Resistin, Elastase, and Lactoferrin as Potential Plasma Biomarkers of Pediatric Inflammatory Bowel Disease Based on Comprehensive Proteomic Screens

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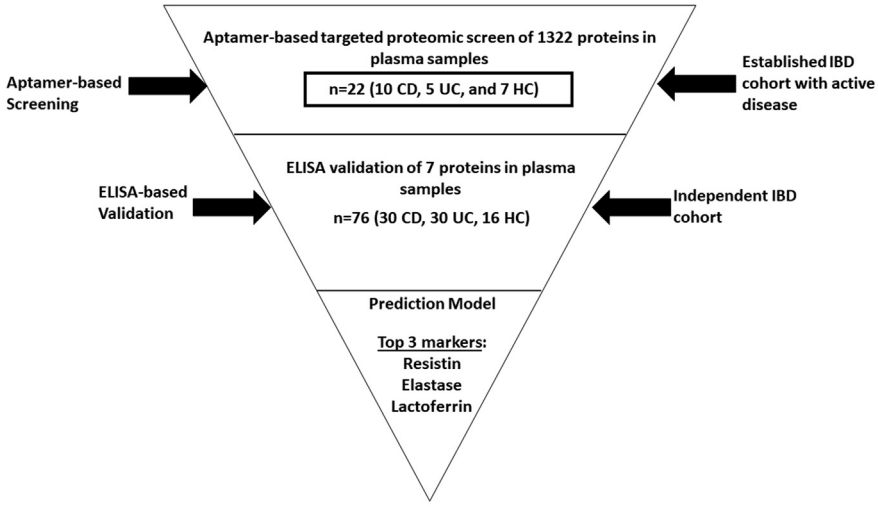
In Brief

Inflammatory bowel disease (IBD) is an immune-mediated chronic inflammation of the intestine, which can present as ulcerative colitis or Crohn's disease. Plasma samples from a pediatric IBD cohort of 22 subjects were interrogated using an aptamer-based screen of 1322 proteins, and the elevated biomarkers identified were further validated by ELISA using an independent cohort of 76 pediatric plasma samples. We have identified circulating resistin, elastase, and lactoferrin as potential plasma biomarkers of IBD in pediatric patients.

Graphical Abstract



Highlight

- Resistin, elastase, and lactoferrin are potential plasma biomarkers of pediatric IBD.

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Resistin, Elastase, and Lactoferrin as Potential Plasma Biomarkers of Pediatric Inflammatory Bowel Disease Based on Comprehensive Proteomic Screens

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Inflammatory bowel disease (IBD) is an immune-mediated chronic inflammation of the intestine, which can present in the form of ulcerative colitis (UC) or as Crohn's disease (CD). Biomarkers are needed for reliable diagnosis and disease monitoring in IBD, especially in pediatric patients. Plasma samples from a pediatric IBD cohort were interrogated using an aptamer-based screen of 1322 proteins. The elevated biomarkers identified using the aptamer screen were further validated by ELISA using an independent cohort of 76 pediatric plasma samples, drawn from 30 CD, 30 UC, and 16 healthy controls. Of the 1322 proteins screened in plasma from IBD patients, 129 proteins were significantly elevated when compared with healthy controls. Of these 15 proteins had a fold change greater than 2 and 28 proteins had a fold change >1.5. Neutrophil and extracellular vesicle signatures were detected among the elevated plasma biomarkers. When seven of these proteins were validated by ELISA, resistin was the only protein that was significantly higher in both UC and CD ($p < 0.01$), with receiver operating characteristic area under the curve value of 0.82 and 0.77, respectively, and the only protein that exhibited high sensitivity and specificity for both CD and UC. The next most discriminatory plasma proteins were elastase and lactoferrin, particularly for UC, with receiver operating characteristic area under the curve values of 0.74 and 0.69, respectively. We have identified circulating resistin, elastase, and lactoferrin as potential plasma biomarkers of IBD in pediatric patients using two independent diagnostic platforms and two independent patient cohorts.

Inflammatory bowel disease (IBD) is an immune-mediated chronic inflammation of the intestine (1–3). While the cause of IBD still remains unclear, factors such as genetic variations, alterations in the immune system, and bacterial interactions may impact the development of IBD. IBD can present as Crohn's disease (CD), affecting any portion of the gastrointestinal tract and all bowel layers, or as ulcerative colitis (UC), mainly affecting the colon and ileum (4). The prevalence of IBD among adults in the United States has increased from 0.9% of the population in 1999 to 1.3% of the population in 2015, with an estimated 3.1 million patients (5). The prevalence of pediatric IBD has also increased from 33 per 100,000 in 2007 to 77 per 100,000 in 2016 (6). Current diagnostic approaches include endoscopic biopsies, imaging, clinical parameters, fecal calprotectin, and inflammatory markers. Biopsies obtained using esophagogastroduodenoscopy and colonoscopy are necessary to confirm the diagnosis (7), but these tests are invasive, costly, and time consuming. Using circulating inflammatory biomarkers as a diagnostic tool may facilitate early detection and treatment in a less aggressive and noninvasive manner (8). These biomarkers are also likely to play a role in surveillance to monitor progression of IBD and related complications, such as colorectal cancer (9).

Shortfalls with current biomarkers include nonspecificity, suboptimal sensitivity, and the inability to differentiate between CD and UC (10). Most protein biomarker studies in IBD to date have focused on limited numbers of biomarker candidates, selected based on prior knowledge of the protein's function in disease (11). This study utilizes a high-throughput aptamer-based targeted proteomic assay to uncover novel plasma biomarkers for pediatric IBD (12, 13). With high dynamic range, sensitivity (femtomolar to micromolar range), accuracy, and reproducibility, this novel targeted screening platform that interrogates >1000 proteins has been applied to several other diseases (14–23). Candidate biomarkers discovered using this novel screening platform were subsequently validated by ELISA in cross-sectional cohort of subjects.

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A recent study has also employed a similar approach to identify plasma biomarkers in adult IBD patients (24). The top biomarkers useful in diagnosis of IBD and differentiating CD and UC differ between adult IBD and pediatric IBD because of the differences in extent of disease manifestation with children having a more spread in proximal colon in UC and have increased large bowel involvement in CD (25, 26). Serological tests of perinuclear antineutrophil cytoplasmic antibody and anti-*Saccharomyces cerevisiae* have been used for diagnosis of UC and CD in adults, but these tests are not effective in pediatric IBD (27). The current study represents the first use of this aptamer-based screen in pediatric IBD plasma samples and also the largest targeted plasma proteomic study in pediatric IBD.

EXPERIMENTAL PROCEDURES

Patients, Sample Collection, and Sample Preparation

All plasma samples were obtained from Emory University School of Medicine, Division of Pediatric Gastroenterology, with informed consent. This study was approved by the institutional review boards of Emory University School of Medicine, Atlanta, GA and the University of Houston, Houston, TX. Plasma samples were sent to the University of Houston for sample processing and analysis after collection at Emory University School of Medicine. The study design and conduct complied with all relevant regulations and criteria set by the Declaration of Helsinki. A cohort of 22 pediatric plasma samples (10 CD, 5 UC, and 7 HC) was used for the aptamer-based screen of 1322 proteins. The biomarker validation by ELISA was performed on a cross-sectional cohort of 76 plasma samples (30 CD, 30 UC, 16 healthy control [HC]). Detailed demographic data are included in Table 1.

Aptamer-Based Screening

Plasma samples were screened to measure the levels of 1322 distinct human proteins using an aptamer-based targeted proteomic assay, as detailed in our previous study (23). After a series of incubation steps, the recovered aptamer oligos were hybridized onto a custom Agilent DNA array overnight, using Agilent buffers (Agilent; catalog no.: 5188-5221) and scanned using a microarray scanner (Agilent; catalog no.: G4900DA) (23). Data were extracted using Agilent Feature extraction software. Along with the plasma samples, a "no protein" buffer blank allowed for the assessment of background signal. Biomarker studies and data analyses were performed at the Houston OMICs Collaborative (https://hoc.bme.uh.edu/), where the relative fluorescence unit values for each protein were normalized using calibrators, and hybridization normalized and median normalized protein expression values were generated. The expression values for all measured proteins are listed in supplemental Table S1. These values were further analyzed, and statistical testing was performed using R scripts in RStudio (Posit, PBC) to evaluate the differences between sample groups.

Cross-Sectional Validation Study Using ELISA

The biomarkers identified by the aptamer-based screen were validated in cross-sectional cohort of 76 samples (30 CD samples, 30 UC samples, and 16 HC samples). Human Catalase ELISA kit (catalog no.: ab171572; Abcam, 1:100 dilution), Human Elastase ELISA kit (catalog no.: ab11955; Abcam, 1:25 dilution), Human Insulin-Like Growth Factor 1 (IGF-1) ELISA kit (catalog no.: ELH-IGF1; Raybiotech, 1:10 dilution), Human Lactoferrin (LTF) ELISA kit (catalog no.: ELH-LTF; Raybiotech, 1:25 dilution), Human Peroxiredoxin ELISA kit (catalog no.: ab185983; Abcam, 1:10 dilution), Human Resistin ELISA kit (catalog no.: ELH-resistin; Raybiotech, 1:10 dilution), and Human tumor necrosis factor alpha (TNF-α) ELISA kit (catalog no.: ELH-TNF-α; Raybiotech, 1:5 dilution) were used as per the manufacturer's instructions. Briefly, diluted plasma samples were added on a precoated 96-well microplate, and antibodies were added after an incubation

TABLE 1
Demographic characteristics of subjects for plasma biomarker studies

Variable	Category	Subjects used for aptamer-based screening			
		HC (N = 7)	CD (N = 10)	UC (N = 5)	All (N = 22)
Gender, n (%)	Female	3 (42.9)	2 (20.0)	3 (60.0)	8 (36.3)
	Male	4 (57.1)	8 (80.0)	2 (40)	14 (63.7)
Race, n (%)	African American	2 (28.6)	3 (30.0)	1 (20)	6 (27.3)
	Caucasian	4 (57.1)	7 (70.0)	2 (40)	13 (59.1)
	Other	1 (14.3)	0 (0.0)	2 (40)	3 (13.6)
		Subjects used for ELISA screening			
		HC (N = 16)	CD (N = 30)	UC (N = 30)	All (N = 76)
Age (years)					
Gender, n (%)	Female	9 (56.2)	12 (40.0)	19 (63.3)	40 (52.6)
	Male	7 (43.75)	18 (60.0)	11 (36.7)	26 (34.2)
Race, n (%)	African American	2 (12.5)	8 (26.7)	2 (6.67)	12 (15.7)
	Caucasian	12 (75.0)	19 (63.3)	19 (63.3)	50 (65.7)
	Hispanic	1 (6.2)	1 (3.33)	5 (16.7)	7 (9.21)
	Asian	0 (0.0)	2 (6.67)	0 (0.00)	2 (2.63)
	Other	1 (6.2)	0 (0.00)	3 (10.0)	4 (5.2)
	Multiracial	0 (0.0)	0 (0.00)	1 (3.33)	1 (1.31)

Data are presented as n (%).

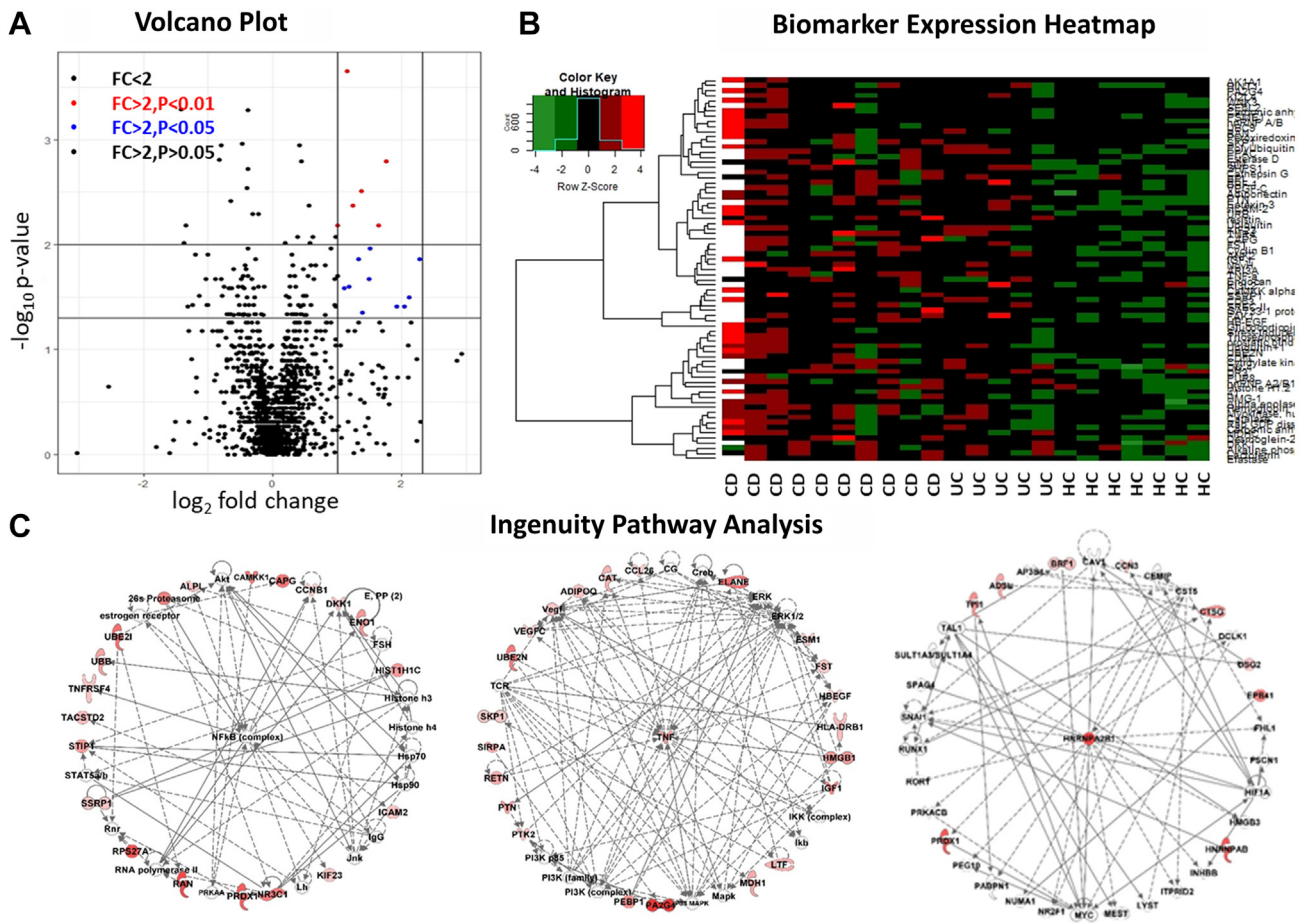

FIG. 1. **Aptamer-based screening of pediatric IBD plasma samples for 1322 proteins.** *A,* volcano plot representation shows the expression of 1322 proteins analyzed in 22 human plasma samples (5 UC, 10 CD, 7 HC), plotting the $\log_2$ of the fold change (FC) *versus* $-\log_{10}$ *p*. Proteins that do not meet the thresholds for biological or statistical significance are represented by *black dots* and proteins with FC >2 and $p < 0.05$ by *blue dots* and those with FC >2 and $p < 0.01$ by *red dots*. The FC thresholds are represented by the *vertical black lines* and *horizontal lines* for *p* value thresholds. *B,* heatmap representation of the top 75 proteins elevated in pediatric IBD plasma samples. *Columns* represent each individual sample, and *rows* correspond to protein levels. Proteins with expression above the mean value for each biomarker are represented by *red*, those with expression below the mean value are in *green*, and those with comparable means are in *black*. *C,* the top three protein clusters identified by integrated pathway analysis (IPA) for the significantly elevated proteins in IBD *versus* HC plasma samples. The proteins elevated in IBD plasma are shaded *red*, *solid arrows* represent documented interactions, and *dashed arrows* represent putative interactions between displayed molecules, as reported in the literature.

period. Then, streptavidin–horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine substrate were added. The plates were read at an absorbance of 450 nm in a microplate reader (ELX808 from BioTek Instruments). Experimentation was performed blinded, so the operator was unaware of which experimental group was being tested. Standard curves on each ELISA plate were used to determine the absolute levels of the protein biomarkers.

Experimental Design and Statistical Rationale

A cohort of 22 pediatric plasma samples were used for the aptamer screens, and an independent cohort of 76 pediatric plasma samples was used for ELISA validation. GraphPad Prism 7 (Dotmatics), Microsoft Excel (Microsoft Corporation), easyROC software (https://github.com/dncR/easyROC), and RStudio were used to plot and analyze biomarker data. Comparisons of biomarker groups were analyzed using Mann–Whitney *U* test, following which *p* values and *q* values were calculated. Correlation analysis was performed using the Spearman and Pearson's methods. For sensitivity, specificity, positive predictive value, negative predictive value, and area under the receiver operating characteristic (ROC) curve (AUC) analysis, the easyROC software was utilized. GraphPad Prism 7 was used to analyze significant differences between the groups tested, using nonparametric Mann–Whitney *U* test.

RESULTS

The overall workflow in this study is depicted in the consort diagram in supplemental Fig. S1. The levels of 1322 proteins were measured in plasma samples from 14 male and 8 female pediatric subjects (10 CD, 5 UC, and 7 HC) using a commercial aptamer-based targeted proteomic platform purchased from SomaLogic. Calibrators were used to normalize the expression of these proteins, and the normalized data

were used for further analysis. About 129 proteins were significantly elevated with $p < 0.05$ in IBD plasma compared with HC plasma.

A volcano plot was used to discern the differences in expression of 1322 proteins in the plasma of pediatric subjects, when comparing $\log_2$ fold change (FC) of protein expression *versus* the negative $\log_{10}$ *p* value (Fig. 1*A*). Each dot represents the average value for that protein (IBD *versus* HC). About 24 proteins exhibited an FC >2 and $p < 0.1$, represented by *red dots*, and 16 proteins exhibited an FC >2 and $p < 0.05$, represented by *blue dots*. While both upregulated and downregulated proteins shed light on the molecular mechanisms of disease, we focused on the upregulated proteins because these proteins had high potential to be noninvasive diagnostic biomarkers. The top 50 downregulated proteins are listed in supplemental Table S2.

The top 75 proteins with FC >1.25 and $p < 0.05$ were used to generate a heatmap to visualize the protein expression clusters among CD, UC, and HC (Fig. 1*B*). The functional networks that the overexpressed plasma proteins in IBD belonged to were examined using ingenuity pathway analysis. The functional networks that interlinked the largest numbers of overexpressed protein biomarkers in IBD included networks related to NF-κB signaling, TNFα signaling, and heterogeneous nuclear ribonucleoproteins A2/B1, as displayed in Figure 1*C*. In these functional networks, the proteins upregulated in IBD plasma are denoted in *red* with the intensity of color being proportional to the FC of these proteins.

Gene Ontology (GO) functional analysis was also performed using the differentially expressed proteins in IBD plasma using DAVID (https://david.ncifcrf.gov/). Figure 2*D* depicts the 10 most significantly enriched GO terms by biological process, molecular function, and cellular component. The cellular location of these proteins from the GO analysis was very interesting because these proteins were mainly associated with extracellular region, extracellular space, extracellular organelle, extracellular vesicle, extracellular exosome, vesicle, vesicle lumen, cytoplasmic vesicle lumen, and secretory granule lumen. This suggests that these biomarkers are mostly extracellular and secretory in nature and are likely released from the site of pathogenesis (colon) into the blood stream.

The expression levels of the top 50 proteins ($p < 0.05$, sorted by FC, listed alphabetically) in the plasma of CD, UC, and HC subjects are displayed by the horizontal dot plot in Figure 2*A* and also detailed in Table 2. A correlation matrix was performed using the top 50 significant proteins (FC >1.25 and $p < 0.05$), and the order of the proteins was based upon hierarchical clustering. Pearson's correlation coefficient was determined for all protein pairs, and the correlations identified as being significant at $p < 0.05$ are represented by *color circles*, *blue* for positive correlation, and *red* for negative correlation as shown in Figure 2*B*. The largest cluster included 14 proteins that were correlated to each other, including peroxiredoxin 1 and IGF-1. The next two largest clusters harbored six to seven proteins each, followed by several smaller clusters composed

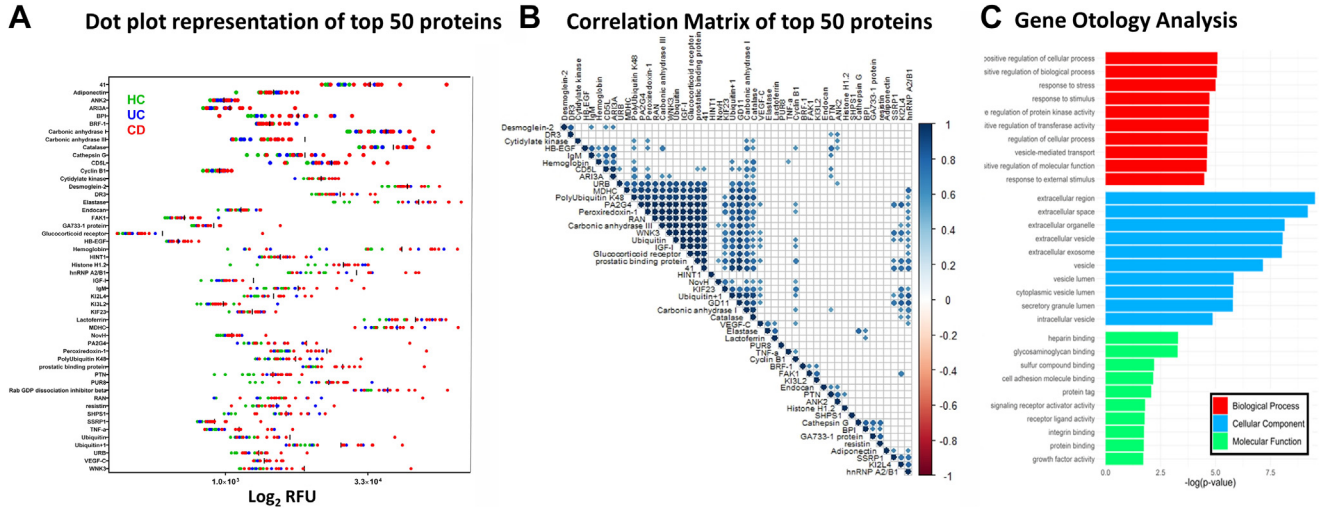

FIG. 2. **Graphical representation of the top 50 plasma proteins elevated in pediatric IBD plasma.** *A,* dot plot representation of top 50 proteins elevated in IBD plasma ($p < 0.05$): each *dot* represents the expression level of the protein in healthy controls (*green*), UC (*blue*), and CD (*red*). *B,* pairwise correlation plot to determine the degree of correlation among the elevated plasma proteins. Each *circle* represents the degree of correlation for the given biomarker pair, with *blue* corresponding to positive correlation and *red* corresponding to negative correlation. Biomarker pairs with low significance ($p > 0.05$) are not considered and left blank. *C,* shown is the Gene Ontology (GO) functional analysis of the proteins elevated in IBD plasma using DAVID. The figure represents the 10 most significantly enriched GO terms in biological process, molecular function, and cellular component. *CD,* Crohn's disease; *HC,* healthy control; *IBD,* inflammatory bowel disease; *UC,* ulcerative colitis.

TABLE 2
Top 50 IBD plasma biomarkers from aptamer-based screening (AUC >0.8)

Protein[a]	Plasma protein level (RFU), mean (median)				FC[b]		
	HC (N = 7)	CD (N = 10)	UC (N = 5)	IBD (N = 15)	IBD/HC	CD/HC	UC/HC
41	15,519 (16,362)	53,550 (37,554)	24,032 (18,743)	43,711 (33,664)	2.82*	3.45*	1.55
Adiponectin	2616 (2452)	3654 (3035)	3549 (3581)	3619 (3544)	1.38*	1.4*	1.36
ANK2	811 (791)	1124 (1091)	992 (976)	1077 (1083)	1.33	1.39	1.22*
ARI3A	724 (719)	1638 (1152)	853 (731)	1358 (1018)	1.87	2.26*	1.18
BPI	2962 (2101)	8018 (5493)	6263 (5088)	7433 (5088)	2.51**	2.71*	2.11
BRF-1	2481 (2352)	3244 (2958)	3336 (2798)	3277 (2878)	1.32*	1.31*	1.34
CA1	30,286 (26,156)	68,602 (59,613)	44,571 (45,718)	60,592 (56,690)	2.00**	2.27	1.47
CA3	2016 (1874)	13,199 (3580)	1963 (1613)	9454 (3078)	4.69	6.55**	0.97
Catalase	39,895 (41,011)	78,262 (78,098)	56,310 (46,673)	70,945 (77,947)	1.78	1.96*	1.41
Cathepsin G	3428 (2806)	10,664 (6445)	5810 (5418)	8930 (6184)	2.61	3.11**	1.69
CD5L	8197 (8554)	19,174 (14,902)	9742 (10,628)	16,030 (12,861)	1.96*	2.34**	1.19
Cyclin B1	770 (759)	972 (967)	903 (923)	947 (964)	1.23**	1.26*	1.17
Cytidylate kinase	8891 (8624)	11,710 (11,100)	10,644 (10,952)	11,329 (11,026)	1.27*	1.32*	1.2
Desmoglein-2	70,831 (63,452)	98,068 (81,565)	82,074 (84,250)	92,737 (84,250)	1.31**	1.38*	1.16
DR3	12,241 (10,361)	19,966 (16,315)	16,546 (14,490)	18,826 (14,490)	1.54**	1.63*	1.35*
Elastase	43,437 (20,913)	161,353 (148,820)	122,748 (108,783)	147,566 (133,949)	3.4	3.71**	2.8*
Endocan	1216 (1192)	1696 (1606)	1495 (1467)	1629 (1584)	1.34*	1.40*	1.23
FAK1	285 (266)	425 (439)	425 (320)	425 (361)	1.49**	1.49**	1.49
FST	843 (823)	1074 (987)	1096 (1027)	1082 (1007)	1.28*	1.27*	1.06
GA733-1 protein	552 (484)	1043 (672)	588 (599)	880 (651)	1.59*	1.89*	1.34
HB-EGF	279 (269)	377 (372)	294 (280)	347 (327)	1.25*	1.35**	1.06
Hemoglobin	23,089 (31,493)	115,140 (121,903)	67,577 (11,986)	99,286 (92,094)	4.3	4.99*	2.93
HINT1	2900 (3050)	4322 (4215)	4524 (3786)	4394 (4001)	1.52*	1.49*	1.56
Histone H1.2	9432 (7638)	22,787 (20,765)	21,781 (18,707)	22,427 (20,305)	2.38	2.42**	2.31
hnRNP A2/B1	7562 (7269)	41,613 (24,747)	15,654 (10,112)	32,960 (15,984)	4.36*	5.50*	2.07
IGF-I	896 (929)	3281 (1208)	1095 (975)	2553 (1130)	2.85**	3.66	1.22
IgM	3608 (3586)	8633 (5320)	4120 (3658)	7128 (4449)	1.98	2.39*	1.14
KI2L4	2205 (2170)	4262 (3824)	3145 (2606)	3863 (3329)	1.75*	1.93**	1.43
KI3L2	741 (710)	954 (911)	1330 (798)	1088 (862)	1.47*	1.29**	1.79
KIF23	1562 (1610)	2213 (2216)	2105 (2411)	2174 (2244)	1.39*	1.42*	1.35
LTF	65,650 (51,155)	124,388 (123,048)	118,878 (121,390)	122,552 (121,390)	1.87**	1.89*	1.81
MDHC	40,178 (39,200)	86,778 (65,595)	64,506 (58,484)	79,354 (59,198)	1.98*	2.16**	1.61
NovH	967 (966)	1385 (1196)	1185 (1113)	1314 (1186)	1.36	1.43	1.23
PA2G4	2035 (2093)	13,001 (3875)	3518 (3381)	9840 (3411)	4.83**	6.39	1.73
Peroxiredoxin-1	4157 (4038)	20,477 (8234)	6542 (4728)	15,832 (8208)	3.81*	4.93*	1.57
PolyUbiquitin K48	3163 (3394)	7550 (5167)	5365 (4169)	6822 (4807)	2.16*	2.39**	1.7
PPAC	3974 (3445)	9927 (6847)	10,045 (12,456)	9969 (9611)	2.51	2.5	1.31
PTN	1766 (1774)	4163 (4001)	3488 (3445)	3938 (3675)	2.23	2.36	1.97*
PUR8	7601 (6173)	14,041 (14,054)	17,292 (13,092)	15,202 (13,800)	2.00*	1.85*	2.27
Rab GDI Beta	33,067 (33,037)	75,693 (61,482)	36,539 (30,230)	62,641 (49,533)	1.89	2.29*	1.1
RAN	2869 (2893)	16,189 (4046)	4654 (2241)	12,344 (3796)	4.3	5.64*	1.62
Resistin	2143 (2199)	4182 (2986)	4341 (3568)	4235 (3078)	1.98**	1.95*	2.03*
SHPS1	2746 (3355)	5321 (4526)	5421 (4684)	5357 (4605)	1.95*	1.94*	1.97
SSRP1	631 (633)	982 (773)	647 (660)	862 (733)	1.37*	1.56	1.03
TNF-a	776 (808)	1996 (1399)	1330 (870)	1758 (1155)	2.27*	2.57**	1.72
Ubiquitin	1581 (1504)	8632 (2528)	2206 (1715)	6490 (2253)	4.11*	5.46*	1.4
Ubiquitin+1	6863 (7096)	25,928 (22,138)	13,443 (8428)	21,766 (18,387)	3.17	3.78*	1.96
URB	1668 (1635)	2926 (2590)	1942 (1806)	2598 (2223)	1.56*	1.75*	1.16
VEGF-C	2243 (2280)	3013 (2812)	2491 (2236)	2827 (2787)	1.26*	1.34**	1.11
WNK3	2098 (2336)	12,596 (3642)	2616 (2166)	9269 (3143)	4.42	6.01*	1.25

[a]Top 50 plasma proteins, based on fold change of IBD *versus* HC, CD *versus* HC, and UC *versus* HC.
[b]Statistical significance *p* values based on Mann–Whitney *U* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

of one to three proteins each. If two or more proteins belonged to the same cluster, only one representative protein was selected for further ELISA validation of the plasma biomarker.

ELISA Validation of Top Plasma Protein Biomarkers

Seven candidates, catalase, elastase, IGF-1, LTF, PRDX1, resistin, and TNF-α, identified from correlation matrix plot were selected for validation using an orthogonal platform (ELISA),

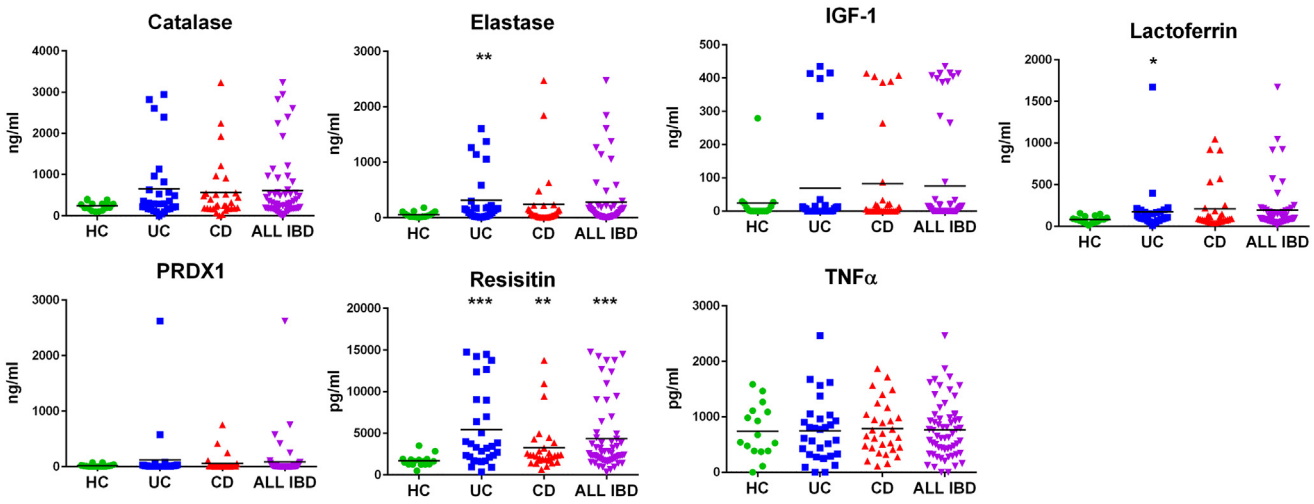

FIG. 3. **Cross-sectional ELISA validation of elevated plasma proteins in IBD plasma.** ELISA validation results for the top seven proteins elevated in pediatric IBD plasma in a validation cohort of 76 plasma samples (30 CD, 30 UC, and 16 HC). *Horizontal lines* in each plot show the mean of each group. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, determined by Mann–Whitney *U* tests. CD, Crohn's disease; HC, healthy control; IBD, inflammatory bowel disease; UC, ulcerative colitis.

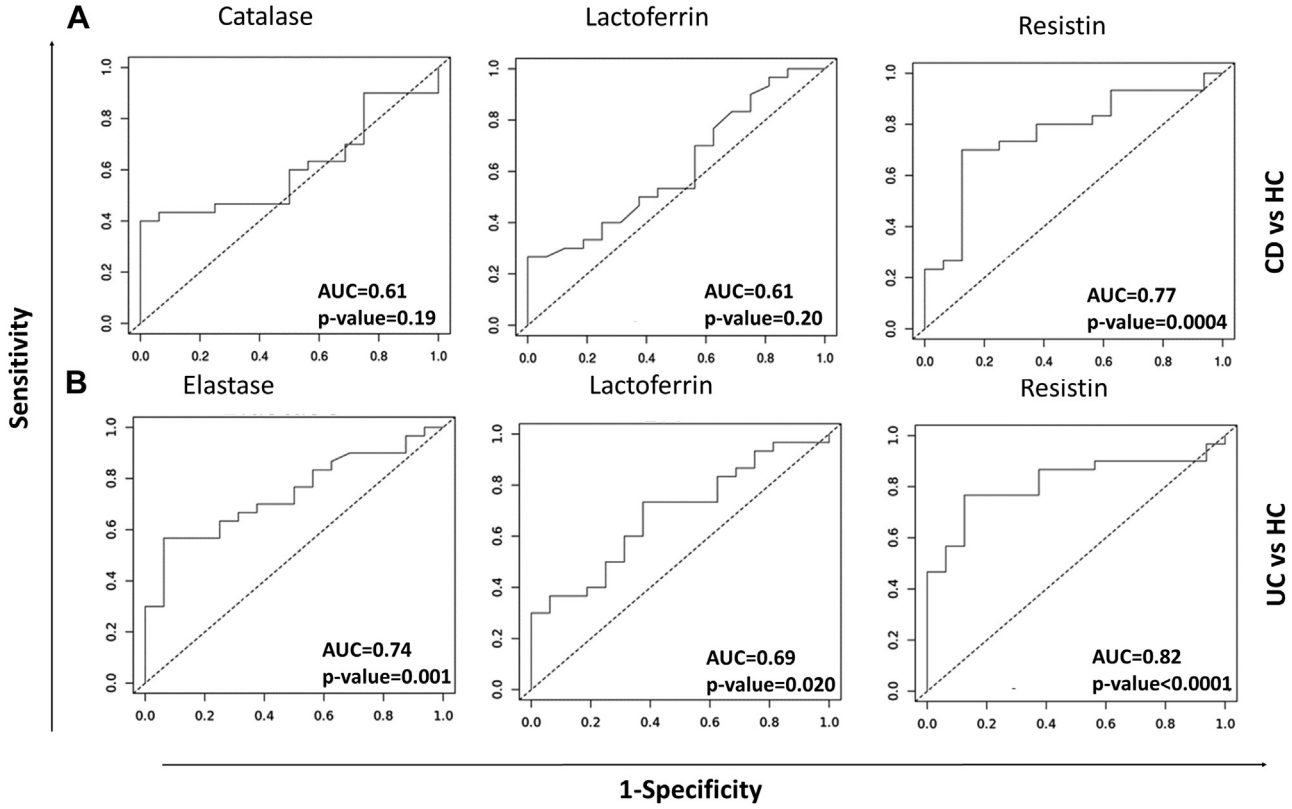

FIG. 4. **Resistin, lactoferrin, and catalase are the top three biomarkers that distinguish UC and CD from HC plasma receiver operating characteristic curves for distinguishing CD from HC and UC from HC, all determined using ELISA.** Shown are the top three plasma protein biomarkers chosen based on highest ROC AUC values for CD (*A*) and UC (*B*) in comparison to HC. ROC AUC and *p* values expressed on each curve. 1 – Specificity is represented on the *x*-axis and sensitivity on the *y*-axis. A higher ROC AUC value indicates greater potential to distinguish two groups from each other, whereas maximizing sensitivity and specificity. AUC, area under the curve; CD, Crohn's disease; HC, healthy control; ROC, receiver characteristic curve; UC, ulcerative coitis.

TABLE 3
Top 7 IBD plasma biomarkers selected for ELISA validation studies[a]

Plasma protein	Plasma protein level, pg/ml, mean (median)				FC	Comparison of UC *versus* HC			FC	Comparison of CD *versus* HC			FC
	HC	UC	CD	IBD	IBD/HC	Cutoff	ROC AUC (CI)[b]	Sensitivity	Specificity	UC/HC	Cutoff	ROC AUC (CI)[b]	Sensitivity	Specificity	CD/HC
Catalase	242 (237)	656 (293)	564 (252)	610 (291)	2.50	488	0.63 (0.36–0.69)	0.37	1.0	2.70	448.00	0.61 (0.32–0.66)	0.40	1.0	2.30
Elastase	57 (39)	317 (146)	241(49)	279 (85)	4.90	131	0.74 (0.45–0.78)	0.57	0.94	5.60**	120.00	0.57 (0.30–0.64)	0.37	0.94	4.20
IGF-1	24 (1.2)	69 (0.65)	83 (2)	76 (0.65)	3.10	285	0.53 (0.32–0.64)	0.17	1.0	2.90	33.00	0.55 (0.35–0.66)	0.27	0.94	3.40
LTF	81 (80)	173 (101)	211 (84)	192 (92)	2.40	85	0.69 (0.43–0.76)	0.73	0.63	2.10*	184.00	0.61 (0.38–0.71)	0.27	1.0	2.60
PRX1	16 (6.3)	118 (4)	56 (4)	87 (4)	5.30	0.89	0.54 (0.32–0.67)	0.77	0.44	7.20	109.00	0.46 (0.26–0.60)	0.13	1.0	3.40
Resistin	1689 (1634)	5456 (3372)	3261 (2314)	4359 (2502)	2.60***	2128	0.82 (0.52–0.84)	0.77	0.88	3.20***	1926.00	0.77 (0.44–0.80)	0.70	0.88	1.90**
TNF-α	744 (609)	748 (695)	787 (677)	768 (677)	1.00	1620	0.47 (0.29–0.62)	0.60	0.5	1.00	412.00	0.52 (0.33–0.67)	0.80	0.31	1.10

[a]The validation cohort of 76 subjects comprised of 30 CD patients, 30 UC patients, and 16 HCs.
[b]Lower and upper limits of confidence interval (CI) calculated by Delong method with 95% confidence level.

using an independent cohort of 76 plasma samples, including 30 from CD, 30 from UC, and 16 from HC subjects. The demographics of these subjects are listed in Table 1. Of the seven proteins tested by ELISA in the validation cohort, elastase, LTF, and resistin were significantly elevated in UC compared with HC, and resistin was also significantly elevated in CD compared with HC (Fig. 3). Interestingly, plasma levels of catalase, elastase, IGF-1, and resistin exhibited a bimodal distribution, with ~15 to 20% of the UC/CD patients exhibiting high levels, as compared with the HCs, who were uniformly negative for these proteins.

The ability of these plasma proteins to discriminate UC *versus* HC and CD *versus* HC was next analyzed using ROC. In comparing UC and HC, the same three plasma proteins, elastase, LTF, and resistin, exhibited ROC AUC values >0.5 (Fig. 4). Serum LTF and resistin had the highest ROC AUC values in comparing CD with HC, with the addition of catalase (Fig. 4 and Table 3). Of the top three ELISA-tested proteins, plasma resistin was the best discriminator between UC from HC (ROC AUC >0.76) and CD from HC (ROC AUC >0.81) (Fig. 4 and Table 3). The mean and median plasma protein values, FC, ROC AUC analysis, sensitivity and specificity metrics of these proteins, as assayed by ELISA, are listed in Table 3. The raw data of the ELISA validations are listed in supplemental Table S3. Of the three proteins that were most discriminatory for identifying UC, plasma elastase and resistin exhibited the highest specificity for UC, whereas plasma LTF and resistin exhibited the highest sensitivity (Table 3). In addition, plasma catalase and IGF-1 exhibited perfect specificity (and positive predictive value) for UC, related to their bimodal distribution (Fig. 3 and Table 3). Of the three proteins that were most discriminatory for identifying CD, all three exhibited good specificity for CD, but only resistin had good sensitivity for CD (Table 3).

We next compared the biomarker hits from this pediatric IBD plasma aptamer screening study against a recently published study performed on adult IBD plasma samples, using the same targeted proteomic platform (24). The results of the comparison are tabulated in Tables 4, 5, and 6. There were six proteins that were statistically significant with similar directions of increase/decrease in both the present pediatric IBD and previous adult IBD plasma proteins (24), including BRF1, cyclophilin F, cytidylate kinase, desmoglein-2, elastase, and resistin, with the latter two being confirmed in this study by ELISA as well in an independent cohort. Several additional proteins were identified as being significant in both studies, although their direction of change in IBD plasma was discordant between the two reports (Tables 4, 5, and 6).

DISCUSSION

Since IBD is a lifelong disease, often treated with intense immunosuppressive therapies, a firm diagnosis supported by

TABLE 4
Comparison between pediatric IBD and adult IBD

Biomarkers identified as significant	Matching FC	Matching *p* value	Matching significance
41		*	
6-Phosphogluconate dehydrogenase	*		
Adiponectin			
ANK2	*		
ARI3A			
BPI		*	
BRF-1	*	*	*
CA1			
CA3		*	
Catalase		*	
Cathepsin G			
CD5L			
CRP			
Cyclin B1	*		
Cyclophilin F	*	*	*
Cytidylate kinase	*	*	*
Desmoglein-2	*	*	*
DR3	*		
Elastase			
Endocan	*	*	*
FAK1	*		
Ferritin	*		
FST			
GA733–1 protein			
Glucagon		*	
H2A3	N/A		
Haptoglobin, mixed type			
HB-EGF			
Hemoglobin		*	
HINT1		*	
Histone H1.2		*	
hnRNP A2/B1			
IGF-I			
IgM	*		
KI2L4			
KI3L2		*	
KIF23			
LTF			
LEAP-1	*		
MDHC		*	
NovH		*	
PA2G4			
PCI			
Peroxiredoxin-1			
PolyUbiquitin K48			
PPAC			
PTN			
PUR8			
Rab GDI beta		*	
RAN			
Resistin	*	*	*
SAA			
SHPS1	*		
SSRP1			
TNF-a			
Ubiquitin		*	
Ubiquitin+1			
URB			
VEGF-C			
WNK3		*	

Abbreviation: N/A, not available (was not present in our study).

Note: *Matching FC based on Log2 values*: both log2 FC >0 or both log2 FC <0 then *; Matching p-values: both *p* value <0.1 then *. *Matching significance*: matching both FC and *p* value. Included the 50 proteins identified as significant in our study and the 11 found in the study by Narzo *et al.* Hemoglobin was identified as significant in both our study and the study by Narzo *et al* (24).

IBD *versus* HC: Exact values for all the data.

endoscopically obtained tissue biopsies and histology is necessary for diagnosis. Since endoscopy is invasive and performed under general anesthesia in children, there is a need for noninvasive markers of clinical activity. Acute phase proteins, including C-reactive protein (CRP), erythrocyte sedimentation rate, FC, differential blood count, iron status, blood sedimentation rate, protein electrophoresis readouts, and fecal neutrophils, constitute some of the many assays currently reported for IBD disease monitoring (28, 29). Fecal calprotectin, a byproduct of neutrophil migration, has been regarded as the gold standard for IBD diagnosis in adults, but there are differences in disease progression and presentation between pediatric and adult IBD patients (25–27). The sensitivity and specificity of fecal calprotectin testing is also dependent on the location of inflammation. Several studies reported lower specificity in CD patients *versus* UC patients and higher specificity for large bowel disease *versus* small bowel disease (30–32). Hence, improved biomarkers are clearly warranted, and biomarkers that are detectable in easily accessible body fluids can be very useful in the monitoring of disease in IBD.

Here, we address the aforementioned need by interrogating plasma from patients with pediatric IBD using a targeted OMICs approach. Targeted aptamer-based screens overcome the hurdle faced by mass spectrometry–based screens in detecting low abundance proteins (24). The current study employs a comprehensive 1322 protein aptamer-based array panel to uncover the proteomic landscape of pediatric IBD plasma. Among the proteins identified to be elevated in IBD plasma, a prominent neutrophil signature was detected, including the top proteins, resistin, LTF, elastase, and catalase. About 20% of the top 75 differentially expressed proteins were associated with neutrophil granules, including alpha enolase, ALPL (bone), BPI, catalase, cathepsin G, CD63, elastase, histone H1.2, LTF, PRX1, resistin, sICAM-2, and TNF-α (33, 34). Of the proteins significantly elevated in IBD plasma based on the proteomic screen, based on cluster analysis, seven proteins were selected and subjected to validation by ELISA using an independent cohort. Of these seven proteins, resistin, elastase,

TABLE 5
Significant biomarkers of pediatric IBD compared with adult IBD parameters

Proteins identified as significant in our study	Parameters in our article		Parameters in the study by Narzo *et al.*	
	Our FC	Log2FC	Log2FC	*p*
41	2.82*	1.49569516262407	−0.48	1.21E-03
Adiponectin	1.38*	0.464668267003444	−0.14	1.65E-01
ANK2	1.33	0.411426245726465	0.13	5.30E-01
ARI3A	1.87	0.903038270112912	−0.10	5.15E-01
BPI	2.51**	1.32768736417605	−0.40	2.23E-02
BRF-1	1.32*	0.400537929583729	0.15	4.48E-03
CA1	2.00**	1	−0.25	2.71E-01
CA3	4.69	2.22958792274065	−0.52	3.80E-03
Catalase	1.78	0.831877241191673	−0.40	3.00E-02
Cathepsin G	2.61	1.38404980679516	−0.09	1.99E-01
CD5L	1.96*	0.970853654340483	−0.14	4.17E-01
Cyclin B1	1.23**	0.298658315564515	0.08	2.98E-01
Cytidylate kinase	1.27*	0.344828496997441	0.06	8.90E-02
Desmoglein-2	1.31**	0.389566811762726	0.12	9.62E-02
DR3	1.54**	0.622930350920177	0.09	4.51E-01
Elastase	3.4	1.76553474636298	−0.15	2.12E-01
Endocan	1.34*	0.422233000683048	0.19	7.16E-04
FAK1	1.49**	0.575312330687437	0.07	5.84E-01
FST	1.28*	0.356143810225275	−0.01	9.93E-01
GA733–1 protein	1.59*	0.669026765509631	−0.17	1.32E-01
HB-EGF	1.25*	0.321928094887362	−0.06	2.35E-01
Hemoglobin	4.3	2.10433665981474	−1.13	1.09E-05
HINT1	1.52*	0.604071323668861	−0.24	8.16E-02
Histone H1.2	2.38	1.25096157353322	−0.54	3.97E-03
hnRNP A2/B1	4.36*	2.1243281350022	−0.08	7.56E-01
IGF-I	2.85**	1.51096191927738	−0.10	1.03E-01
IgM	1.98	0.985500430304885	0.11	6.31E-01
KI2L4	1.75*	0.807354922057604	−0.05	7.46E-01
KI3L2	1.47*	0.55581615506164	−0.25	1.42E-02
KIF23	1.39*	0.475084882948783	−0.07	5.50E-01
LTF	1.87**	0.903038270112912	−0.09	7.72E-01
MDHC	1.98*	0.985500430304885	−0.36	9.87E-03
NovH	1.36	0.443606651475615	−0.28	7.73E-07
PA2G4	4.83**	2.27202318906105	−0.26	1.18E-01
Peroxiredoxin-1	3.81*	1.9297909977186	−0.20	2.20E-01
PolyUbiquitin K48	2.16*	1.11103131238874	−0.20	2.16E-01
PPAC	2.51	1.32768736417605	−0.15	6.52E-01
PTN	2.23	1.15704371014558	−0.10	4.23E-01
PUR8	2.00*	1	−0.20	4.47E-01
Rab GDI Beta	1.89	0.918386234446348	−0.39	4.52E-03
RAN	4.3	2.10433665981474	−0.21	4.42E-01
Resistin	1.98**	0.985500430304885	0.47	1.98E-10
SHPS1	1.95*	0.963474123974886	0.12	6.89E-01
SSRP1	1.37*	0.454175893185802	−0.01	9.89E-01
TNF-a	2.27*	1.18269229751619	−0.06	3.14E-01
Ubiquitin	4.11*	2.03913839390696	−0.30	4.55E-03
Ubiquitin+1	3.17	1.66448284036468	−0.32	1.16E-01
URB	1.56*	0.641546029087524	−0.10	1.88E-01
VEGF-C	1.26*	0.333423733725192	−0.04	7.93E-01
WNK3	4.42	2.14404636961671	−0.29	6.60E-02

All p values less than 0.1.
*$p < 0.05$.
**$p < 0.01$.

and LTF showed significant increase in IBD plasma and exhibited the highest ROC AUC values of 0.767, 0.613, and 0.608, respectively. Importantly, these findings resonate well with previous proteomic analysis of plasma from adult IBD patients, which also revealed significant elevations of resistin and elastase in IBD (Fig. 4 and (21)).

TABLE 6
Significant biomarkers of adult IBD compared with pediatric IBD parameters

Identified as significant in the study by Narzo *et al.*	Parameters in our article			Parameters in the study by Narzo *et al.*	
	FC	Log2FC	*p*	Log2FC	*p*
6-Phosphogluconate dehydrogenase	2.89	1.53	0.16	2.07	6.66E-16
CRP	0.93	−0.10	0.73	1.18	6.45E-12
Cyclophilin F	0.41	−1.28*	0.07	−1.03	5.51E-11
Ferritin	0.44	−1.18	0.19	−1.2	4.36E-10
Glucagon	0.49	−1.03*	0.04	1.06	2.02E-14
H2A3	N/A	N/A	N/A	−1.33	9.21E-15
Haptoglobin, mixed type	0.61	−0.71	0.12	1.06	8.28E-06
Hemoglobin	4.3	2.10	0.09	−1.13	1.09E-05
LEAP-1	0.42	−1.25	0.12	−1.49	1.99E-11
PCI	0.9	−0.15	0.37	1.67	2.22E-11
SAA	0.97	−0.04	0.78	2.12	9.15E-10

Resistin although originally associated with adipocytes is highly expressed in neutrophils and macrophages and is released by neutrophil granules at the site of inflammation and attracts other immune cells (35). Levels of resistin in plasma have been shown to be positively correlated with CRP levels, inflammatory cytokines, and IBD disease activity (36, 37). Resistin has been found to be significantly elevated in serum from antinuclear antibody–positive patients compared with antinuclear antibody–negative individuals and has been proposed as a marker for autoimmune gastrointestinal inflammatory diseases (38). In the present study, resistin was the only protein, of all the ELISA-validated proteins, to have shown significance when comparing CD and UC plasma with HCs, with a *p* of less than 0.01 (Fig. 3). In the current study, plasma resistin was significantly elevated in UC and CD with FCs of 3.2 and 1.95, respectively, with ROC AUC value of 0.82 for UC *versus* HC and 0.77 for CD *versus* HC groups. Moreover, it was the only plasma protein that exhibited high sensitivity and specificity for both CD and UC (Table 3).

Elastase has been previously proposed as a marker for monitoring disease activity in IBD, and plasma levels of elastase have been reported to be higher in patients with active disease compared with patients in remission and HCs (39). Neutrophil migration in colon is highly prevalent in IBD, and inhibitors of neutrophil-elastase have been shown to reduce immune cell infiltration and ulceration in colon (40, 41). In our current study, elastase was significantly elevated in UC and CD with FCs of 5.6 and 4.2, respectively, with ROC AUC value of 0.74 for UC *versus* HC and 0.57 for CD *versus* HC groups. Of interest, plasma elastase exhibited higher specificity for both UC and CD, compared with plasma resistin (Table 3).

LTF is a glucoprotein that reflects the activity of neutrophils and inflammation (42). Fecal LTF has been reported to be correlated with intestinal inflammation and disease activity in IBD and has demonstrated high sensitivity and specificity for UC and CD and has been proposed as a better marker than CRP for pediatric patients in early stages of IBD (42, 43). It has been proposed that since LTF correlates very well with inflammation, fecal LTF can be used to distinguish between inflammatory and non-IBD and exclude irritable bowel syndrome (42). In the current study, LTF was significantly elevated in UC and CD with FCs of 2.1 and 2.6, respectively, with ROC AUC value of 0.69 for UC *versus* HC and 0.61 for CD *versus* HC groups (Table 3).

Catalase is an antioxidant enzyme that protects cells against reactive oxygen species and is a granulocyte autoantigen targeted by autoantibodies in IBD patients (44). It plays critical role in protecting neutrophils from their metabolic products during phagocytosis (45). Catalase has also been identified as an elevated marker, which could distinguish active IBD from non-IBD stool samples with high sensitivity and specificity (46–48). In the present study, plasma catalase was significantly elevated in UC and CD with FCs of 2.7 and 2.3, respectively, and when comparing CD and HCs. Although plasma catalase demonstrated an acceptable ROC AUC value of 0.61, it exhibited high specificity (100%), and high positive predictive value (100%) for both UC and CD (Table 3).

Recently, a comprehensive proteomic screen of IBD stools (not plasma) has been reported (49). In that report, several stool proteins exhibited ROC AUC values, sensitivity and specificity values exceeding 0.90 (and some even exceeding 0.95) in distinguishing UC/CD from HC subjects, clearly outperforming the plasma biomarkers uncovered in this study. Moreover, several stool proteins in that study were demonstrated to have the potential to track disease activity over time (49). Taken together, it is tempting to speculate that stool biomarkers may be superior in diagnosing and monitoring IBD compared with plasma biomarkers, although the latter may be more amenable for repeat monitoring and point-of-care assays.

This study can be improved by adding additional samples from different ethnic groups and increasing the sample size in order to boost statistical power. With additional biomarkers, a combination panel of biomarkers can be employed to predict

disease with even higher accuracy and specificity. Longitudinal studies are needed to assess the utility of the identified plasma markers in monitoring disease activity. Mechanistic studies are also warranted to explore the potential pathogenic roles of the identified proteins. Finally, further OMICS studies may help identify biomarkers that can distinguish CD from UC.

DATA AVAILABILITY

All data are contained within the article.

Supplemental data—This article contains supplemental data.

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Abbreviations—The abbreviations used are: AUC, area under the curve; CD, Crohn's disease; CRP, C-reactive protein; FC, fold change; GO, Gene Ontology; HC, healthy control; IBD, inflammatory bowel disease; IGF-1, insulin-like growth factor 1; LTF, lactoferrin; ROC, receiver operating characteristic; TNF-α, tumor necrosis factor alpha; UC, ulcerative colitis.

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