GM-CSF Antibodies and Peripheral T_{reg} Cells as Potential Markers of Disease Activity to Guide Therapeutic Decisions in Pediatric IBD

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Abstract

Objectives: 1. To measure and compare granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies and peripheral regulatory T cells (Tregs) cells as non-invasive biomarkers in active inflammatory bowel disease (IBD) and remission in pediatric IBD. 2. To determine the ability of these biomarkers to predict severity/type and choice of therapy in pediatric IBD.

Methods: We hypothesized that patients with severe IBD requiring biologics will have higher circulating GM-CSF antibodies and lower Foxp3+ Tregs at baseline, with subsequent improvement in response to therapy. 28 patients were prospectively analyzed. Blood collected pre- and post-treatment was analyzed for GM-CSF antibodies by ELISA and Tregs by flow cytometry. Clinically, PCDAI and PUCAI were scored. Wilcoxon Rank Sum tests were used to compare baseline values between groups, and paired signed-rank tests used to compare pre- and post-treatment values.

Results: Median GM-CSF antibodies trended higher (0.50 µg/mL vs. 0.37) and Tregs lower (5.23% vs. 6.71%) in the biologic cohort with no significant difference between cohorts. In response to biologics, 37% showed decrease in GM-CSF antibodies and 75% showed increase in Tregs. GM-CSF antibodies trended higher in Crohn’s disease (CD) than ulcerative colitis (UC) (0.81 vs. 0.31, p=0.073). Activity indices showed significant improvement in the biologic cohort of UC patients (p=0.04).

Conclusion: GM-CSF antibodies and peripheral Tregs may reflect disease activity and thereby guide IBD therapy. Although no individual biomarker could predict need for biologics, their trend favors their use in a predictive model to identify the most effective treatment strategy in pediatric IBD.

Keywords: Biomarkers; GM-CSF antibodies; Tregs; Biologics; Pediatric IBD

Introduction

What is known

- The incidence of inflammatory bowel disease (IBD) in pediatric population has doubled in the past 2 decades.
- IBD is recognized as immune dysregulation that fails to maintain gut homeostasis between intraluminal intestinal microbiota, host inflammatory mediators and host regulatory immune response [1,2].
- Granulocyte-macrophage colony-stimulating factor (GM-CSF) has an important role in the pathogenesis of intestinal immune and inflammatory processes and the regulation of gut homeostasis.

What is new

- Levels of GM-CSF antibodies and peripheral Tregs may have value in predicting the choice of therapy in pediatric IBD.
- A combination of biomarkers and their potential for incorporation in a predictive model may be more useful than any individual biomarker, to predict the need for early biologic therapy in pediatric IBD.

The incidence of inflammatory bowel disease (IBD) in pediatric population has doubled in the past 2 decades. With more intensive research delving into the pathogenesis of the disease process, IBD is increasingly being recognized as an immune dysregulation that fails to maintain gut homeostasis between intraluminal intestinal microbiota, host inflammatory mediators and the host regulatory immune response [1,2].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has an important role in the pathogenesis of intestinal immune and inflammatory processes and the regulation of gut homeostasis [3-6]. Prior studies in a mouse model showed that GM-CSF-deficient mice developed more severe colitis in

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response to mucosal injury [2,3,7]. GM-CSF antibodies in high titers are associated with reduced intestinal GM-CSF bioactivity, reduced neutrophil bacterial killing, increased intestinal permeability, and more severe mucosal injury in IBD [5,8]. Recent studies have correlated higher GM-CSF antibody levels with stricturing disease or luminal narrowing as evidenced by magnetic resonance enterography [5,9,10].

The human immune system is pivotal in maintaining a dynamic balance between intestinal luminal microbiota and host protective immune responses by using complex regulatory mechanisms [11]. The balance between host and intestinal flora is maintained through the cooperation of various regulatory mechanisms that prevent the immune system from reacting toward harmless exogenous antigens present in the intestine. Among the cells mediating dominant tolerance, Foxp3+regulatory T cells (Tregs) play an essential role in intestinal homeostasis [11-14]. Foxp3+Tregs are reportedly reduced in peripheral blood in patients with active IBD when compared to patients in remission [11,15].

In most adult patients, generally steroids and aminosaliclylates for induction of remission is successful, making the step-up approach a viable option to avoiding the cost and potential complications of biologics [16-22]. However, children with IBD often have a severe presentation and a predilection for colitis. It is also important to note that early mucosal healing is correlated with longer remission periods in IBD and reduced steroid dependence, thereby justifying use of biologics when steroids fail to induce mucosal healing at diagnosis [16,18,23,24].

Although follow-up endoscopy to verify mucosal healing is the strongest predictor of drug efficacy, it is expensive to perform repeated colonoscopies in children to determine response to therapy [21]. Additionally, fecal calprotectin, a marker of neutrophil infiltration, has been shown to be helpful in assessing disease activity but requires stool collection, a return visit to clinic, and mailing out the sample to a reference lab [25-28]. For these reasons, we proposed to determine the predictive value of 2 new non-invasive biomarkers in the blood that could be drawn at the time of examination.

We conducted a prospective translational study comparing GM-CSF antibodies and peripheral Tregs as biomarkers to predict response to first-line medications versus step-up to biologics for remission in pediatric IBD. Our primary aim was to measure these non-invasive biomarkers in active IBD and remission, to determine if a significant increase in GM-CSF antibodies and/or a decrease in peripheral Tregs predicted a need for step-up therapy to biologics in IBD. Our secondary aim was to assess the trend of these biomarkers in response to therapy and disease activity.

Methods

All pediatric patients with active IBD seen at the University of Texas Health Science Center at Houston, Pediatric GI clinic and Children’s Memorial Hermann Hospital in the Texas Medical Center were enrolled from November 2014 to March 2016. The study was approved by the Institutional Review Board (IRB # HSC-MS-14-0711) and the Institutional Biosafety Committee at the University of Texas Health Science Center at Houston.

Eligibility

We included all pediatric patients aged 4-18 years presenting with symptoms suggestive of IBD and elevation in blood inflammatory markers e.g. erythrocyte sedimentation rate (ESR), C-reactive protein (CRP). All patients were endoscopically diagnosed with Crohn’s Disease or Ulcerative Colitis with biopsy confirmation. We excluded those who had recently required steroids within the past 2 months, or any other form of immunosuppression. We also excluded patients with evidence of enteric infection or infestation detected by laboratory screening. Written parental/legal guardian informed consent and subject assent (for children 7 years of age or older) was obtained prior to study enrollment.

Activity index

Patients and caregivers were provided with a questionnaire to obtain a validated Activity Index (Figure 1).

That objectively assesses their disease severity as mild or moderate-severe based on points allotted to severity of abdominal pain, frequency of bowel movements, blood in stools, night-time awakenings, limitation of daily activities,
weight loss and constitutional symptoms of fever, joint pains, fatigue, skin rash or mouth sores. These questions allowed calculation of a PCDAI (Pediatric Crohn’s Disease Activity Index) or a PUCAI (Pediatric Ulcerative Colitis Activity Index) along the recent NASPGHAN guidelines [29]. The Activity Index was monitored after treatment to document clinical remission (Tables 1 and 2).

Table 1 Activity index PCDAI (Abbreviated PCDAI).

| History (Recall, 1 week) | Score |
|--------------------------|-------|
| Abdominal Pain           |       |
| 0 - None                 |       |
| 5=Mild: Brief, does not interfere with activities |       |
| 10=Moderate/Severe: Daily, longer lasting, affects activities, nocturnal |       |
| Patient Functioning, General Well-Being | |
| 0=No limitation of activities, well |       |
| 5=Occasional difficulty in maintaining age appropriate activities, below par |       |
| 10=Frequent limitation of activity, very poor |       |
| Stools (per day)         |       |
| 0=0-1 liquid stools, no blood |       |
| 5=Up to 2 Semi formed with small blood, or 2-5 liquid |       |
| 10=Gross bleeding, ≥ 6 liquid, or nocturnal diarrhea |       |
| Examination              |       |
| Abdomen                  |       |
| 0=No tenderness, no mass |       |
| 5=Tenderness, or mass without tenderness |       |
| 10=Tenderness, involuntary guarding, definite mass |       |
| Perirectal Disease       |       |
| 0=None, asymptomatic tags |       |
| 5=1-2 indolent fistula, scant drainage, no tenderness |       |
| 10=Active fistula drainage, tenderness, or abscess |       |
| Weight                   |       |
| 0=Weight gain or voluntary weight stable/loss |       |
| 5=Involuntary weight stable, weight loss 1%-9% |       |
| 10=Weight loss ≥ 10% |       |
| Extra intestinal Manifestations | |
| (Fever ≥ 38.5°C for 3 days over past week, definite arthritis, uveitis, E. nodosum, P. gangrenosum) |       |
| 0=None |       |
| 5=1 |       |
| 10 ≥ 2 |       |
| Total Score              |       |

Table 2 Activity index PUCAI (Abbreviated PUCAI).

| Item                                      | Points |
|-------------------------------------------|--------|
| Abdominal pain                            |        |
| No pain                                   | 0      |
| Pain can be ignored                       | 5      |
| Pain cannot be ignored                    | 10     |
| Rectal bleeding                           |        |
| None                                      | 0      |
| Small amount only, in < 50% of stools     | 10     |
| Small amount with most stools             | 20     |
| Large amount (> 50% of stool content)     | 30     |
| Stool consistency of most stools          |        |
| Formed                                    | 0      |
| Partially formed                          | 5      |
| Completely unformed                       | 10     |
| Number of stools per 24 hours             |        |
| 0-2                                       | 0      |
| 3-5                                       | 5      |
| 6-8                                       | 10     |
| > 8                                       | 15     |
| Nocturnal stools (any episode causing wakening) |    |
| No                                        | 0      |
| Yes                                       | 10     |
| Activity level                            |        |
| No limitation of activity                 | 0      |
| Occasional limitation of activity         | 5      |
| Severe restricted activity                | 10     |
| Total maximum score                       | 85     |

Study design

Routine labs and stool studies including complete blood count with differential, electrolyte panel, ESR, CRP and fecal calprotectin were ordered at the initial visit as part of routine standard of care. Blood was collected for the research study on endoscopic and pathological confirmation of IBD. The blood was than analyzed for GM-CSF antibodies and peripheral Tregs by enzyme-linked immunosorbsent assay (ELISA) and flow cytometry respectively.

Plasma was stored immediately at -80 degrees C until performing ELISA to detect the levels of GM-CSF auto antibodies. The specimens were shipped to Cincinnati Children’s Hospital Medical Center, Cincinnati, OH for ELISA testing to detect GM-CSF antibody levels.

For Flow cytometry, fresh blood samples in EDTA-treated tubes were centrifuged for 15 minutes at 350 g to separate plasma from blood cells. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using Ficoll-Paque (GE Healthcare), and immediately stained with T cell surface CD markers, CD4 (T helper) and CD25 (high expression in Treg population) and intracellular Foxp3+ (specific marker for
functional Tregs [30]. To ensure highly reliable results, all samples were processed on the same day of phlebotomy for surface and intracellular staining without freezing. The cells were then analyzed by flow cytometry (FACS Caliber; Becton Dickinson) and FlowJo software (TreeStar, Inc) [30-34].

Patients were subsequently followed prospectively to assess response to first-line medications (most often, mesalamine and/or steroids) and stratified into 2 groups: non-biologic (responding to first-line therapy) and biologic group. The latter included those treated with escalation of care to biologics: anti-TNF-alpha (Remicade or Humira). GM-CSF antibodies, peripheral Tregs and fecal calprotectin were monitored in both groups in remission, at the completion of induction phase. Differences in these biomarkers between the groups at diagnosis, and the change in these biomarkers after treatment were analyzed statistically at the end of the study. The study methods have been summarized (Figure 2).

**Results**

We enrolled 31 patients in the study, of whom 28 were diagnosed with IBD on endoscopy and biopsy confirmation. Of the 28 patients, 14 had CD (50%). Eleven out of 23 patients (48%) required biologics, while 52% patients responded to first-line medications. Of the 12 who responded to first-line medications, 1 required only mesalamine for induction of remission while 3 required addition of a topical steroid.

The remaining 8 required oral prednisone for clinical remission, followed by primary maintenance with immunomodulators such as azathioprine. Of the 11 requiring biologics, 8 had Crohn’s disease (72%) while 75% of the non-biologic group had ulcerative colitis. Figure 3 shows the flow diagram for study participants.

![Figure 3 Participant flow diagram.](image)

A total of 18 of the 28 patients completed the study, with both pre- and post-treatment samples. We were able to obtain baseline blood samples and to follow another 5 patients to assess response to first-line medications or step-up to biologics; however, these 5 failed to return for a post-treatment sample after remission.

**Statistical analysis**

Wilcoxon Rank Sum tests were used to compare baseline GM-CSF antibodies and Tregs in the non-biologic vs. biologic groups. The percent decrease in GM-CSF antibodies and increase in Tregs in response to therapy was analyzed by the Wilcoxon paired signed-rank test. The Mann-Whitney test was used to compare the therapeutic change in biomarker levels between the biologic and non-biologic cohorts. Multivariate logistic regression analysis was done to see if any individual laboratory variable including the two study biomarkers or a combination of variables predicted a need for early biologic therapy. A two-sided p-value of 0.05 was considered significant.

**Table 3** shows the demographic, clinical and laboratory variables in the non-biologic (first-line medications) and biologic groups. Study subjects’ ages ranged from 5 to 18 years; 61% were male and 39% female. Caucasians comprised 73% of the biologic group while Hispanics comprised 50% of the non-biologic group.

Of the patients in the biologic group, 46% had penetrating or perianal Crohn’s Disease. Forty-five % of the biologic group showed a ≥ 90% improvement in activity indices following treatment, compared to 75% in the non-biologic group (p=0.21). As seen in **Table 3**, there are no significant differences in laboratory parameters, including ESR, CRP or hemoglobin between the biologic and non-biologic cohorts.

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Albumin <3.5 was seen in 73% of the biologic group compared to 33% of the non-biologic group (p=0.10). Fecal calprotectin was also noted to be ≥ 1000 in 82% of the biologic group compared to 42% of the non-biologic group (p=0.08). Initial biomarker levels were compared between the biologic and non-biologic groups (Figure 4).

Table 3 Baseline demographic, clinical and laboratory characteristics of the non-biologic and biologic groups.

| Variable                              | Non-biologic group (n=12) | Biologic group (n=11) | p-value |
|---------------------------------------|---------------------------|-----------------------|---------|
| Mean Age (years)                      |                           |                       | 0.852   |
| Gender                                |                           |                       | 0.214   |
| Male                                  | 5                         | 8                     |         |
| Female                                | 7                         | 3                     |         |
| Ethnicity                             |                           |                       | 0.015   |
| Caucasian                             | 3                         | 8                     |         |
| African American                      | 2                         | 3                     |         |
| Hispanic                              | 6                         | 0                     |         |
| Asian                                 | 1                         | 0                     |         |
| IBD subtype                           |                           |                       | 0.039   |
| Crohn’s Disease                       | 3                         | 8                     |         |
| Ulcerative Colitis                    | 9                         | 3                     |         |
| Penetrating/Perianal Crohn’s          | 0                         | 5                     | 0.014   |
| Improvement in Activity Index         |                           |                       | 0.214   |
| ≥ 90%                                 | 9                         | 5                     |         |
| <90%                                  | 3                         | 6                     |         |
| ESR                                   |                           |                       | 0.68    |
| >20 mm/hr                             | 6                         | 7                     |         |
| ≤ 20 mm/hr                            | 6                         | 4                     |         |
| CRP                                   |                           |                       | 0.371   |
| >2.9 mg/L                             | 7                         | 9                     |         |
| ≤ 2.9 mg/L                            | 5                         | 2                     |         |
| Calprotectin                           |                           |                       | 0.089   |
| ≥ 1000 µg/g                           | 5                         | 9                     |         |
| <1000 µg/g                            | 7                         | 2                     |         |
| Hemoglobin                            |                           |                       | 0.414   |
| <11 g/dL                              | 4                         | 6                     |         |
| ≥ 11 g/dL                             | 8                         | 5                     |         |
| Albumin                               |                           |                       | 0.1     |
| <3.5 g/dL                             | 4                         | 8                     |         |
| ≥ 3.5 g/dL                            | 8                         | 3                     |         |

GM-CSF antibodies were numerically higher in biologics than non-biologics group, though not statistically significant (median 0.50 µg/mL, IQR 0.16-5.06 in biologics vs. median 0.37 µg/mL, IQR 0.24-1.14 in non-biologics; p=0.57).

Tregs, although numerically lower in the biologic group, were also not found to significantly differ, when comparing the two groups (median 5.23%, IQR 1.55-7.38 in the biologic group vs. median 6.71%, IQR 1.72-8.24 in non-biologic group; p=0.62).

GM-CSF antibodies were found to be higher in Crohn’s disease than ulcerative colitis, as seen in Figure 5. This approached statistical significance in our study (median 5.23%, IQR 1.55-7.02 in CD vs. median 5.07%, IQR 1.55-8.29 in UC; p=0.74).

Following induction therapy, GM-CSF antibodies declined in 39% of all treated patients and specifically declined in 38% of the biologic group. The decline in GM-CSF was more in the biologics group although the median change was similar between the groups (median change 0.05 µg/mL, IQR 0.55-4.52 in biologics vs. median change 0.02 µg/mL, IQR 0.06-0.22 in non-biologics; p=0.78). Tregs increased following induction therapy in 67% of all treated patients and specifically in 75% of the biologic group.

The rise in Tregs was more in the biologics group although the median change was similar between the groups (median change 1.23%, IQR 1.95-3.61 in biologics vs. median change 0.87%, IQR 4.67 to 3.17 in non-biologics; p=0.65). The change in biomarker levels in response to therapy is shown in Figure 6.
Figure 7 shows a representative flow cytometry plot of Foxp3+ Tregs before and after therapy.

Figure 4 Initial biomarker levels between biologic and non-biologic groups.

Figure 5 Biomarker levels in Crohn’s disease versus ulcerative colitis.

An ROC analysis was done for baseline GM-CSF antibodies and Tregs to predict need for biologics. GM-CSF antibodies ≥ 4.3 µg/mL showed the highest accuracy with 100% specificity but low sensitivity of 36.4% (AUC of 0.68) as seen in Figures 8 and 9.

Tregs ≤ 7.4% showed the highest accuracy with a sensitivity of 81.8% but lower specificity of 41.7% (AUC of 0.62) (Figures 10 and 11).

Fecal calprotectin at diagnosis didn’t show any significant difference between the biologic and non-biologic groups (median 1280 µg/g, IQR 1072 to >2000 in biologics group vs. median 403.9 µg/g, IQR 215 to >2000 in non-biologics group; p=0.13). Calprotectin did not differ between the CD and UC groups (median 1224 µg/g, IQR 422 to >2000 in CD vs. median 949 µg/g, IQR 225 to >2000 in UC; p=0.25). Laboratory parameters routinely used in clinic were compared between biologic and non-biologic cohorts and in CD vs. UC.

ESR and CRP were significantly higher and albumin significantly lower in CD compared to UC (median ESR 32.5 mm/hr in CD vs. 8.5 mm/hr in UC; p=0.01, median CRP 23.2 mg/L in CD vs. 2.9 mg/L in UC; p=0.007 and median albumin 3 g/dl in CD vs. 4 g/dl in UC; p=0.020). No differences in these parameters were noted between the biologic and non-biologic groups. A multivariate logistic regression analysis did not show any individual biomarker to significantly predict a need for early biologic therapy.
Discussion

Inflammatory bowel disease results from an immune dysregulation involving Tregs, as well as a dysfunction of the innate immune system, which includes neutrophils and macrophages. GM-CSF is required for priming of monocyte/macrophage and neutrophil antimicrobial functions. An altered bioactivity of GM-CSF, therefore, likely plays a role in the innate immune dysregulation in CD. This immune dysfunction leads to increased bowel permeability and progression of disease type from primarily inflammatory, to penetrating and stricture disease later in the process [2,9]. The impaired intestinal barrier function and gut homeostasis is associated with reduced GM-CSF bioactivity and monocyte/macrophage function, resulting in high titers of endogenous anti-GM-CSF antibodies (GM-CSF Ab) with increasing gut permeability [5].

Foxp3+Tregs play an important role in maintenance of intestinal homeostasis through IL-10 and TGF-β1-dependent mechanisms [35]. Tregs can produce IL-10 and TGF-β. IL-10 acts on innate immune cells to reduce inflammation, whereas TGF-β inhibits effector cells directly and promotes epithelial homeostasis. In addition, TGF-β can synergize with other cytokines to promote the differentiation of Th17 cells that promote IL-17 and IL-22, which also contributes to the barrier function [11,36]. Tregs are characterized by high levels of the transcription factor Foxp3, which is considered to confer their regulatory activity [37].

There is a proven underlying pathogenesis involving a complex interplay between host genes, microbial and food antigens and the genetically mediated immune response of the host to these antigens, which influences the severity of presentation in IBD. GM-CSF antibodies and Tregs are likely to play a central role in this pathogenesis. Current available blood markers like ESR and CRP and stool markers of inflammation such as fecal calprotectin are elevated in response to the
inflammation associated with IBD [25]. GM-CSF antibodies and peripheral Tregs are an integral part of the immunopathogenesis of the disease process, hence are impacted earlier in the disease process and may have potential for more accuracy and reliability as predictive markers of response to therapy [8]. This was the basis for our hypothesis that increased GM-CSF antibodies and/or decreased Tregs may predict a need for earlier biologic therapy for improved mucosal healing in IBD.

This study suggests, though does not prove, that higher GM-CSF antibodies may be associated with a need for biologic therapy. GM-CSF antibodies were also found to be higher in Crohn’s Disease compared to Ulcerative Colitis. Furthermore, antibody levels declined in response to therapy with a greater decline in the biologic group. Prior studies have shown a statistically significant difference in levels of GM-CSF antibodies when measured during active disease compared to values obtained at remission [2,5,8-10]. Nylund et al suggested that disruption of the intestinal barrier in IBD could lead to permeation of luminal antigens and increased bacterial translocation. This stimulates memory B cells to upregulate GM-CSF autoantibodies [2]. Dabritz, et al. reported a clear increase in GM-CSF antibodies in the months preceding a relapse where they studied patients longitudinally with serial measurements of GM-CSF antibody levels. They theorized that fluctuating antibody levels during remission versus relapse affect myeloid functions and result in an impaired modulation of gut homeostasis and gut mucosal barrier function [8].

Jurickova, et al. showed a significant correlation and inverse relation between GM-CSF antibodies and bacterial killing; the latter decreasing as serum GM-CSF autoantibodies increase [5]. This is seen progressively with a rise in GM-CSF antibodies from 1 to 10 µg/mL and studies in adults have shown levels >5 µg/mL to be the cut-point where GM-CSF antibodies start to inhibit neutrophil anti-bacterial function [8]. Gathungu, et al. showed an association of GM-CSF antibodies ≥ 4 µg/mL with aggressive ileal disease [10]. An ROC analysis in our study showed that GM-CSF antibodies ≥ 4.3 µg/mL had the highest accuracy of predicting need for biologics.

There were 5 patients in our study who had GM-CSF antibody levels >5 µg/mL. Of these, 4 patients had radiological evidence of luminal narrowing, intestinal strictures or microperforation with intra-abdominal abscess and all of them had Crohn’s disease. Dykes et al showed similar results with GM-CSF antibodies ≥ 1.6 µg/mL associated significantly with luminal narrowing and CD patients being 5 times as likely to have a stricture on CTE or MRE [9]. Han et al also found elevated GM-CSF antibodies to be associated with a 2-fold higher rate of stricturening/penetrating disease behavior [7].

One patient had more than a 90-fold increase in GM-CSF antibody levels following therapy and clinical remission. This followed a prolonged course of refractory IBD that finally responded to biologics. The only plausible explanation seemed to be an early indication of relapse in this patient who shortly thereafter, went on to receive a higher dose of biologics with increased frequency of administration. This phenomenon was also noted by Dabritz et al in their study on GM-CSF antibody levels in patients with relapse, where they explain an elevated serum GM-CSF antibody titer in patients in clinical remission to represent an early stage of increased intestinal permeability and bacterial translocation [8]. Thus increasing GM-CSF antibody levels in a patient in clinical remission, may herald a secondary loss of response with a subsequent relapse, especially in CD that involves transmural inflammation.

Our study also suggested that lower Tregs may be associated with a need for biologic therapy. There was an increase in Tregs after treatment in 75% of the biologic group. Although not statistically significant, this increase was greater after biologic therapy, similar to prior studies [32,38]. Wang, et al. also reported higher Tregs in inactive versus active CD and UC, however this did not reach statistical significance [15]. Tregs were also found to trend lower in patients with Crohn’s disease, comparable to prior studies [39,40]. An ROC analysis in our study showed Tregs ≤ 7.4% to have the highest accuracy to predict a need for biologics. Saruta et al reported peripheral Tregs ranging from 5%-8% in active versus 9%-12% in inactive disease [41]. Li, et al. reported Tregs ranging from 3%-6% in active IBD that increased to 8%-16% following anti-TNFα therapy [38].

Tregs are anti-inflammatory and are proposed to mediate many of the beneficial effects on gut inflammation [31,32,35,42]. The severity of disease activity in IBD is inversely correlated with the frequency of peripheral Tregs [15,41,43,44]. This contraction of the peripheral pool of Tregs is postulated to be due to an insufficient counter-regulation to inflammation, rather than their flowing out from the circulation and accumulating in inflamed intestinal tissue [40]. There is, in turn, a significant increase in frequency of peripheral Tregs in response to treatment with biologics [39]. The increased apoptotic activity of Tregs in active IBD declines with response to anti-TNFα therapy, and their numbers increase. Six out of 18 patients in our study did not have an increase in Tregs after treatment. Lack of improvement in Tregs after treatment has been attributed to alternate immune pathways in the intestinal mucosa, not involving Foxp3+Tregs during remission, in prior studies [39].

Fecal calprotectin did not significantly differ in the biologic and non-biologic cohorts or in CD versus UC in this study. Calprotectin, a gut inflammatory marker released from neutrophils at the site of intestinal inflammation, has been valuable as a screening tool preceding invasive endoscopy. However, Henderson, et al. who studied calprotectin extensively in the pediatric population found that the level was not influenced by disease type (CD vs. UC) or location [28]. Previous studies have also shown that calprotectin has poor correlation with the clinical activity indices although better correlation with endoscopic indices of severity [26,45].

PCDAI and PUCAI were used in all our patients throughout the study to obtain an objective assessment of clinical severity and response to therapy. Forty-five% of the biologic group showed a ≥ 90% improvement in activity indices following treatment, compared to 75% in the non-biologic group. This is probably a reflection of the severity of disease in the biologic group that was less responsive to therapy, compared to the
milder spectrum of disease in the non-biologic group. Clinical remission and improvement in activity indices was noted to be significant in the biologic cohort only in UC patients (p=0.04). CD patients did not show a significant improvement, indicating that severe CD may require earlier institution of anti-TNF therapy to bring about a significant clinical improvement. Several prior studies have shown short-term response rate with biologics in CD and failure rates ranging from 18 to 46% at 1 and 5 year follow up [16]. Although several factors may cause loss of response including host and drug pharmacokinetics and the development of antibodies to infliximab, the decreased response of CD to anti-TNF agents may reflect the underlying severe transmural inflammation, as well as the delay in starting biologic therapy that results after numerous unsuccessful attempts to treat flares with steroids. This delays mucosal healing further, which may contribute to lesser efficacy of biologics than their potential, in the long run.

Our study showed that higher levels of GM-CSF antibodies and lower levels of peripheral Tregs were associated with a greater need for biologics reflecting disease severity in pediatric IBD. Thus these biomarkers help guide treatment choices from the variety of treatment options available, based on individual disease activity. However, the lack of statistical significance may be attributed to the small sample size in this study. Despite a small sample size, the trends of these novel biomarkers in our study of pediatric patients paralleled those in adult IBD studies with statistical significance. It is also important to note that most prior studies investigating these blood biomarkers were larger studies in adult patients, who may have had more severe inflammation and an advanced disease process, allowing for a greater difference in biomarker levels between cohorts. The biologic cohort also had a larger proportion of Caucasians while there were a larger number of Hispanics in the non-biologic cohort. The effect of this demographic disparity on biomarker levels in this study, however, is unknown, despite studies reporting higher serological markers like p-ANCA in the Hispanic population compared to Caucasians [46].

Although sample size was a limitation of this study, we suggest it had a few strengths. Unlike prior studies comparing mostly between active and inactive IBD or remission and relapse, this study was, to our knowledge, the first to compare groups based on the type of therapy needed, reflecting disease severity. We aimed to evaluate the ability of these biomarkers to guide therapy decisions in our IBD patients. Designed as a prospective study, we were able to collect all clinical variables for an accurate scoring of the activity indices, to objectively assess disease severity and monitor patients for remission. Longer follow-up periods would add more value to assess loss of response and clinical relapse.

Conclusion

This prospective translational study of novel biomarkers in pediatric IBD, although limited by a small sample size, underscores the hypothesis that blood biomarkers like GM-CSF antibodies and Tregs, that play an important role in the underlying immuno-pathogenesis of IBD, may have value in guiding IBD therapy based on disease severity. GM-CSF antibodies ≥ 4.3 μg/mL and Tregs ≤ 7.4% may be used in combination to improve the predictive value of these biomarkers. The greater sensitivity of Treg cells and higher specificity of GM-CSF antibodies may be better combined in a predictive model using both these biomarkers to reflect disease severity. They may also help to identify CD from UC, complementing the serology tests used for evaluation of indeterminate colitis which is very common in the pediatric population. Although the findings in this study of new non-invasive biomarkers were not statistically significant, it does not preclude this work from serving as a pilot study, with promise for larger studies with more robust ROC analyses to improve the predictive accuracy of these non-invasive biomarkers. A combination of such validated biomarkers may help develop a predictive model, to guide management strategies and identify the most effective treatment in personalized treatment regimens. Our pilot study may serve as the basis for future studies, with combinations of these same or similar novel biomarkers involved in pivotal inflammatory pathways in IBD, to help develop a commercially marketable panel of biomarkers that would be more readily available in pediatric hospital and clinic settings. Guided by these biomarkers, we may be able to provide appropriate first-line therapy to those expected to respond, avoiding the potential adverse effects and higher cost of biologics. Conversely, institution of early anti-TNF therapy where indicated, may induce early remission especially in our CD patients, to reduce steroid-resistance and promote mucosal healing. This will ultimately improve quality of life scores and reduce hospitalizations and healthcare cost.

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