

Low Mass Blood Peptides Discriminative of Inflammatory Bowel Disease (IBD) Severity: A Quantitative Proteomic Perspective*[§]

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Breakdown of the protective gut barrier releases effector molecules and degradation products into the blood stream making serum and plasma ideal as a diagnostic medium. The enriched low mass proteome is unexplored as a source of differentiators for diagnosing and monitoring inflammatory bowel disease (IBD) activity, that is less invasive than colonoscopy. Differences in the enriched low mass plasma proteome (<25 kDa) were assessed by label-free quantitative mass-spectrometry. A panel of marker candidates were progressed to validation phase and “Tier-2” FDA-level validated quantitative assay. Proteins important in maintaining gut barrier function and homeostasis at the epithelial interface have been quantitated by multiple reaction monitoring in plasma and serum including both inflammatory; rheumatoid arthritis controls, and non-inflammatory healthy controls; ulcerative colitis (UC), and Crohn’s disease (CD) patients. Detection by immunoblot confirmed presence at the protein level in plasma. Correlation analysis and receiver operator characteristics were used to report the sensitivity and specificity. Peptides differentiating controls from IBD originate from secreted phosphoprotein 24 (SPP24, $p = 0.000086$, 0.009); whereas those in remission and healthy can be differentiated in UC by SPP24 ($p = 0.00023$, 0.001),

α -1-microglobulin (AMBP, $p = 0.006$) and CD by SPP24 ($p = 0.019$, 0.05). UC and CD can be differentiated by Guanylin (GUC2A, $p = 0.001$), and Secretogranin-1 (CHGB $p = 0.035$). Active and quiescent disease can also be differentiated in UC and CD by CHGB ($p \leq 0.023$) SPP24 ($p \leq 0.023$) and AMBP (UC $p = 0.046$). Five peptides discriminating IBD activity and severity had very little-to-no correlation to erythrocyte sedimentation rate, C-reactive protein, white cell or platelet counts. Three of these peptides were found to be binding partners to SPP24 protein alongside other known matrix proteins. These proteins have the potential to improve diagnosis and evaluate IBD activity, reducing the need for more invasive techniques. Data are available via ProteomeXchange with identifier PXD002821. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M115.055095, 256–265, 2016.

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Inflammatory bowel disease (IBD)¹ is a life-long relapsing and remitting inflammatory disorder primarily affecting the gastrointestinal tract and can be subdivided into the main groups of Crohn’s disease (CD) and ulcerative colitis (UC) (1). Current treatment focuses on reducing and controlling inflammation. There is no cure and the majority of IBD patients remain under medical care and management for life. With increasing prevalence around the world, clinical assays that can provide accurate diagnosis, discrimination between CD and UC, and determination of disease activity are being sought to achieve effective treatment and management. The clinical presentations of both subtypes are similar and invasive diagnostic investigations, specifically colonoscopy and histopathological evaluation of the inflamed gut wall, remains the gold standard for diagnosis and assessment of activity (2–5). Current diagnostic antibody markers such as anti-*Saccharomyces cerevisiae* antibody (ASCA) and peri-nuclear anti-neutrophil cytoplasmic antibody (P-ANCA) or combinations of genetic susceptibility markers and serological markers provide increased specificity (6–10). Despite this, acute phase

¹ The abbreviations used are: IBD, inflammatory bowel disease; MRM, multiple reaction monitoring; ESI, electrospray ionization; DDA, data dependent acquisition, m/z , mass-to charge ratio; rT, retention time; FDA, Food and Drug Administration.

TABLE I

Both pooled and individual analysis was carried out for 109 participant samples with the described characteristics. Crohns disease = CD, Ulcerative colitis = UC, controls consisted of healthy patients and inflammatory RA controls

Group	CD	UC	Healthy Controls	RA Controls
Discovery	n = 20	n = 20	n = 10	–
Verification	n = 56	n = 27	n = 14 ^a	n = 12
Total samples	59	32	14	12
Mean Age (\pm S.D.)	37 (\pm 15)	39 (\pm 15)	31 (\pm 11)	56 (\pm 14)
Males (%)	30 (51%)	10 (31%)	3 (21%)	3 (33%)
Females (%)	29 (49%)	22 (69%)	11 (79%)	6 (67%)
Disease activity at collection				
Remission	27 (47%)	11 (34%)		
Mild activity	18 (30%)	9 (28%)		
Moderate activity	8 (13%)	5 (16%)		
Severe activity	6 (10%)	7 (22%)		

^a Two samples were obtained from inflamed synovium from otherwise healthy individuals.

proteins such as C-reactive protein (CRP), fecal calprotectin in addition to the erythrocyte sedimentation rate (ESR) and other clinical activity indicators are more typically used in practice to monitor disease progression in addition to colonoscopy (11). Unbiased discovery in patient plasma samples has the potential to capture both the reactive pathways that result in symptoms as well as identify novel causal proteins that may have initiated disease onset and the biological switch to autoimmune complications of IBD (12, 13). The regulation of homeostasis between the intestinal epithelial cells, mucosal surface, and the immune system that contribute to exacerbated inflamed response are less well characterized and would benefit from the *posteriori* knowledge of the global “omics” approach to explore emerging causal and reactive proteins and peptides for further validation. Discovery of new protein markers through proteomic technology has already expanded the knowledge of IBD (14–19) and can be used to improve the diagnostic accuracy, long-term management, and treatment of a host of different diseases (20, 21).

We have specifically focused on the differential protein profiles of 1–25 kDa fraction between IBD and healthy human plasma samples. Such partitioning of proteins enabled powerful enrichment of low mass and poorly abundant proteins (22). Using a shotgun proteomic approach, this large scale survey of proteins has highlighted the increase in inflammatory and acute phase proteins that are known to plague the illness and in addition has revealed novel peptides and proteins that can be used to discriminate IBD from controls, and UC from CD. These proteins have been investigated further using accurate and sensitive quantitative techniques of multiple reaction monitoring (MRM) for low-concentration peptides (23) applicable to verification phase Tier 2 multiplexed MRM assay development within the FDA-National Cancer Institute (NCI) biomarker pipeline (24). The on-column amounts of each protein from this biomarker panel were evaluated for individual samples, and Western blots have also been used to confirm presence.

MATERIALS AND METHODS

Experimental Design and Rationale—This study was approved by the Sydney Local Health District Human Research Ethics Committee (Approval code: CH62/6/2011 - 154). A total of 109 participants were included in this study. CD, UC, and RA patients were recruited from IBD and rheumatology ambulatory clinics, and control patients from those with no gastrointestinal disease and/or those undergoing endoscopy with normal findings. Biomarker specificity was tested using RA inflammatory controls as the disease shares some Th1/17 response pathways with IBD. IBD diagnoses were confirmed by histological and endoscopic criteria and RA by rheumatoid arthritis classification criteria with at least six months duration. All subjects had their phenotype confirmed by a gastroenterologist with radiologic and/or endoscopic evidence within 30 days from blood sampling as part of their routine care. Disease-specific activity indices for CD, UC, and RA (Crohn’s Disease Activity Index (CDAI), UC Partial Mayo Score (PMS), and 28-Joint Disease Activity Score, respectively) with paired biochemical markers of inflammation (C-reactive protein (CPR) and erythrocyte sedimentation rate (ESR)) were collected. This study included 83 patients suffering from IBD; 27 UC patients (7 severe: PMS >5, 14 mild to moderate: PMS 3–5, 11 remission: PMS \leq 2); 56 CD patients (6 severe: CDAI >450, 18 mild: CDAI <150, 27 remission: CDAI <150, and 8 moderate: CDAI 150–300); 14 healthy controls and 12 inflammatory RA controls. All patients were recruited from Concord Repatriation Hospital, and Bankstown-Lidcombe Hospital, in New South Wales, Australia. A second plasma sample was collected for some patients if their disease activity changed. Medications were noted at time of sample collection. Subject details and disease characteristics were obtained at time of recruitment and are summarized in Table I. This study consisted of a broad Discovery phase followed by validation and verification of peptide markers. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (25) via the PRIDE partner repository with the data set identifier PXD002821.

Collection and Storage of Plasma and Serum—Patient blood samples were collected using standard venipuncture techniques for plasma (EDTA-vacutainers) or Serum (gold-top), and centrifuged at 4000 rpm for 10 min at room temperature. Plasma or serum was extracted and separated into 100 μ l aliquots and stored at -80°C .

Low Mass Plasma Enrichment—Pooled plasma samples were created from 30 μ l aliquots of patients based on their defined clinical activity. Fifteen microliters of (10%) protease inhibitor (Roche, Basel, Switzerland) was added to 150 μ l of the mixed pools of plasma resulting in 10 pools of grouped samples comprising of

the groups: C (2 groups, $n = 10$), UC (4 groups, $n = 20$), CD (5 groups, $n = 25$). Prior to enrichment, the pooled samples were diluted 1:1 with 165 μl of 180 mM Tris/20 mM EACA/4 M urea buffer, pH 10.2. A five-chamber cartridge assembly using the ProteomeSep (MF10) for protein and peptide separation was prepared using 5, 25, 45, 65, and 125kDa polyacrylamide membranes (NuSep, Frenchs Forest, Sydney, Australia), and a 1 kDa regenerated cellulose membrane (Millipore, Merck Millipore, Darmstadt, Germany, MA), as previously described (22). Here, only the 1 to 25 kDa fraction is further reported.

Digestion and LC-MS/MS—The 1 to 25 kDa fractions were concentrated with C18 stage tips (ThermoScientific) according to the manufacturers recommendations except that the elution buffer consisted of 80% CH_3CN , 0.1% Formic acid. Peptides were resolubilized with 50 μl of 50 mM NH_4HCO_3 , pH 8.0. Subsequently, trypsin was added at an enzyme to protein ratio of $\sim 1:100$, and incubated overnight at 37 °C. Following digestion, 4 μl of formic acid was added and the samples were dried. Peptides were resuspended in 10 μl of 2% CH_3COOH , 0.1% formic acid prior to LC-MS/MS. One μl (10%) of 1–25 kDa was injected onto a nano-LC using an Ultimate 3000 HPLC and autosampler (Dionex, Dionex, Sunnyvale, USA, Amsterdam, Netherlands) for analysis. The samples were loaded onto a micro C18 precolumn (500 $\mu\text{m} \times 2$ mm, Michrom Bioresources, Michrom Bioresources, Auburn, USA, Auburn, CA) with Buffer (98% H_2O , 2% CH_3CN , 0.1% TFA) at 10 $\mu\text{l}/\text{min}$. After a 4-min wash the pre-column was switched (Valco 10-port valve, Dionex) into line with a fritless nano column (75 $\mu\text{m} \times 10$ cm) containing reverse phase C18 media (5 μm , 200Å Magic, Michrom Bioresources). Peptides were eluted using a linear gradient of Buffer A (98% H_2O , 2% CH_3COOH , 0.01% HFBA) to Buffer B (98% CH_3CN , 2% H_2O , 0.01% HFBA) at 250 nl min^{-1} over 60min. An LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany) was used to analyze the plasma fractions. The column tip was positioned ~ 0.5 cm from the heated capillary ($T = 200$ °C) of the LTQ-FT, and 1800V was applied to a low volume tee (Merck, Darmstadt, Germany). The instrument was operated in data dependent mode, with positive ions generated by electrospray. A survey scan of m/z 350–1750 was acquired in the FT ICR cell. Collision induced dissociation was used by the linear ion trap in which up to eight of the most abundant ions (>2000 counts) with charge states of $\geq (M + 2H)^{+2}$ were successively isolated and fragmented. Mass to charge (m/z) ratios selected for MS/MS were dynamically excluded for 60 s. There were a minimum of three replicates carried out for each of the pooled groups.

Protein Identification—For label-free quantification MS peak intensities were analyzed using ProgenesisQ1, LC-MS data analysis software v2.4 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Ion intensity maps from each run were aligned to a reference sample and ion feature matching was achieved by aligning consistent ion m/z and retention times, normalized against total intensity (sample specific log-scale abundance ratio scaling factor), and compared between groups by one-way analysis of variance (ANOVA, $p \leq 0.05$ for statistical significance). Type I errors were controlled for by false discovery rate (FDR) with q value significance set at <0.01 (26, 27). MS/MS spectra were searched against the Uniprot database (release 15, Nov 2009, containing $>497,000$ sequence entries). “Mascot Daemon/extract_msn” (Matrix Science, London, England) was used with 4ppm peptide tolerance and 0.6 Da fragment tolerance, “All-species” and “no enzyme,” and variable modifications to: cysteine (acrylamide); methionine (oxidation); serine, threonine, tyrosine (phosphorylation), selected to generate peak lists, which were submitted to the database search program Mascot (Matrix Science). Only peptides with anion score >20 were considered for protein identification. Enrichment pathway analysis was achieved using Ingenuity software (Qiagen,

Limburg, Netherlands) using the protein list described in supplemental Table S1.

Peptide Quantitation by MRM—Candidate peptides were selected from the enriched low mass proteome. Skyline software v1.4 (MacCoss Lab, WA, Seattle, USA) and MS/MS spectra from previous LC-MS/MS experiments were used to generate an MRM method consisting of 4–7 transitions per peptide. Multiple peptides were chosen from candidate proteins and refined by iterative experimentation, optimization of declustering potential, collision energies. Synthetic light and $^{13}\text{C}/\text{N}^{15}$ -labeled heavy peptides for each candidate peptide were obtained from Sigma-Aldrich at greater than 95% purity and dissolved in 50% CH_3CN , 2% CH_3COOH , 0.1% TFA to a 1 mg/ml concentration. Generation of standard curves are described by Yau et al. 2014 (23).

A plasma or serum volume of 2 μl from each patient (58 $\mu\text{g}/\mu\text{l}$, $\pm 7\%$) was added to 48 μl of 50 mM NH_4HCO_3 , pH8 and digested as described earlier. Peptides were captured using 3 passes through C18 Stage tip (ThermoScientific) and dried down. Samples were then resuspended in heavy labeled peptides to a final heavy peptide concentration of 50fmol/ μL each, and a final volume of 10 μl in 0.1% formic acid. Two μl injections of each sample were analyzed in a 4000Q-Trap mass spectrometer (AB SCIEX, MA, Framingham, USA) coupled to an Ultimate 3000 HPLC and autosampler system for the selected transitions. MRM data were processed using Skyline software (28). For each target peptide, quantitation was performed by ratio comparison of total transition peak areas between samples, normalization of peak areas to their heavy isotope internal standard, and concentration calculated according to the constructed standard curves for each peptide.

SPSS 21 (IBM, Englewood Cliffs, USA, NY) was used for statistical analysis to determine: distribution normality (Log_{10} was used to transform all peptides except SPP24: VNSQSLSPYLFR); means were calculated by One-Way ANOVA, correlations were assessed using Spearman Rho 2 tailed significance, and receiver operating characteristic (ROC) curves used to calculate sensitivity and specificity.

Confirmation by Western Analysis—Confirmation of three markers were investigated further using Western blot assays to confirm the MRM findings. Western blotting was carried out on control ($n = 4$), UC ($n = 7$), and CD ($n = 8$) patient samples using 2 μl of each subject's plasma, transferred onto PVDF membranes of pore size 0.2 μm (Merck Millipore, Darmstadt, Germany). Membranes were blocked with 10% skim milk for one hour and incubated overnight at 4 °C with primary antibody (polyclonal) in blocking solution. Anti-protein (Abcam, Cambridge, UK; and LSBio, Seattle, WA) diluted in blocking solution, were used for the proteins: SPP24 (1:400, immunogen details not provided), CHGB (1:500, C-terminal 15 residues), and AMBP (1:500; residues 200–300). Stabilized goat anti-rabbit horseradish peroxidase (Thermo Fisher Scientific Inc., Rockford, USA IL) diluted 1:10,000 (from 10 $\mu\text{g}/\text{ml}$) in blocking solution was used as a secondary antibody. Membranes were washed three times with chemiluminescence buffer and visualized using enhanced chemiluminescence detection reagents. The membranes were incubated in Pierce Super-Signal West Femto solution (Thermo Scientific) for 5 min and exposed to x-ray film (GE Healthcare, Buckinghamshire, UK) for 5–60 min to obtain the signal.

Binding Study—Tosylactivated magnetic beads (Thermo Scientific) were used as per manufacturer's instructions. Synthetic SPP24 peptide, or SPP24 Antibody was bound to the magnetic beads. Pooled whole serum samples grouped into IBD (10 patients) and healthy controls (5 patients) were analyzed for binding partners following trypsin digestion of eluted proteins using an Orbi-trap MS instrument (Thermo Electron, Bremen, Germany) as described by Coumans et al (29). MS ion abundance was analyzed using Q1 for proteomics (non-linear dynamics) as previously described. Nonredundant NCBI data-

TABLE II

Functional pathway enrichment of proteins significantly altered in IBD shows: A, Top canonical pathways characterized by alterations in lipid metabolism and signaling; and B, the five most enriched molecular and cellular functions based on the number of proteins identified significantly differing between IBD and control samples; C, Functional pathway enrichment of changes in IBD compared to control patients for SPP24 binding partners. Data is generated from normalized average abundances of significant differences ($p < 0.05$) between Control, UC and CD pooled plasma identifications

	p value	# Molecules/total molecules in pathway
	Fishers Exact Test	
A) Significant canonical pathways		
LXR/RXR Activation	4.28E-33	20/121
FXR/RXR Activation	8.64E-33	20/127
Acute phase response signaling	2.96E-22	16/169
Atherosclerosis signaling	6.71E-19	13/123
Clathrin-mediated endocytosis signaling	4.05E-18	14/184
IL-12 Signaling and Production in Macrophages	2.19E-18	13/135
Production of nitric oxide and reactive oxygen species in macrophages	1.39E-16	13/179
Role of tissue factor in cancer	2.03E-03	3/107
Complement system	3.24E-03	2/36
Actin cytoskeleton signaling	1.32E-02	3/210
B) Significant molecular and cellular functions		
	p value	# molecules
Lipid metabolism	4.33e-13-1.08e-3	24
Molecular transport	4.33e-13-1.08e-3	29
Small molecule biochemistry	4.33e-13-1.08e-3	27
Cell-to-cell signaling and interaction	9.38e-13-1.09e-3	25
Free-radical scavenging	3.50e-9-1.44e-4	13
C) Significant molecular and cellular functions of SPP24 binding partners		
Top hit	Name	p value range
Disease or disorder	Cancer	2.56E-3-4.32E-1
	Gastrointestinal disease	2.32E-3-4.32E-10
Molecular and cellular functions	Cell-to-cell signaling	2.7E-3-1.59E-7
	Lipid metabolism	2.52E-3-1.89E-6
Physiological system	Tissue morphology	2.32E-3-4.08E-7
	Immune cell trafficking	2.36E-3-4.72E-7
Identified associated network functions		Cancer, gastrointestinal disease Carbohydrate, lipid metabolism Cell cycle, connective tissue

base (downloaded 29 January 2015 containing 57,851,050 sequence entries) was searched with the following parameters; semi-tryptic, variable modifications (Cysteine oxidation, Methionine oxidation), error tolerance of 4ppm peptide and 0.4 Da fragment tolerance. Functional Pathway enrichment analysis using Ingenuity software was also carried out.

RESULTS

The IBD's Have a Distinct Low-mass Plasma Proteome—Greater than 1400 MSMS spectra were linked to 2300 features in the different pooled groups of control, CD, and UC; matching the criteria: significance $p < 0.05$, FDR adjusted p value $q < 0.01$, and a fold change ≥ 3 . Only data achieving a power of $> 80\%$ was further evaluated. There were 45 proteins that differed in relative abundance between the groups. Sixteen proteins were highest in abundance in IBD (for both UC and CD) compared with control, whereas 32 proteins were highest in UC patients and nine proteins were highest in CD patients. These proteins are listed in supplemental Table S1. Pathway enrichment analysis revealed lipid metabolism (LXR) ($p = 4.3E-33$), acute phase response signaling ($p = 3.0E-22$), IL-12 signaling and macrophages ($p = 2.2E-18$) among the top canonical pathways; and cell-to-cell signaling and transport among the significant molecular and cellular functions (Table II). Described here is the further verification of GUC2A,

SPP24, AMBP, CHGB, for their ability to distinguish between our groups as well as severity in UC and CD patients.

Increased SPP24 and AMBP Are Able to Differentiate IBD and Control Patients—The abundance of peptides in samples from controls: healthy patients (C), inflammatory control rheumatoid arthritis (RA); and patients suffering from IBD were determined by relative quantitation (ion count), absolute quantitation (MRM) and Western detection and are shown in Fig. 1. Significant differences for SPP24 peptides VSAQQVQGVHAR ($p = 0.005$) and VNSQSLSPYLFR ($p = 0.004$), between controls and IBD were observed and elevated in IBD (Fig. 1A). SPP24 shows close to 10 fold difference at the protein level (Fig. 1B). SPP24 (VSAQQVQGVHAR $p = 0.002$), VNSQSLSPYLFR $p = 0.004$) and AMBP (HHGPTITAK $p = 0.01$) can also be used to distinguish between healthy and quiescent disease in UC, and SPP24 (VSAQQVQGVHAR ($p = 0.017$), VNSQSLSPYLFR ($p = 0.048$)) in CD (Fig. 1C, 1D). Western analysis of 2 patients each for control, UC and CD also revealed lower levels of SPP24 present in control patients compared with IBD and lower levels of AMBP in control versus patients in remission in UC (Fig. 1E).

CHGB and GUC2A Are Able to Differentiate UC and CD Patients—The level of the peptide ADQTVLTEDK from CHGB ($p = 0.035$), and VTVQDGNFSFSLESVK from GUC2A ($p =$

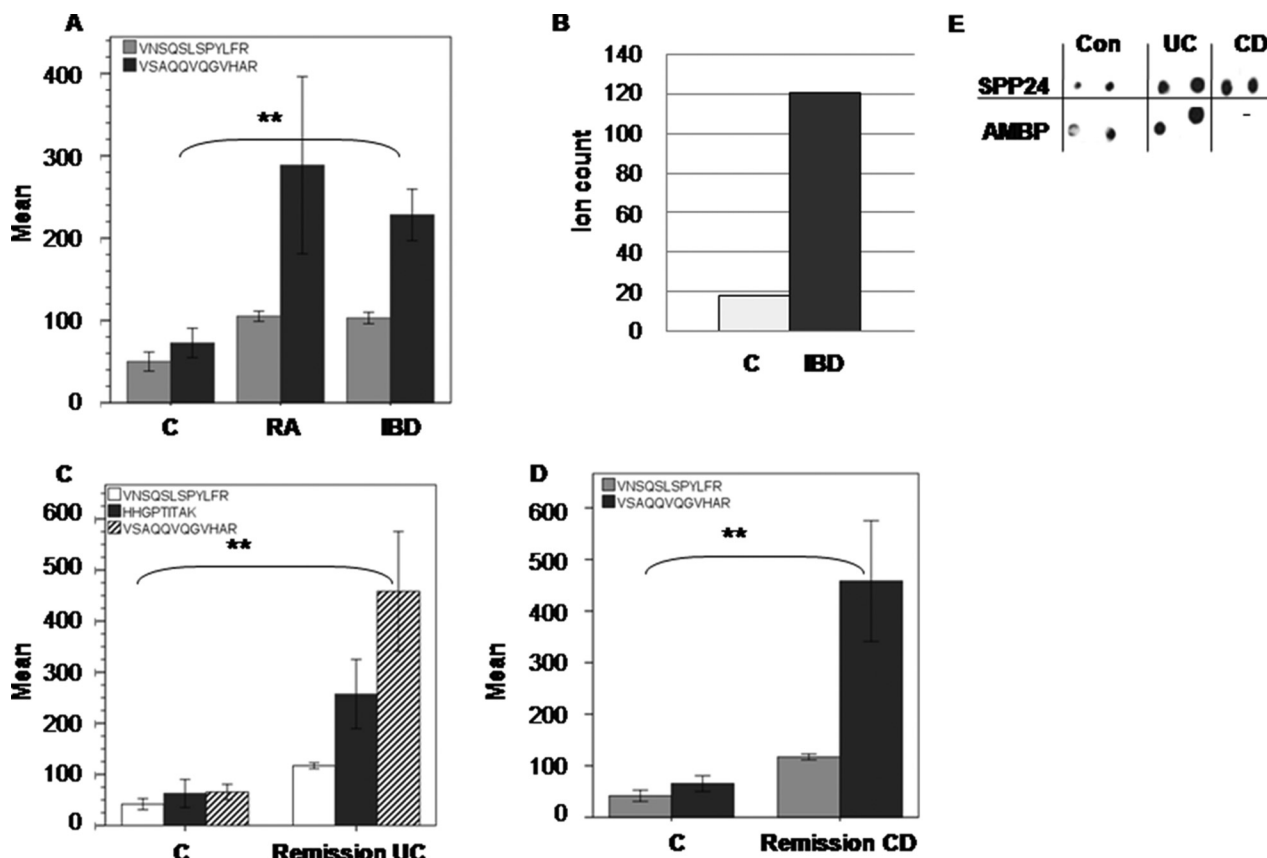


FIG. 1. Peptides from SPP24 using: A) MRM means of individual patient measurements shown in pg amounts, B) Label-free relative ion-count values of pooled samples, C–D) peptides from SPP24 and AMBP show elevation in quiescent disease compared with healthy patients and E) Antibody detection in whole serum for the proteins SPP24 showing differential abundance between control, UC and CD patients, and for AMBP showing difference in abundance between control and UC remission patients. Error bars indicate ± 1 SE, ** denotes $p < 0.01$.

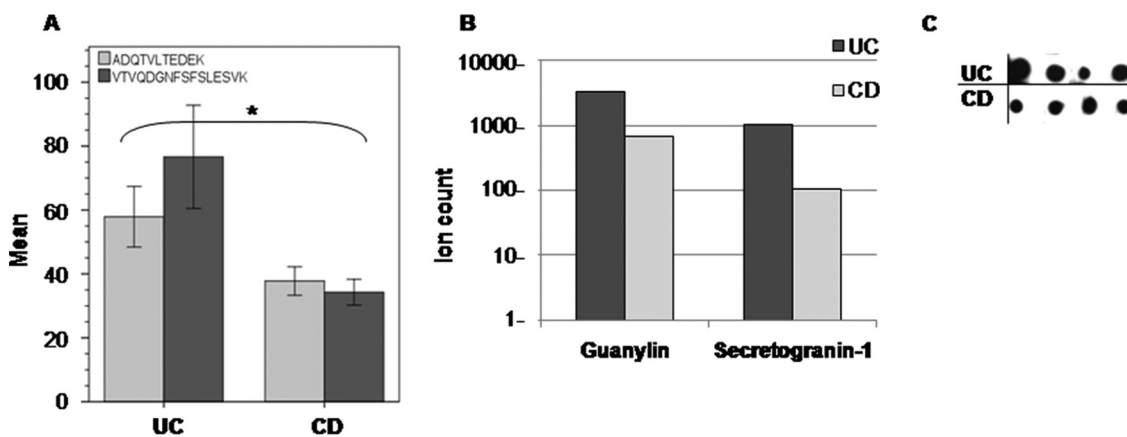
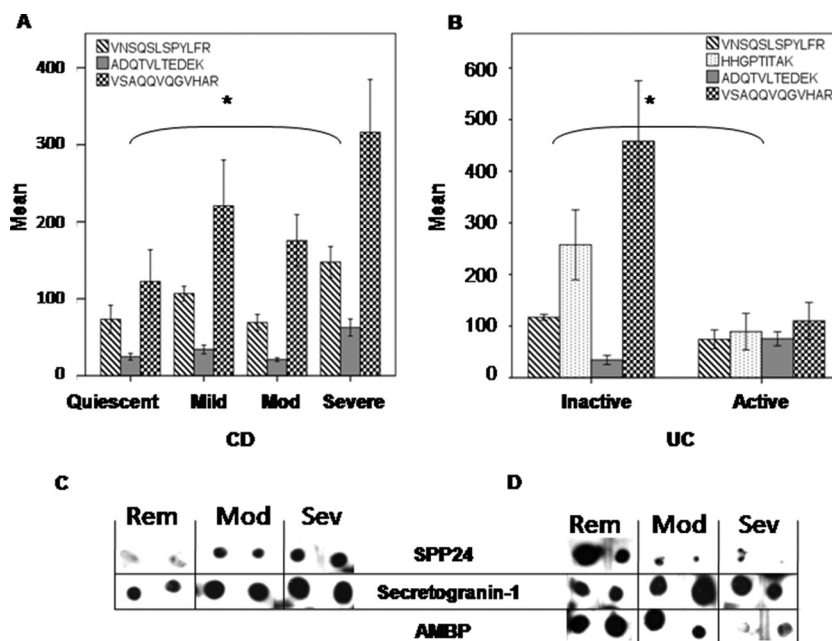


FIG. 2. Peptides from CHGB and GUC2A using: A) MRM mean pg amounts of individual measurements; B) ion count technique. C) Antibody detection of whole serum for the protein CHGB for 4 UC and CD patients. Error bars indicate ± 1 SE, * denotes $p < 0.05$.

0.005), are elevated in serum from patients suffering from UC as compared with the level of the same peptides in patients suffering from CD (Fig. 2A). This is also confirmed by ion count (Fig. 2B) and marginally observed increases at the protein level for CHGB in UC relative to CD detected by Western analysis (Fig. 2C).

IBD Severity can be Differentiated by SPP24, CHGB, and AMBP—The abundance of peptides in samples from patients suffering from CD of varying severity from clinical remission (CDAI<150), moderate/mild (CDAI>150) and severe (CDAI>450) cases were grouped. Severity scoring was based on the (Crohn’s) Disease Activity Index (CDAI), or Mayo Score

FIG. 3. Significantly differential peptides discriminating severity in CD and activity in UC patients: A) mean individual patient pg amounts for peptides from SPP24 and CHGB significantly increase with increasing severity based on CDAI score where quiescent is defined as a score <150 , mild to moderate >150 and severe >450 in CD patients. B) SPP24, AMBP peptides decrease with increasing activity compared with remission, while CHGB is increased in active UC patients compared with remission based on PMS score where inactive is defined as ≤ 2 , active >2 . Antibody detection for individual patients is shown for C) SPP24 and CHGB in CD, and D) SPP24, CHGB and AMBP in UC patients. Bars indicate $\pm 1SE$, * denotes $p < 0.05$.



(PMS) in UC; a broadly used and accepted measure of disease activity (30). Across these groups, peptides varying significantly include: VSAQQVQGVHAR and VNSQSLSPYLFR (Quiescent to severe, $p = 0.048$ and 0.023 ; moderate to severe, $p = 0.036$ and 0.047 respectively) from SPP24, ADQTVLTEDK (Quiescent to severe, $p = 0.012$) from CHGB. These peptides are elevated in the serum of patients with increased severity as compared with the level of the same peptides in serum from patients in remission; Fig. 3A, 3C. Similarly in UC significant differences in the abundance of the peptides VNSQSLSPYLFR (inactive to active $p = 0.019$, $p = 0.03$ across all groups) from SPP24 and ADQTVLTEDK from CHGB (inactive to active $p = 0.039$), and HHGPTITAK (inactive to active $p = 0.045$) from AMBP were observed when patients were grouped as quiescent and active based on PMS score; Fig. 3B, 3D. Overall, severity in general for IBD could be differentiated compared with quiescent levels using CHGB ($p = 0.01$), regardless of UC and CD status.

ROC curves reflect the differences between the peptide abundances for the conditions examined. The area under curve and probability shows the predictive performance and potential clinical utility of each marker across the conditions. These results and on-column derived cut-off values are summarized in Table III. Correlation analysis of the peptides was carried out using Spearman's Rho with both peptides from SPP24 correlating positively (0.574 , $p < 0.00001$), AMBP peptide correlating positively with SPP24 (VSAQQVQGVHAR: 0.688 , $p = <0.0001$; VNSQSLSPYLFR 0.405 , $p < 0.01$); GUC2A correlating positively with CHGB (0.651 , $p < 0.001$), AMBP (0.294 , $p < 0.05$) and with SPP24 VNSQSLSPYLFR (0.431 , $p < 0.001$). There was no significant correlation of any of these peptides to the current inflammatory tests of ESR, CRP, white cell count and platelet count (supplemental Table S2).

Both GUC2A and AMBP as well as the peptide VNSQSLSPYLFR have been shown to bind to the peptide VSAQQVQGVHAR and/or SPP24 antibody for a partner binding study. The binding partners included α -2-macroglobulin among other cell-to-cell signaling molecules, molecules involved in lipid metabolism, tissue morphology and gastrointestinal disease Table IIC (and supplemental Table S3, supplemental Table 4).

DISCUSSION

The etiology of IBD remains uncertain. However, the gut epithelium is heavily implicated as the battle-front as it responds to various environmental assaults to launch an immune response that is often systemic (31–33). The capacity to maintain epithelial cell integrity relies on the synergy and coordinated regulation of the largest endocrine system in the body. The gastrointestinal tract has a surface area of $\sim 100\text{m}^2$ and is integrally linked via effector systems (epithelium, secretory epithelium and endocrine cells and vasculature) to the enteric nervous system (gut-brain axis) (34). The cells lining the intestinal tract include enterocytes, secretory cells, goblet and enteroendocrine cells. Tight junctions working with actin and cytoskeletal proteins hold the enterocytes in place, and the mucus layer containing bioactive compounds, hormones such as gastric inhibitory peptide, serotonin and gastrin, and the immune cells provide both a chemical and physical barrier.

This study highlighted the modulation of many acute phase inflammatory response proteins between healthy and IBD sufferers. These inflammatory response proteins can be common to other conditions, such as rheumatoid arthritis. Confoundingly, there are also many overlapping clinical features within the IBD's, and extensive similarities between proteins

TABLE III
Receiver Operator Curve data for 5peptides are able to distinguish differences between IBD, severity and control samples

Test	Protein	Peptide	Area	p value	Sensitivity %	Specificity %	On-column cut-off
↑ IBD vs. Healthy	SPP24	VSAQQVQGVHAR	0.9	4.1×10^{-4}	82	73	70.9
		VNSQSLSPYLFR	0.9	2.5×10^{-4}	90	73	49.0
↑ UC vs. CD	GUC2A	VTVDGNFSFSLESVK	0.8	6.0×10^{-3}	67	89	49.6
		ADQTVLSTEDEK	0.7	8.0×10^{-2}	67	78	51.9
↑ Quiescent vs. Healthy (UC)	SPP24	VSAQQVQGVHAR	1.0	2.0×10^{-3}	100	91	153.3
		VNSQSLSPYLFR	1.0	4.0×10^{-3}	80	91	106.3
↑ Quiescent vs. Healthy (CD)	SPP24	HHGPTITAK	0.9	1.1×10^{-2}	80	81	101.3
		VSAQQVQGVHAR	0.8	4.8×10^{-2}	78	73	70.9
↑ Quiescent vs. active (UC)	SPP24	VNSQSLSPYLFR	0.8	1.7×10^{-2}	78	73	50.6
		VSAQQVQGVHAR	0.9	2.8×10^{-2}	67	100	156.8
↓ Quiescent vs. active (UC)	SPP24	VNSQSLSPYLFR	0.8	6.8×10^{-2}	67	100	95.5
		HHGPTITAK	0.8	1.0×10^{-1}	60	83	192.8
↑ Severe vs. Quiescent (CD)	CHGB	ADQTVLSTEDEK	0.9	4.5×10^{-2}	83	100	58.4
		VSAQQVQGVHAR	0.8	6.8×10^{-2}	67	90	268.5
	SPP24	VNSQSLSPYLFR	0.8	2.8×10^{-2}	83	60	112.9
		ADQTVLSTEDEK	0.8	1.5×10^{-2}	83	75	39.7

present in the UC and CD analysis. The identified differential peptides cement the role of neuroendocrine and enteric nervous systems as measured via hormones, peptides, protein fragments, naturally occurring within the circulating plasma. The nature and context of these peptides in the enriched low mass fraction of plasma may be related to the associated increase in vascular permeability (35, 36), as well as the activated binding and packaging of peptides into complexes in the endoplasmic reticulum (ER) tagged for secretion (37). At the interface between the epithelium and lymphatic/circulatory system, the altered secretion of lysozyme, defensins, IgA and mucins as well as the catabolism and breakdown of (intracellular) proteins become sequestered into the circulatory system and can be indicators of disease, phenotype and severity. Our data has shown increased levels of kininogen in IBD patients, along with other acute phase proteins such as fibrinogen, β -2-microglobulin, α -2-macroglobulin, haptoglobin and the apolipoproteins. In particular α -2-macroglobulin has previously been implicated as an intra and extracellular chaperone of vesicular traffic in the ER (37, 38). All proteins and small peptides have been measured initially from the enriched low mass component of plasma as well as in unfractionated plasma and serum using MRM. They have been quantitated as unique peptides from pooled and individual patients and confirmed using antibody detection, LC-MS/MS, and gel slice identification from Western blots. The importance of fragmented forms of larger inflammatory marker proteins have been noted in the literature and include C-reactive protein, ApoE, nucleolin, and collagen among others; these fragments are released from tissue into the circulation (39, 40).

Proteins are Modulated in Response to a Disruption in Gut Barrier Function: Enteric Nervous System and Immune System Messaging—For IBD patients, the aberrant inflammatory response is thought to be triggered by a break down in immune homeostasis, exposure to intestinal flora in the gas-

trointestinal tract as a consequence of a disruption to the mucus barrier (32), and autophagy of breakdown proteins as well as microbial attack. This manifests itself as a chronic and increasingly aberrant reaction against commensal flora. Homeostasis and intestinal motility can be maintained by the secretory proteins chromogranin-A (CHGA) and secretogranin-1(CHGB) (41, 42). These proteins are co-stored with other hormones and released in response to inflammation as part of the innate immune response (43) and have both pro-inflammatory and anti-inflammatory properties (44). Plasma CHGA levels have been found to be significantly higher in IBD patients over healthy subjects (35). It has also been reported this correlates positively with tumor necrosis factor- α (TNF- α) levels; particularly for patients with carcinoid tumor (42, 45). Increased levels of CHGA derived peptides have also been linked to extracellular Ca^{2+} influx by calmodulin regulated phospholipase A2 (46), while Wagner *et al.*, have shown increased fecal levels of CHGA, CHGB and secretoneurin in collagenous colitis (41). Higher levels of fecal CHGB in UC patients in remission as compared with healthy controls were observed by Strid *et al.* (47). These secretory proteins play a role as chemical messengers between the enteric nervous system and the immune system and therefore may act as potential markers for other neurological and psychiatric disorders (48). Their fragment forms have bioactive properties against microbial infection (49), homeostatic regulation (50), and angiogenesis (51), and are modulated in many inflammatory diseases. Here we have shown that circulating levels of CHGB are significantly increased for patients suffering from UC compared with CD. A positive correlation was observed between the levels of CHGB and GUC2A.

Homeostasis in the enteric system is also maintained by the absorption and secretion of salt and water between the mucosal surface and the circulatory system. This regulation is achieved by low Ca^{2+} activation of guanylate cyclase via release of GUC2A. GUC2A is a neuroendocrine peptide hor-

more produced by the epithelium and secreted by goblet cells locally in the mucosa (52). Increases in intracellular cGMP by guanylin causes Cl^- and HCO_3^- secretion into the lumen and results in loss of fluid and diarrhea (53). It is interesting to note that GUC2A has structural homology to the heat stable enterotoxin (STa) and opportunistic gut infections generating STa act on the same guanylin receptor to produce similar symptoms. In a small study comparing the levels of GUC2A, Kuhn *et al.* (2008), have previously found no difference in levels for IBD compared with controls. However, potentiators of GUC2A have been found to be useful for the treatment of a variety of gastric disorders (54). Here, GUC2A was found to increase with severity in CD sufferers with higher overall means quantitated in the IBD's over both healthy and inflammatory controls. Peptides originating from the HMW-chain (position 22–115) of GUC2A were found to be a binding partners to SPP24 in binding studies and this is also supported by its positive correlation with levels of SPP24 protein in the enriched low mass fraction of plasma. GUC2A also had a positive correlation to levels of AMBP in the low mass fraction. Peptides from the position of 20–203 in the α -1-microglobulin region of AMBP (1 of 3 cleavage products) were also shown to be binding partners to SPP24. Serine proteases are released by intestinal cells in response to inflammation through protease activated receptors (PARS) which causes the damage to surrounding tissue that is so prevalent in CD and UC long term sufferers. Alpha-1-microglobulin is an inhibitor of these events and is released into the plasma. Levels of α -1-microglobulin have been shown to result in increased apoptosis (55–57). Lipopolysaccharide (LPS) stimulated release of TNF- α is also attenuated by the binding of α -1-microglobulin to LPS (58). Here we have shown that levels of α -1-microglobulin are increased in remission compared with control for UC patients and higher in these patients than in UC active disease. Sufferers in clinical remission are known to display the histological features of inflammation which correlate with endoscopic appearance (59). This AMBP cleavage product detectable in blood is able to highlight clinical remission and could therefore provide an alternative measure over colonoscopic evaluations. Correlation between the levels of CHGB, GUC2A, and AMBP in the enriched low mass fraction may be related to their binding affinity to SPP24 and in particular the VSAQQVQGVHAR playing a role as receptor in this affinity.

Restricting the release of TNF- α to limit the deleterious effects of inflammation is often essential. Mast cell and macrophage secretory granules contain many bioactive peptides and proteases bound together within the serglycin proteoglycan with decreased levels of serglycin correlating to increased release of TNF- α (60). These granules release their serglycin-bound proteases when the gut barrier is disrupted through many trigger pathways including the activation of the complement-receptor mediated pathway (61, 62). Serglycin and the associated release of lysozyme also correlates with

our finding of increased levels of these two proteins in IBD (63, 64). A study has shown increased expression of lysozyme and neutrophil defensins in secretory vesicles of mucosal epithelial cells of active UC patients (1, 2) and the associated serological anti-neutrophil antibodies (ANCA) as well as ASCA have been described as useful markers to differentiate between IBD and non-IBD cases (3, 6) SPP1 (osteopontin) and SPP24 are key regulators of immune function. The mature form of SPP24 contains 3 domains including a cystatin-like domain (55). Both peptides, VSAQQVQGVHAR (residues 121–133) and VNSQSLSPYLFR (residues 50–61), sit within the cystatin-like domain positionally appearing quite similar in structure to cystatin and its active binding site. In particular VSAQQVQGVHAR may be uniquely geared to activate and or bind α -2-macroglobulin along with other response related proteins either at the site of intestinal damage or within the circulation. This domain also binds transforming growth factor- β (TGF- β) resulting in increased apoptosis (55). SPP24 has also been observed by us and others (65) to exist in a high mass complex bound to α -2-macroglobulin and other proteins in serum (supplemental Table S3). SPP24 is present in smooth muscle, epithelial cells and is released from macrophages and T-cells in response to heightened levels of TNF- α . Increased levels of SPP24 trigger macrophage infiltration and release of Interferon-Gamma (IF- γ) and Interleukin-12 (IL-12). Recent studies show SPP24 down-regulates TGF- β with significant implications for bone growth (66). The elevation in CD and reduction in UC in blood serum of SPP24 in quiescent disease and severe inflammation also has the potential to be used to monitor disease progression.

Together, the identification of clinically relevant blood markers may aid in the stratification of disease as well as predict for severity while reducing the need for invasive procedures. The sensitivity and specificity have been demonstrated for these peptide markers and can be used to differentiate IBD from healthy, UC from CD, and quiescent disease from IBD. In association with other measures these markers may prove to be useful for the IBD's.

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☐ This article contains supplemental Tables S1 to S4.

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