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Identification of Pathway-Specific Serum Biomarkers of Response to Glucocorticoid and Infliximab Treatment in Children with Inflammatory Bowel Disease

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OBJECTIVE: Serum biomarkers may serve to predict early response to therapy, identify relapse, and facilitate drug development in inflammatory bowel disease (IBD). Biomarkers are particularly important in children, in whom achieving early remission and minimizing procedures are especially beneficial.

METHODS: We profiled protein and micro RNA (miRNA) in serum from patients pre- and post-therapy, to identify molecular markers of pharmacodynamic effect. Serum was obtained from children with IBD before and after treatment with either corticosteroids (prednisone; $n = 12$) or anti-tumor necrosis factor-α biologic (infliximab; $n = 7$). Over 1,100 serum proteins were assayed using aptamer-based SOMAscan proteomics, and 22 miRNAs analyzed by quantitative real time PCR. Concordance of longitudinal changes between the groups was used to identify markers responsive to treatment. Bioinformatic analysis was used to build insight into mechanisms of changes in response to treatment.

RESULTS: We identified 18 proteins and three miRNAs responsive to both prednisone and infliximab. Eight markers that decreased are associated with inflammation and have gene promoters regulated by nuclear factor (NF)-κB. Several that increased are associated with resolving inflammation and tissue damage. We also identified six markers that appear to be steroid-specific, three of which have glucocorticoid receptor binding elements in their promoter region.

CONCLUSIONS: Serum markers regulated by the inflammatory transcription factor NF-κB are potential candidates for pharmacodynamic biomarkers that, if correlated with later outcomes like endoscopic or histologic healing, could be used to monitor treatment, optimize dosing, and enhance drug development. The pharmacodynamic biomarkers identified here hold potential to improve both clinical care and drug development. Further studies are warranted to investigate these markers as early predictors of response, or possibly surrogate outcomes.

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INTRODUCTION

The onset, symptoms, and progression of inflammatory bowel disease (IBD) are highly variable and unpredictable. A variety of phenotypes exist; extra-intestinal inflammation may manifest as uveitis, arthritis, or growth failure in children. Therapies for IBD are focused on the induction and maintenance of remission, and the prevention of longer-term complications of chronic inflammation, such as relapsing disease, steroid-dependence, malnutrition, growth-stunting in children, and colorectal cancer. There is a disconnect between patient symptoms and mucosal inflammation, and increasing evidence in adults with IBD shows that long-term clinical outcomes are not improved by treating to symptom remission, but are improved by directly targeting mucosal inflammation.[1,2] Practically, using ileocolonoscopy and imaging to monitor disease response requires waiting 3–6 months for cycles of repair to occur, followed by re-assessment of healing by endoscopy or imaging, adjusting therapy based on these results, and then repeating the evaluation again.[3] Though this may currently be the optimal approach available, acceptance of repeated colonoscopy as a widespread clinical practice or as a clinical trial endpoint may be limited by patient discomfort, procedural risk, anesthesia, and high cost. This is a particularly important issue in pediatric clinical care, and a barrier to recruitment in pediatric clinical trials.[4]

There is a need for biomarkers to predict response to therapy and optimize treatment regimens to improve quality of life. Such biomarkers are particularly important in children.[5] Facing a long or lifetime duration of disease encompassing important stages of development, the disease manifestations, side-effects of current therapies, and exposure to repeated invasive procedures may have greater negative impacts on children and their families. In addition to treatment decisions, serum biomarkers may inform earlier dosing, safety, and

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efficacy decisions in pediatric clinical trials. Currently, a small number of clinically-utilized biomarkers of disease response are available to the IBD clinician, as recently reviewed in Sands *et al.*[6]

New candidates have been identified as potential blood-based biomarkers in IBD, but the effect of specific treatments on the prospective change of these biomarkers has not been investigated. These include proteins, as well as micro RNAs (miRNAs), which are emerging as promising treatment-responsive biomarkers. Recently, serum SERPINA1 (α-1-antitrypsin) was shown to differentiate between mild and more severe forms of adult ulcerative colitis (UC), and appears to be superior to C-reactive protein in this regard.[7,8] In two recent studies, circulating miRNAs were measured in patients with Crohn's disease (CD) and/or UC. One study found 24 serum miRNAs differentially expressed in children with CD.[9] The other study found three sets of peripheral blood miRNAs that distinguish between active CD, UC, and healthy adult patients.[10] Bridging specific biomarkers to drug response (for example, pharmacodynamic biomarkers) will be a necessary step in establishing a biomarker as a treatment monitoring tool or as an outcome measure in clinical trials. In this exploratory study, our goal was to carry out a broad discovery of pharmacodynamic biomarkers that show response to corticosteroid and anti-tumor necrosis factor-α (TNFα) biologic treatment of pediatric IBD. Our hypothesis was that serum inflammatory proteins and miRNAs that lie in the pathway of drug effect, and that change in the same direction after treatment with both classes of drugs, are candidates for further study as potential surrogate outcome measures.

METHODS

Ethics statement. All work was conducted according to relevant national and international guidelines, reviewed and approved by the Institutional Review Board of Children's National Health System.

Patients, treatment, and serum collection. Patients ranged in age from 9 to 19 years, and were all cared for at Children's National Health System, a quaternary-care, free-standing children's hospital in Washington, DC, USA that serves ~400 IBD patients. Patients with IBD were randomly recruited for this study, which was designed for the collection of serum samples pre- and post-treatment to evaluate longitudinal changes in proteins and miRNAs. Demographics and clinical characteristics of the two patient groups are provided in Table 1. All were outpatients, treated as indicated per their gastroenterologist; follow-up serum samples were drawn at the time of a clinically indicated lab-draw. There were no entry disease scores or criteria. Most patients were evaluated by ileocolonoscopy or magnetic resonance enterography prior to starting therapy. Two patients were started on infliximab to treat perianal disease. One child was started on infliximab for growth failure. Two patients reported no symptoms, but therapy was escalated to infliximab because of elevated serum inflammatory markers and endoscopic inflammation. For this study, we selected patients treated with infliximab who were not recently exposed to corticosteroids or immunomodulators.

Table 1 Patient clinical characteristics, treatment dosing and schedule, clinical scores, and serum inflammatory markers

Study description	Prednisone cohort	Infliximab cohort
Number of patients	12	7
Age (years)	12.2 ± 2.4	15.7 ± 1.8
Males:females	6 M:6 F	4 M:3 F
Patient diagnoses	10 CD:2 UC	7 CD
Disease years	11 newly dx'd 1 CD 4.1 yrs*	2.1 ± 1.6
Paris classification disease location[53]		
L1 (distal 1/3 ileum)	1	
L1,L4b (distal 1/3 ileum, upper GI distal to ligament of Treitz		1
L2 (colonic)	2	1
L2, p (colonic, perianal disease)	1	
L3 (ileocolonic)	4	1
L3,p (ileocolonic, perianal disease)	1	4
L3,L4a (ileocolonic, upper GI proximal to the ligmant of Treitz	1	
Treatment time (weeks)	9.4 ± 4.4	6.1 ± 0.3
Treatment regimen	Daily prednisone	Two infusions, 5 mg/kg
Dose range	0.6–1 mg/kg	2 weeks apart
CRP levels (mg/dl, normal 0.06–0.79)		
Pre-treatment	3.7 ± 3.1	1.1 ± 1.0
Post-treatment	0.5 ± 0.8	0.6 ± 0.5
P-value	0.003	0.2
ESR (mm/h, normal 0–20)		
Pre-treatment	44.4 ± 32.0	31.0 ± 19.7
Post-treatment	22.5 ± 14.9	12.3 ± 6.5
P-value	0.06	0.07
PCDAI		
Pre-treatment	36.5 ± 16.4	16.4 ± 11.4
Post-treatment	11.0 ± 7.8	9.6 ± 12.1
P-value	0.06	0.07
PUCAI (2 UC patients treated with prednisone)		
Pre-treatment	70, 85**	
Post-treatment	15, 40**	

CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GI, gastrointestinal; PCDAI, Patient Crohn's Disease Activity Index; UC, ulcerative colitis.
(*patient received 5-ASA prior to study; values are mean ± s.d.; paired *t*-test). (**two patient scores).

Corticosteroid-treated patients received daily oral prednisone at standard 1 mg/kg dosing up to a maximum of 40 mg daily. All but one steroid-treated patient was newly diagnosed, without baseline exposure to other medications. The one previously diagnosed patient with CD had only been exposed to mesalamine at diagnosis. None of the infliximab-treated patients were treated with concurrent steroids or immunomodulators, and received standard induction dosing of infliximab (5 mg/kg/dose) at time 0 and week 2.

As our goal was to identify pharmacodynamic effects of therapy, we chose a longitudinal approach. We focused, in this initial discovery study, on obtaining an early read-out of physiologic biomarker change after administration of therapy. We chose not to wait for a longer period to fully assess clinical or endoscopic response or remission (typically 12–52 weeks of treatment), as this would have the potential to introduce more variability and dilute a pharmacological effect. Two blood samples were obtained from each patient, one before

treatment (baseline control), and one after treatment with either prednisone or infliximab (paired sample for longitudinal analysis). Duration of steroid treatment ranged from 3 to 18 weeks. For infliximab, post-drug samples were drawn at 6 weeks after treatment initiation, just prior to the third induction dose. Pediatric Crohn's Disease Activity Index and Pediatric Ulcerative Colitis Activity Index were recorded at both pre- and post-treatment time points, as were C-reactive protein and erythrocyte sedimentation rate.

Proteomic profiling. Proteins were profiled using a SOMAscan assay (SomaLogic; Boulder, CO, USA) that analyzed either 1,120 unique proteins (prednisone group; 1st-generation panel), or 1,300 proteins (infliximab group; 2nd-generation panel), as previously described.[11,12] In total, 70 µl of serum per patient was used to measure all proteins in a multiplex manner. Each aptamer targets a specific protein and is tagged with a unique DNA sequence permitting its quantification. Protein-aptamer levels were quantified on Agilent hybridization arrays in relative fluorescent units and analyzed using SomaSuite version 1.0 (SomaLogic). Protein levels were log transformed and tested to verify normality. Eleven patients were included in the prednisone group (nine CD, two UC) and seven patients in the infliximab group (seven Crohn's). The two treatment groups were analyzed separately using paired *t*-tests, comparing baseline to post-treatment values. A *P*-value of 0.05 was set as the significance threshold, without adjustment for multiple comparisons. To reduce false-positive discovery in this setting, we used an evidence-based approach where results from the two groups were cross-referenced. Proteins that significantly changed in the same direction in each of the two separate groups were considered candidate biomarkers.

miRNA profiling. RNA was isolated using TRIzol with overnight isopropanol precipitation. The same patient samples were analyzed for miRNA, with the addition of one more CD patient in the prednisone group. A total of 12 patients were included in the prednisone group (10 CD and 2 UC) and 7 in the infliximab group (7 CD). We used separate aliquots of the same patient serum samples used for SOMAscan, plus one additional prednisone-treated patient. We performed targeted quantification of 24 miRNAs selected for their prior detection in either inflammation or steroid-responses (primer/probe sets in Supplementary Table S1).[13] Complementary DNA was synthesized using multiplexed RT primers, pre-amplified using TaqMan PreAmp Master Mix (Life Technologies, Carlsbad, CA), and miRNAs quantified using individual TaqMan assays on an ABI 7900HT.[14] miRNAs were normalized to the geometric mean of multiple control genes, followed by statistical comparison of baseline to post-treatment values by paired *t*-test.[15,16] Control miRNAs (hsa-miR-342-3p and hsa-miR-150-5p) were previously identified as stable control reference miRNAs in IBD patient serum.[9]

Bioinformatics. We examined regulation of gene promoters for each biomarker candidate to gain insight into mechanisms of their response to treatment. Promoter binding by the inflammatory transcription factor nuclear factor (NF)-κB or the glucocorticoid receptor (GR) steroidal transcription factor was examined using chromatin immunoprecipitation sequencing (ChIP-seq) data from ENCODE. ChIP-seq data from ENCODE and Factorbook was used to query physical binding of candidate gene promoters.[17,18] Data/images were produced using UCSC Genome Browser Release 4 with alignment to human reference genome GRCh37/hg19.[17,19] Within ChIP-seq peaks, sequences were downloaded and analyzed using UCSC and/or JASPAR version 5.0a.[20] ChIP-seq data sets were obtained from TNFα-induced human immune cells (RelA), and dexamethasone-induced human lung, liver and endometrial cell lines (NR3C1). Heat maps were generated with Hierarchical Clustering Explorer Version 3.5 (Human Computer Interaction Laboratory; University of Maryland, College Park, MD, USA). To examine interactions between the identified molecular markers, we performed gene pathway analysis using Ingenuity Pathway Analysis, IPA Version 26127183 (QIAGEN, Hilden, Germany).

RESULTS

Proteomic responses to treatment. Aptamer-based proteomics was used to profile serum protein levels pre- and post-treatment (Figure 1). For prednisone-treated children, 213 proteins showed significant changes (Supplementary Table S2). For infliximab-treated children, 94 proteins showed significantly different levels after treatment (Supplementary Table S3). Eighteen proteins showed a significant change in

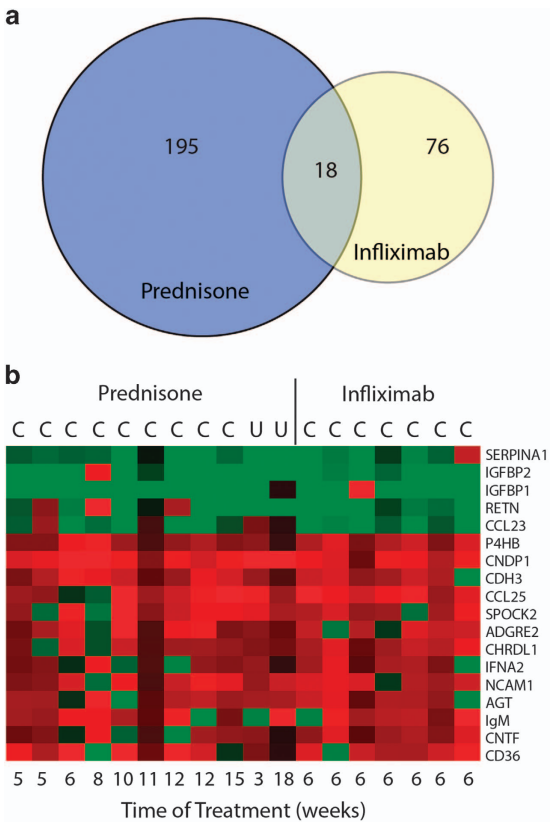


Figure 1 Summary of serum protein changes. (**a**) Venn diagram of proteomics results analyzing serum changes of proteins in the Prednisone and Infliximab treatment groups. (**b**) Heat map visualization of the fold changes of the 16 overlapping protein markers within each patient. (red = increased, green = decreased).

Table 2 Differential expression of serum proteins by both treatment groups (prednisone and infliximab), indicating the direction of change, and a description of the general protein function

		Glucocorticoid cohort			Infliximab cohort			
Gene	**↑ or ↓**	***P*-value**	**Pre (RFU)[a]**	**Post (RFU)[a]**	***P*-value**	**Pre (RFU)[a]**	**Post (RFU)[a]**	**Description**
SERPINA1	↓	<0.001	6.90 ± 0.26	6.52 ± 0.17	0.017	6.77 ± 0.15	6.65 ± 0.07	Increased in inflammation
IGFBP2	↓	0.003	10.88 ± 0.47	10.17 ± 0.34	0.006	6.90 ± 0.40	6.40 ± 0.48	Increased in inflammation
IGFBP1	↓	0.006	8.34 ± 0.65	7.01 ± 0.82	0.025	7.92 ± 0.86	6.92 ± 0.87	Increased in inflammation
RETN	↓	0.025	8.49 ± 0.53	8.13 ± 0.43	0.004	8.31 ± 0.45	8.06 ± 0.36	Increases cytokines
CCL23	↓	0.030	8.23 ± 0.31	7.97 ± 0.21	0.020	7.93 ± 0.37	7.66 ± 0.37	Chemotactic for monocytes
P4HB	↑	<0.001	6.02 ± 0.05	6.14 ± 0.07	0.001	6.10 ± 0.09	6.22 ± 0.09	Integrin binding
CNDP1	↑	<0.001	9.02 ± 0.35	9.61 ± 0.40	0.005	8.26 ± 0.44	8.55 ± 0.50	Improved cancer prognosis
CDH3	↑	<0.001	9.76 ± 0.20	10.01 ± 0.17	0.044	10.34 ± 0.16	10.47 ± 0.15	Cell adhesion
CCL25	↑	0.006	7.53 ± 0.51	7.92 ± 0.68	0.005	7.86 ± 0.55	8.21 ± 0.57	Protective in colitis
SPOCK2	↑	0.011	6.48 ± 0.29	6.81 ± 0.16	0.018	6.71 ± 0.24	6.84 ± 0.19	ECM binding
ADGRE2	↑	0.021	5.90 ± 0.25	6.15 ± 0.32	0.036	6.62 ± 0.23	6.79 ± 0.16	Promotes cytokine release
CHRDL1	↑	0.021	7.69 ± 0.14	7.81 ± 0.12	0.010	7.84 ± 0.16	7.98 ± 0.14	BMP4 antagonist
IFNA2	↑	0.024	5.27 ± 0.12	5.33 ± 0.09	0.023	4.98 ± 0.09	5.04 ± 0.10	JAK/STAT signaling
NCAM1	↑	0.026	8.89 ± 0.31	9.11 ± 0.21	0.030	9.10 ± 0.23	9.26 ± 0.20	Cell adhesion
AGT	↑	0.033	8.67 ± 0.21	8.84 ± 0.20	0.022	8.86 ± 0.14	8.98 ± 0.20	Vasoconstriction
IgM	↑	0.034	7.45 ± 0.39	7.64 ± 0.50	0.036	7.94 ± 0.28	8.10 ± 0.16	B-cell antibody
CNTF	↑	0.042	6.47 ± 0.13	6.53 ± 0.12	0.004	5.73 ± 0.08	5.79 ± 0.11	Protective in inflammation
CD36	↑	0.046	8.05 ± 0.33	8.33 ± 0.44	0.039	8.76 ± 0.32	8.96 ± 0.28	Pro-resolution M2

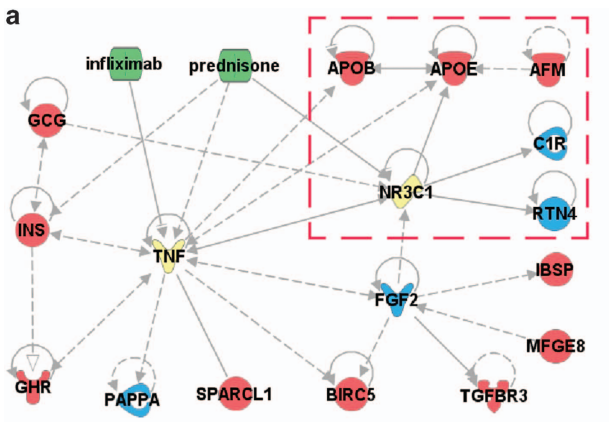
BMP4, bone morphogenetic protein 4; ECM, extracellular matrix; JAK/STAT, Janus kinase and a Signal Transducer and Activator of Transcription; M2, M2 macrophage; RFU, relative fluorescent units.
[a]Values are log transformed mean ± s.d.

the same direction for both prednisone and infliximab therapy (Table 2). Of these, five proteins showed a significant decrease in response to treatment. These proteins have known functions associated with inflammation, including α-1-antitrypsin (SERPINA1), insulin-like growth factor binding protein 1 and 2 (IGFBP1 and IGFBP2), resistin (RETN), and C-C motif chemokine 23 (CCL23).

The specific SOMAscan panels utilized for the two groups were slightly different, in that the prednisone-treated group was tested using a 1,120 protein panel, whereas the infliximab-treated group was tested with a newer 1,300 protein panel. Thus, ~180 additional proteins were assayed in the infliximab group. Eleven of these significantly changed with infliximab, but were not able to be cross-referenced in the prednisone-treated data set (Supplementary Table S4). These include CD177 antigen (CD177), chitinase-3-like protein 1 (CHI3L1), protein S100A12 (S100A12), and C-X-C motif chemokine 9 (CXCL9), which are inflammatory proteins that decreased with infliximab.

To identify potential steroid-specific responses, we used bioinformatics to screen differentially expressed proteins for established interactions with either the GR or TNFα. We found that the genes encoding three of these proteins were direct targets of the GR (Figure 2). These include apolipoprotein E, complement subcomponent C1r, and reticulon 4. An additional two, apolipoprotein B and afamin, were direct targets of apolipoprotein E. Other markers were more indirectly connected to the GR or were regulated by TNFα as well. Because they changed exclusively in response to prednisone, and are known to be regulated by the GR, these five proteins may represent steroid-specific biomarkers.

We did not aim to correlate biomarkers with established clinical or biochemical response following this rather short-treatment period. However, we did note that a majority of patients in both groups did show expected improvement in clinical and biochemical measures following treatment. Prednisone treatment ($n=12$) resulted in a significant



b

	Glucocorticoid cohort			Infliximab cohort		
Gene	p-value	Pre (RFU)[a]	Post (RFU)[a]	p-value	Pre (RFU)[a]	Post (RFU)[a]
APOB	<0.001	8.16 ± 0.64	8.57 ± 0.55	0.113	8.06 ± 0.40	8.20 ± 0.33
APOE	<0.001	9.62 ± 0.33	10.17 ± 0.30	0.248	11.65 ± 0.41	11.77 ± 0.19
AFM	<0.001	9.79 ± 0.45	10.19 ± 0.27	0.404	10.18 ± 0.14	10.23 ± 0.13
C1r	0.009	5.34 ± 0.44	4.85 ± 0.51	0.818	4.71 ± 0.33	4.69 ± 0.30
RTN4	0.009	10.84 ± 0.28	10.62 ± 0.31	0.884	10.70 ± 0.14	10.71 ± 0.23

Figure 2 Potential steroid-specific protein markers. To identify potential steroid-specific biomarkers, we used Ingenuity Pathway Analysis to screen proteins significantly altered by prednisone but not by infliximab. (**a**) Three proteins were found to be directly regulated by the glucocorticoid receptor (GR, or NR3C1) but not by TNF. An additional two were found to be regulated by one of its direct targets, but not by TNF. These five proteins (marked by a red box) were selected as potential steroid-specific markers. (**b**) Proteomic expression data for these five targets demonstrates that these were affected by prednisone treatment but not by infliximab treatment. ([a]Values are log transformed mean ± s.d.; red = increased, blue = decreased, green = drug, yellow = direct drug targets, NR3C1 = glucocorticoid receptor).

improvement in C-related protein ($P<0.005$) and pediatric Crohn's disease index ($P<0.001$, $n=10$ Crohn's patients), along with a trend of improvement in erythrocyte sedimentation rate ($P=0.06$). Infliximab treatment ($n=7$) led to a trend of improvement in each of these three measures (C-related

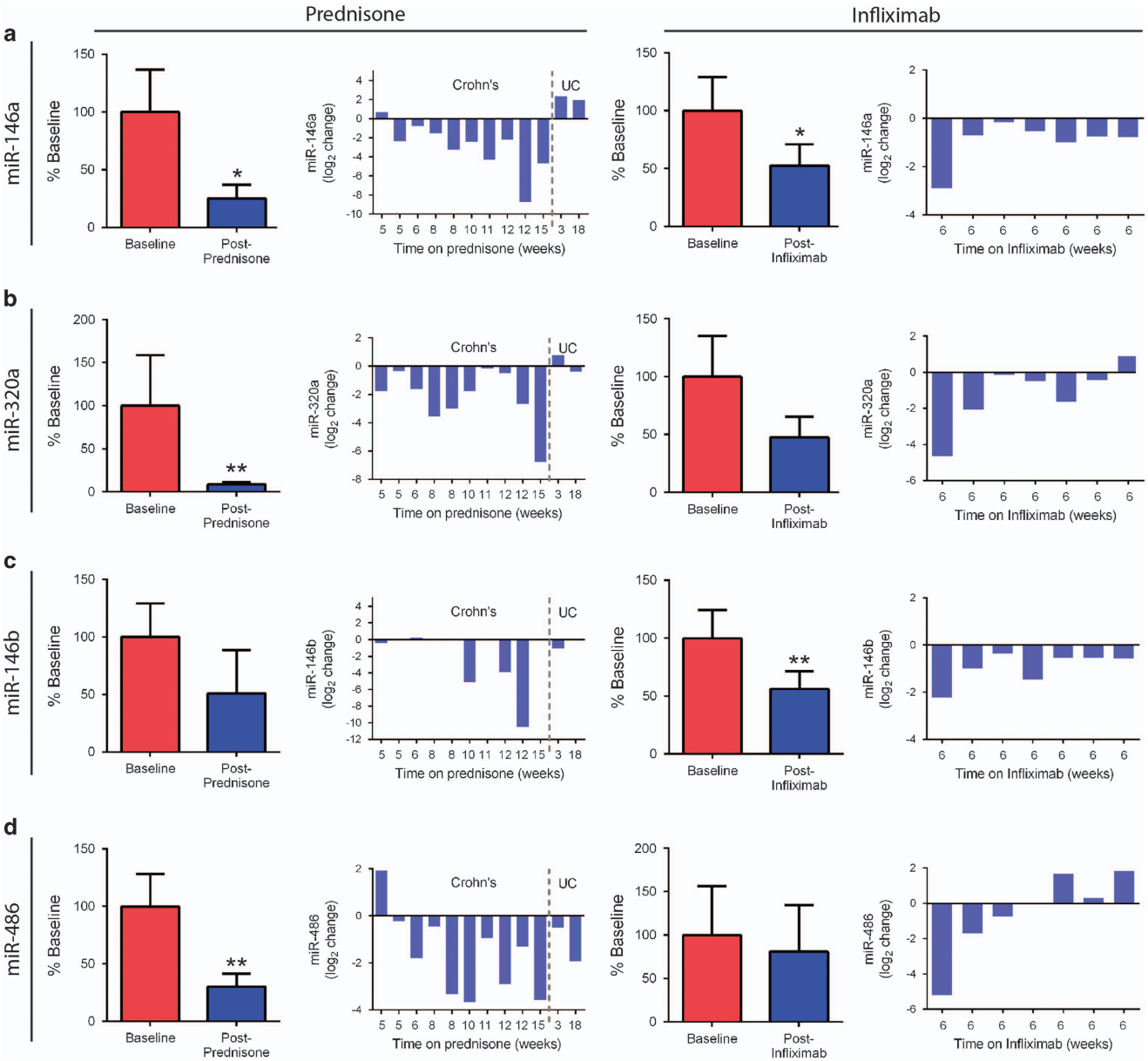
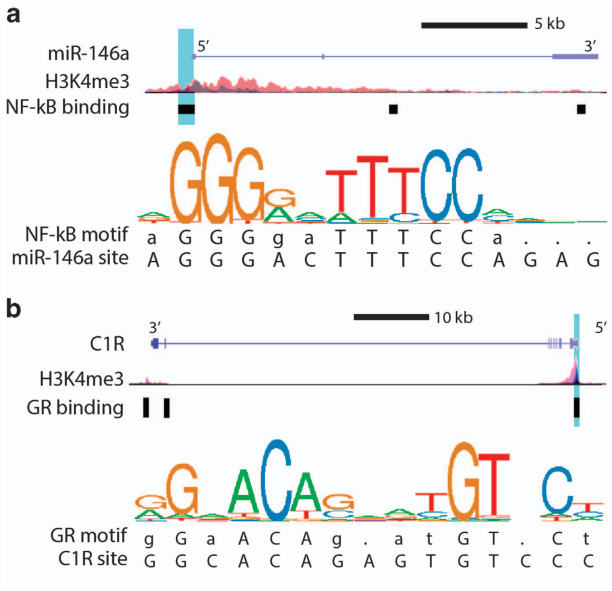


Figure 3 Serum microRNA levels change with prednisone or infliximab treatment. Levels of miRNAs were assayed by qRT-PCR. Bar graphs of the percent baseline levels for each treatment group, as well as change in expression for each individual patient, are provided for each miRNA. (**a**) miR-146a in response to prednisone (**a**) and infliximab (a'). miR-320a in response to prednisone (**b**) and infliximab (b'). miR-146b in response to prednisone (**c**) and infliximab (c'), note; four post-treatment and two baseline samples had miR-146b levels below detection threshold. (**d**) miR-486 in response to prednisone (d) and infliximab (d'). (*$P<0.05$, **$P<0.01$; paired *t*-test).

protein, $P=0.2$; Pediatric Crohn's Disease Activity Index, $P=0.09$; erythrocyte sedimentation rate, $P=0.07$). Non-statistically significant trends also likely reflect our small sample size.

miRNA responses to treatment. We identified four miRNAs that responded to treatment using quantitative real time PCR (Figure 3). Of these, three decreased with both prednisone and infliximab: miR-146a, miR-320a, and miR-146b. These miRNAs have been associated with inflammation. Notably, in individual patients, it appeared there may be a relationship between the size of effect and the duration of prednisone treatment. In addition, the two UC patients appeared to show a different pattern of miRNA response to treatment. Interestingly, other groups have found differences in serum miRNAs between Crohn's and UC, which may be consistent with our observations.[10] A fourth miRNA, miRNA-486, showed a significant change in response to prednisone ($P<0.01$), but not to infliximab. These data provide a panel of miRNAs that are responsive to two anti-inflammatory drugs with different mechanisms of action.

Bioinformatic analysis of gene regulation pathways. We found in ChIP-seq data and established literature that the majority of markers, which decreased with both drugs have gene promoters directly bound by NF-κB (Figure 4).[18,21–26]



c

Molecular marker	Expression Regulated by	Source	Drug changed	Up or Down
SERPINA1	NF-κB	Wang 2012	Both	Down
IGFBP1	NF-κB	Lang 1999	Both	Down
IGFBP2	NF-κB	Ben-Shmuel 2013	Both	Down
RETN	NF-κB	Singh 2010	Both	Down
CCL23	NF-κB	Shin 2007	Both	Down
miR-146a	NF-κB	Wang 2012	Both	Down
miR-146b	NF-κB	Wang 2012	Both	Down
miR-320a	NF-κB and GR	Wang 2012	Both	Down
CD36	M2	Martinez 2006	Both	Up
CCL25	M2, NFkB	Zeyda 2010, Wang 2012	Both	Up
P4HB	GR and NFkB	Wang 2012	Both	Up
RTN4	GR and NF-kB	Wang 2012	Prednisone	Down
C1R	GR	Wang 2012	Prednisone	Down
miR-486	GR	Wang 2012	Prednisone	Down

Figure 4 Promoter analysis of serum markers. (**a**) Schematic of the gene locus for miR-146a, illustrating the binding site and sequence of a promoter element bound by NF-κB. (**b**) Schematic of the gene locus for C1R, illustrating the binding site and sequence of a promoter element bound by GR. (**c**) Summary of promoter analysis and literature data indicating the marker and known elements associated with its regulation. (H3K4me3 = histone modification associated with active gene promoters, Blue highlight = site of promoter bound by transcription factor, with relevant sequence provided below consensus motif sequence logo, M2 = expressed by pro-resolution M2 macrophages).

This includes the protein SERPINA1, which is known to be elevated in IBD, and three miRNAs (miR-146a, 146b, and 320a). When we assessed steroid receptor binding, we found genes for three of the steroid-specific markers possess promoter elements directly bound by the GR, including complement subcomponent C1r and miR-486 (Figure 4). Together, this indicates that gene expression regulation for these markers is consistent with their response to treatment.

We found that 11 of the identified proteins are established to have connections to each other through interactions with NF-κB, TNFα, and/or the GR (Figure 5). This further establishes these molecular changes as part of a systemic network response to effective anti-inflammatory treatment.

DISCUSSION

We have identified 18 proteins and 3 miRNAs that are responsive to both prednisone and infliximab treatment. Both

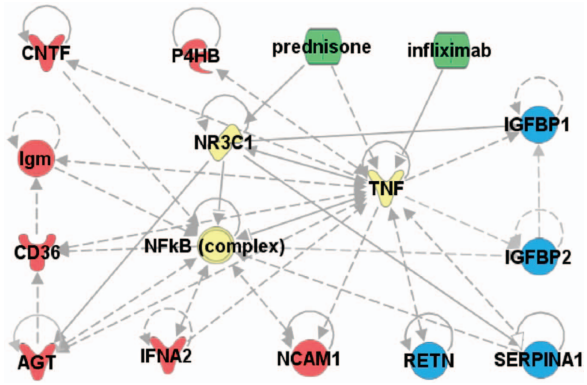


Figure 5 Pathway analysis of serum markers that change in response to treatment. Gene pathways of serum markers that responded to treatment were analyzed using Ingenuity Pathway Analysis. Established gene network connections were present between 11 of the 18 drug-responsive protein markers and NF-κB, NR3C1, or TNF. (red = increased, blue = decreased, green = drug, yellow = direct drug targets, NR3C1 = glucocorticoid receptor).

of these drugs target inflammatory TNFα and NF-κB signaling, but they do so via distinctly different mechanisms. Infliximab is an antibody that specifically binds to and inactivates the TNFα cytokine (ligand), blocking its NF-κB-mediated antiapoptotic effects.[27] In contrast, prednisone is a corticosteroid that binds to and activates the GR. Activated GR then binds to NF-κB complexes to inhibit downstream TNFα-mediated inflammation.[28] However, prednisone-activated GR shows many additional activities outside of NF-κB transrepression, as it also binds directly to hundreds of DNA elements that affect additional gene expression pathways.[29] By overlaying molecular changes in serum from patients treated with these partially overlapping drug mechanisms, we have identified ~20 serum-based pharmacodynamic markers of anti-inflammatory therapies.

All five of the NF-κB-regulated proteins that decrease with treatment have roles in inflammation. The first is α-1-antitrypsin, or SERPINA1, a serine protease inhibitor that induces T-regulatory cell expansion.[30–32] Notably, serum SERPINA1 already shows potential as a diagnostic biomarker in IBD.[7,8] A second protein, CCL23, is a chemokine known to be elevated in both IBD and rheumatoid arthritis.[33,34] NF-κB regulates CCL23, which then increases expression of pro-inflammatory cytokines, chemokines and adhesion molecules.[35] Another protein marker, resistin, or RETN, is an adipocytokine that shows species-specific tissue expression and is induced by TNFα in human macrophages.[25,36] Corticosteroids are known to affect RETN, though these effects may be time-specific.[37,38] Finally, both IGFBP1 and IGFBP2 decrease with prednisone and infliximab. The IGF-binding proteins regulate endocrine actions of insulin-like growth factors, and IGFBP1 is primarily produced by the liver during states of inflammation. IGFBP2 and IGFBP3 have been shown to decrease after high-dose corticosteroids in Crohn's disease and UC.[39] Our results suggest these effects on IGFBP proteins may not be steroid-specific.

Twelve proteins increased with treatment of IBD by both drugs. Many of these have a role in resolving tissue damage. CNTF is a polypeptide hormone, which acts as a growth and survival factor to reduce inflammatory tissue destruction in a

variety of injury types.[40,41] Cadherin 3, or p-cadherin, is involved with cell adhesion in epithelial tissues and may be involved in wound repair.[42] CCL25 and CD36 are particularly interesting as potential efficacy markers in IBD. Both are expressed by M2 macrophages associated with resolving inflammation. CCL25 is required for protection against chronic colitis in animal models.[43] CD36 is required, together with lipolysis, for activation of M2 pro-resolution macrophages.[44] Together, these markers may reflect the repair of intestinal tissue damage in IBD.

We identified three miRNAs that are responsive to both drugs and are known to be induced by inflammatory signaling. Notable among these is miR-146a, which is elevated in several inflammatory diseases and is induced by NF-κB in immune and muscle cells.[13,45–47] Interestingly, miR-146a (on chromosome 5) has both acute anti-inflammatory affects[45] and chronic pro-inflammatory effects,[48] and it is proposed that these contrasting effects may help in the staging of the inflammation/resolution process.[49] The miR-146 family also includes miR-146b (on chromosome 10), which we find is affected by both treatments as well. These two miRNAs have shared gene targets, although timing of their expression may differ. A treatment response of this miR-146 family is consistent with studies in muscular dystrophy, where we find both prednisone and a novel dissociative steroid (VBP15) reduce TNFα-mediated induction of miR-146b and miR-146a.[13,50] Though less studied, miR-320a is linked to inflammatory disease as well. In colon biopsies from adults with UC, reduced miR-320a is associated with reduced inflammation.[51] Together, measuring levels of these miRNAs could help assess inflammatory disease, as well as therapeutic response.

The pharmacodynamic biomarkers identified here hold potential for improving clinical care, and for streamlining development of new treatments. However, there are several limitations to our study. This is an exploratory, proof-of-concept study with limited patient numbers, variable sample timing, and heterogeneous patient populations. Because our statistical approach did not adjust for multiple comparisons, there is potential for type II error and false discovery. Further studies are necessary to replicate these findings, and to develop these markers as surrogate outcome measures. Summary data have been included in the Supplementary Data section.

Moving forward, a prospective study with more acute and less-variable blood sampling, hours or days after treatment, will define which biomarkers show the earliest responses to treatment, as well as validate biomarker discovery. Acutely responsive biomarkers should then be bridged to objective measures of intestinal inflammation and downstream clinical outcomes, with larger numbers of pediatric IBD patients followed out over a longer-term outcome period. This subset of biomarkers could then be used as outcome measures in drug development for dose finding studies (Phase 2a), and as surrogate biochemical outcome measures to support efficacy (Phase 2b or Phase 3 studies). In addition to biologics and small molecule inhibitors for IBD therapy, novel anti-inflammatory drugs with improved safety profiles are in development for IBD and other chronic inflammatory disorders.[50,52] Based on the successes of this pilot and feasibility study, drug-specific responses to treatment can be measured using specific serum biomarkers. Unanswered questions about the most clinically appropriate biomarkers to track various components of a complex disease like IBD (for example, the type and degree of the inflammatory process, and the state and activity of the healing process) remain. The time-line of the responses, the sensitivity and specificity of response over time compared to established (invasive) clinical measures, cost, and other issues must be addressed.

CONFLICT OF INTEREST

Guarantor of the article: Laurie S. Conklin, MD.
Specific author contributions: Heier, Conklin, Hathout, Fiorillo, Damsker, and Hoffman contributed to the study concept and design. Heier, Chaisson, Hathout, and Conklin acquired the data. Gordish-Dressman performed statistical analysis. Heier and Conklin drafted the manuscript. Hoffman and Fiorillo offered critical revision for intellectual content. Funding was obtained by Conklin, Damsker, Heier, and Hoffman.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- Objective evaluation is necessary to adequately assess disease response in inflammatory bowel disease (IBD).
- Current disease evaluation tools either use symptom-based indices that poorly reflect underlying inflammatory disease, or use invasive, costly, and slow methods like colonoscopy.

WHAT IS NEW HERE

- We used novel aptamer-based proteomics methods to discover serum proteins that respond to each of two different anti-inflammatory drugs with known efficacy in IBD.
- Serum miRNAs are now emerging as stable, well-conserved serum biomarkers, and we found several miRNAs responsive to anti-inflammatory treatments.
- Bioinformatic analyses showed that eight markers that decrease are associated with inflammation and have gene promoters regulated by NF-κB, a key inflammatory transcription factor.

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