM synthase and receptor protein transcripts, it may allow for confirmation of QSM translocation from the intestines to the bloodstream.

In summary, we have demonstrated that AHLs are found in human blood serum and these levels are different in healthy individuals and patients with CD, correlating with intestinal inflammation. Although further investigation is necessary into QSM profile changes over time and verifying potential mechanisms of AHL elevation, we believe that the knowledge from this initial investigation will be critical in the design and development of noninvasive platforms for disease management in CD and potentially other bacterially mediated disorders.

CONFLICTS OF INTEREST

Guarantors of the article: Sylvia Daunert, PharmD, PhD, MS.

Specific author contributions: G.O., S.K.D., and S.D. planned the study and designed the experiments. M.Q. and M.A. collected human specimens and participant information. G.O. performed laboratory experiments, and S.K.D., S.D., M.Q., and M.A. assisted with data interpretation. G.O. wrote the manuscript with input from the other authors.

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