Article

Potential Role of Methotrexate Polyglutamates in Therapeutic Drug Monitoring for Pediatric Inflammatory Bowel Disease

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Abstract: Inside cells, the immunomodulator methotrexate (MTX) undergoes the addition of glutamates to form methotrexate polyglutamates (MTX-Glu)—promising biomarkers of systemic exposure and treatment response to MTX in rheumatology. MTX-Glu are underexplored in Inflammatory Bowel Disease (IBD), with no data in pediatrics. In this cross-sectional secondary analysis, we assessed the relationships between MTX-Glu and MTX dose and treatment response in pediatric IBD. Twenty-one children with IBD, receiving maintenance therapy with infliximab (IFX) and MTX, had $MTX\text{-}Glu_{1-6}$ concentrations and IFX troughs/antibodies measured and disease activity assessed for comparison in remission vs. active IBD using non-parametric tests, with associations explored using Spearman's correlation (ϱ) and regression analyses; SASv9.4 ($\alpha = 0.05$). Total and long-chain MTX-Glu correlated with MTX dose ($\varrho = 0.51$ and 0.56, respectively; $p \leq 0.02$). In children with Crohn's disease ($n = 19$), short-chain $MTX\text{-}Glu_{1-2}$ were 2.5-fold higher in remission vs. active disease, approaching statistical significance ($p = 0.066$), with no statistical differences in IFX trough ($p = 0.549$) between groups. Our study highlights a potential role for long-chain MTX-Glu in the therapeutic drug monitoring of MTX in IBD. It is the first study in pediatric IBD and, although statistical significance was not reached, our findings also suggest that higher short-chain MTX-Glu levels may be associated with IBD treatment response to MTX in children.

Keywords: methotrexate; methotrexate polyglutamates; infliximab; inflammatory bowel disease; Crohn's disease; ulcerative colitis; pediatric

1. Introduction

Methotrexate (MTX) is a folic acid analog used as monotherapy, or in combination with anti-TNF-α agents, for the treatment of autoimmune disorders [1]. To exert its action, MTX enters cells via folate transporters and receptors, and undergoes the addition of glutamic acid (Glu) residues by folylpolyglutamate synthetase to form methotrexate polyglutamates ($MTX\text{-}Glu_n$) [2], where *n* represents the number of glutamic acid residues, with $MTX\text{-}Glu_1$ denoting the parent form of MTX. The addition of glutamic acid to MTX occurs sequentially, and the metabolites formed are classified based on the length of the glutamate side chain, where glutamate $Glu_{1 \text{ and } 2}$ are considered short-chain and Glu_{3-6} long-chain [3]. Unlike $MTX\text{-}Glu_{1-2}$, $MTX\text{-}Glu_{3-6}$ are not effluxed from the cell efficiently and, therefore, reflect steady-state intracellular methotrexate concentration. In addition, the polyglutamated metabolites of MTX have a differential activity profile compared to the parent drug, with similar inhibitory activity against dihydrofolate reductase, but increased direct inhibitory activity against folate-dependent enzymes involved in purine

and pyrimidine biosynthesis [4]. In rheumatology, erythrocyte concentrations of MTX-Glu$_n$ have been associated with systemic exposure to MTX and MTX treatment efficacy [3,5–7]. Data are lacking in inflammatory bowel disease (IBD), where erythrocyte MTX-Glu$_n$ levels could offer a clinically useful biomarker for MTX responsiveness and/or a measure for therapeutic drug monitoring—currently unavailable for this drug despite its frequent use. The aim of this prospective, single-center, cross-sectional, secondary analysis of pediatric patients receiving stable doses of infliximab (IFX) and MTX for IBD was to explore the relationships between MTX-Glu$_n$ and MTX dose and treatment response in IBD.

2. Results

Children included in this secondary analysis completed a convenience-sampling cross-sectional investigation of IFX pharmacokinetics across different pediatric autoimmunity diagnoses [8]. Of the 21 children enrolled (median age 16 years (IQR 12, 17), 38% F, 90% Crohn's disease), 10 had active disease (six mild, three moderate, and one severe) and 11 were in remission (Paris Disease Classification in Table 1). One child with active disease (mild), and undetectable IFX trough, had anti-IFX antibodies. The two study groups were otherwise comparable in age, at 16.0 (13.0, 17.0) vs. 14.5 (9.0, 19.0) years, and disease duration, at 2.5 (1.8, 5.2) vs. 3.8 (1.1, 4.5) years; both $p \geq 0.8$. Clinically prescribed MTX doses for patients varied from 5 to 25 mg weekly, with 86% oral administration. To account for inter-individual variability in MTX dose, as well as variability in patient age and size in our pediatric cohort (5–21-year-olds), the MTX dose was adjusted for total body weight (mg/kg). Adjusted for weight (mg/kg), the MTX dose correlated significantly with long-chain MTX-Glu$_{3-5}$ ($\varrho = 0.56$; $p = 0.009$) and MTX-Glu$_{Total}$ ($\varrho = 0.51$; $p = 0.018$), but not short-chain Glu$_{1-2}$ ($\varrho = 0.27$, $p = 0.244$); see Figure 1. MTX-Glu$_6$ was undetectable across the study population.

Table 1. Paris Classification. Age at diagnosis: A1a (0–<10 years); A1b (10–<17 years). Location: L1: distal 1/3 ileum +/− limited cecal disease; L2: colonic; L3: ileocolonic; L4a: upper disease proximal to ligament of Treitz; L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum. Extent: E1: ulcerative proctitis; E2: Left-sided UC (distal to splenic flexure); E3: extensive (hepatic flexure distally); E4: pancolitis (proximal to hepatic flexure). Behavior: B1: nonstricturing nonpenetrating; B2: structuring; B3: penetrating; B2B3: both penetrating and stricturing disease, either at the same or different times; *p*: perianal disease modifier. Severity: S0: never severe; S1: ever severe. Growth: G0: no evidence of growth delay; G1: growth delay.

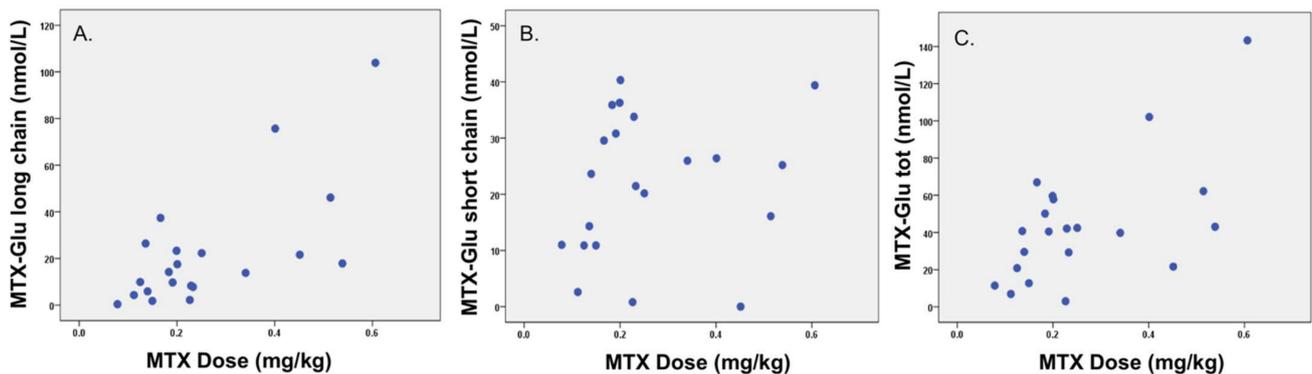|		Location	Behavior	Growth
Remission (n = 11)	A1a 36%	L1 25% L2 0%	B1 50% B2 0%	G0 100%
		L3 50% L4b 25%	B3p 50% S1 0%	G1 0%
	A1b 64%	L1 17%	B1 57%	G0 43%
		L2L4a 17%	B2B3 14%	
		L3 33%		G1 43%
		L3L4aL4b 17% L4aL4b 17%	B3 14%	Missing Data 14%
		E2 17%	S1 14%	
Active (n = 10)	A1a 40%	L1L4a 25% L3 25%	B1 50% B2 25%	G0 50% G1 25%
		L3L4aL4b 25% E4 25%	B3 0% S1 25%	Missing Data 25%
	A1b 60%	L1 33% L2 17%	B1 66% B2B3p 17%	G0 100%
		L3L4a 33% L3L4aL4b 17%	B3 17% S1 0%	G1 0%

Figure 1. Spearman's correlation (ϱ) between methotrexate polyglutamate (MTX-Glu) concentrations and methotrexate (MTX) dose for long-chain MTX-Glu$_{3-5}$, $\varrho = 0.56$; $p = 0.009$ (**A**); short-chain Glu$_{1-2}$, $\varrho = 0.27$; $p = 0.244$ (**B**); and total MTX-Glu (MTX-Glu$_{tot}$), $\varrho = 0.51$; $p = 0.018$ (**C**).

MTX-Glu$_n$ concentrations were not statistically different between children with IBD in remission vs. active disease ($p > 0.1$; data not shown). However, when MTX-Glu$_n$ concentrations were compared only in children with Crohn's disease ($n = 10$ remission vs. $n = 9$ active), an increase greater than two-fold in short-chain MTX-Glu$_{1-2}$ concentrations (nmol/L) was observed in remission (27.77 (16.10, 35.90)) vs. active Crohn's disease (10.90 (2.60, 25.20)), approaching statistical significance ($p = 0.066$; Figure 2). The two Crohn's disease study groups were receiving comparable dosing of both MTX (0.20 (0.17, 0.23) vs. 0.23 (0.15, 0.45) mg/kg weekly) and IFX (9.24 (7.45, 10.04) mg/kg every 4.0 (4.0, 6.0) weeks vs. 9.56 (8.49, 10.03) mg/kg every 5.0 (4.0, 6.0) weeks); $p \geq 0.554$ (Table 2). Median and IQR IFX troughs (μg/mL) were within therapeutic range (i.e., $\geq$3–7 μg/mL [9]) in both groups, and not statistically different between groups (25.28 (5.35, 38.96) vs. 15.54 (7.11, 24.15)); $p = 0.540$ (Table 2). However, when IFX was included as a covariate in a logistic regression model, the differences in short-chain MTX-Glu$_n$ between remission and active Crohn's disease became less apparent ($p = 0.130$).
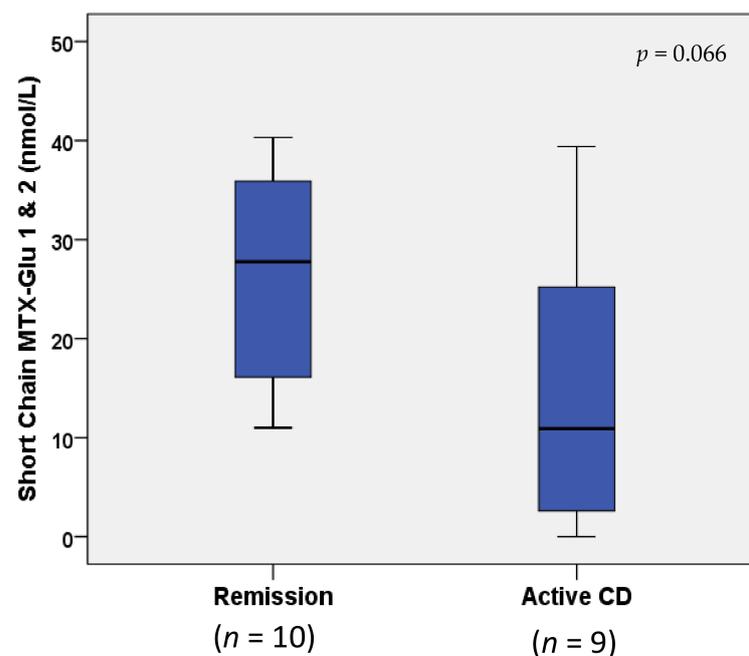

Figure 2. Box and whisker plot comparison of erythrocyte MTX-Glu$_{1-2}$ concentrations in remission vs. active Crohn's disease. Horizontal line in boxes represents median value, with whiskers representing the range.

Table 2. Patient characteristics for children with Crohn's disease: IFX: infliximab, MTX: methotrexate, $_{adj}$IFX: dose or concentration adjusted for mg/kg IFX received and time since drug administration. All patients received weekly MTX.

	Remission (*n* = 10)	Active (*n* = 9)	
	Median [IQR]	**Median [IQR]**	***p*-Value**
Age (years)	16.0 [13.0, 17.0]	17.0 [11.0, 19.0]	0.902
Weight (kg)	54.6 [50.2, 75.1]	52.3 [39.9, 80.1]	0.653
Hemoglobin (g/dL)	13.0 [12.1, 13.8]	12.2 [11.3, 13.8]	0.438
White Blood Cell Count ($\times 10^3$/mcL)	5.8 [5.0, 6.7]	7.4 [5.0, 10.1]	0.347
Platelet Count ($\times 10^3$/mcL)	264 [237, 299]	325 [243, 394]	0.713
ESR (mm/h)	8.0 [6.0, 10.0]	41.0 [18.0, 42.0]	0.006
CRP (mg/dL)	0.5 [0.5, 0.8]	1.4 [0.8, 2.2]	0.028
Albumin (g/dL)	4.2 [3.6, 4.3]	4.0 [3.8, 4.1]	0.657
IBD duration (years)	3.05 [1.93, 5.21]	4.18 [1.14, 4.53]	0.775
IFX trough (µg/mL)	25.28 [5.35, 38.96]	15.54 [7.11, 24.15]	0.540
IFX dose (mg)	500 [500, 700]	500 [400, 600]	0.738
IFX dose (mg/kg)	9.24 [7.45, 10.04]	9.56 [8.49, 10.03]	0.838
IFX interval (weeks)	4.0 [4.0, 6.0]	5.0 [4.0, 6.0]	0.554
Days since IFX dose (days)	32.0 [28.0, 42.0]	35.0 [28.0, 42.0]	0.967
$_{adj}$IFX dose (mg/kg/day)	0.30 [0.18, 0.33]	0.24 [0.22, 0.36]	0.967
$_{adj}$IFX Trough (µg/mL per mg/kg/day IFX)	69.06 [36.56, 127.63]	55.03 [36.04, 66.78]	0.653
Weekly MTX dose (mg)	12.50 [10.00, 17.50]	10.00 [10.00, 17.50]	0.701
Weekly MTX dose (mg/kg)	0.20 [0.17, 0.23]	0.23 [0.15, 0.45]	0.653

Cut-off values for MTX-Glu$_n$ concentrations previously associated with treatment response in rheumatology [5] were probed, and none were found to discriminate remission from active disease in IBD or in the IBD subset with Crohn's disease only (data not shown).

3. Discussion

To the best of our knowledge, this is the first study of MTX-Glu$_n$ levels in pediatric IBD and it offers valuable insights for future investigations. First, our data demonstrate that long-chain MTX-Glu$_n$ levels correlate with methotrexate dose and could, therefore, serve as

markers of systemic MTX exposure, offering a therapeutic drug monitoring option for MTX. Second, in children with Crohn's disease, short-chain $MTX-Glu_n$ levels were over two-fold higher in patients with disease in remission, compared to active disease, suggesting that short-chain MTX-Glu may have a role in Crohn's disease treatment responsiveness to MTX.

Although our observation of higher short-chain MTX-Glu levels in CD in remission compared to active CD did not reach statistical significance ($p = 0.066$), this is likely due to limitations in the sample size and further obscured by participants receiving concomitant IFX therapy, as suggested by our multivariable regression analysis. Nevertheless, the observed trend in short-chain MTX-Glu suggests that efficient intracellular drug uptake and/or primary intracellular conversion of $MTX-Glu_1$ to short-chain $MTX-Glu_2$ may be associated with the treatment responsiveness of pediatric Crohn's disease to MTX. An alternative explanation for this observation is that primary polyglutamation may be impaired in active disease, perhaps through altered intracellular drug uptake or inflammatory cytokine-mediated modulation of enzymes in the polyglutamation pathway (e.g., folypolyglutamate synthase or gamma glutamyl hydrolase, responsible for glutamation and deglutamation, respectively) [10]. Future studies should be aimed at understanding such sources and implications of inter-individual variability in polyglutamation for the treatment of pediatric IBD, where equipoise remains regarding up-front identification of patients who benefit most from MTX therapy (alone or in combination with biologics).

Ours is the first study of $MTX-Glu_n$ in pediatric IBD and our findings regarding long-chain $MTX-Glu_n$ echo findings in juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA)—two chronic, autoimmune, inflammatory conditions also treated with MTX. Becker et al. previously demonstrated increased $MTX-Glu_{3-5}$ formation as a function of MTX dose, route of administration, and duration of drug exposure in patients with JIA. Specifically, MTX dose escalation resulted in a preferential increase in long-chain $MTX-Glu_n$, at the expense of short-chain $MTX-Glu_n$ [11]. A study in RA also demonstrated a positive association of MTX dose with long-chain $MTX-Glu_3$, $MTX-Glu_4$, $MTX-Glu_5$, and $MTX-Glu_{3-5}$, while observing a weaker association between dose and total $MTX-Glu_{1-5}$ [12], analogous to the observations in our study.

Previous studies in rheumatology have also commented on the relationship between $MTX-Glu_n$ and treatment response [3,5–7]. No statistically significant differences in individual or total $MTX-Glu_n$ concentrations were observed in children with IBD in remission vs. active disease in our study; however, MTX therapy is felt to be less effective for ulcerative colitis than Crohn's disease [13,14]. Looking only at children with Crohn's disease ($n = 19$), a positive trend toward higher short-chain $MTX-Glu_n$ concentrations was observed in children with remission vs. active disease, approaching statistical significance ($p = 0.066$). The two Crohn's disease study groups were otherwise comparable for age and disease duration, without statistically significant differences in IFX troughs between groups. Although a trend toward greater IFX troughs (both absolute and adjusted for clinical variability in IFX dose and interval) was observed in children with remission vs. active Crohn's disease, this trend did not approach statistical significance ($p = 0.549$), and mean and median IFX troughs for both groups fell well within the therapeutic range (i.e., ≥3–7 µg/mL [9]); see Table 2. Nevertheless, we acknowledge that concomitant IFX therapy may be a confounding variable in our MTX-Glu analyses, as supported by observations of a less obvious difference in short-chain $MTX-Glu_n$ between Crohn's disease in remission and active disease after incorporating IFX into a multivariable logistic regression model. While some previous studies of IFX and MTX have shown a questionable benefit of combination therapy for Crohn's disease [15], our findings raise the possibility that the benefit of combination therapy may be related to the intracellular $MTX-Glu_n$ concentration achieved, emphasizing the need for further investigation of $MTX-Glu_n$ monitoring in IBD.

Although published rheumatology data to date are felt insufficient to recommend the definite implementation of $MTX-Glu_n$ therapeutic drug monitoring in clinical practice [16], a systematic review of the rheumatology literature from 2015 provides supportive evidence for the role of $MTX-Glu_n$ as biomarkers of autoimmune disease response to MTX treat-

ment [7]. In a study of MTX-naïve patients with RA, short-chain MTX-Glu$_2$ correlated with an improvement in clinical assessments over 16 weeks of MTX therapy. [3] In another study of RA, de Rotte et al. proposed concentrations of MTX-Glu$_2$ > 22 nmol/L and MTX-Glu$_{Total}$ > 74 nmol/L as predictors of moderate/good clinical response to MTX. [5] Cut-off values to discriminate MTX responders from non-responders could not be established in our study, but this is likely secondary to limitations in sample size.

Previous studies of MTX-Glu$_n$ in IBD are sparse and limited to adult observational studies smaller than ours, which may explain why prior studies also failed to demonstrate a statistically significant association between MTX-Glu$_n$ and IBD treatment response. [17,18]. In a cross-sectional study of 12 adults, Fischer et al. observed higher long-chain MTX-Glu$_{3–5}$ in patients with Crohn's disease in remission vs. active disease, but the trend did not reach statistical significance. [17] Brooks et al. found long-chain MTX-Glu$_{4–5}$ to correlate inversely with MTX efficacy in 18 adults with Crohn's disease, with higher levels noted in patients experiencing gastrointestinal adverse effects [18]. However, findings of an inverse relationship between MTX-Glu$_n$ and MTX efficacy are not substantiated in most rheumatology literature [5–7], except for one study that found a positive correlation for MTX-Glu$_n$ with MTX dose, but a negative correlation with treatment outcomes in RA [19].

Studies of MTX-Glu$_n$ in children are rare and, to the best of our knowledge, ours is the first pediatric study in IBD. In a pediatric study of JIA, higher concentrations of long-chain MTX-Glu$_n$ (MTX-Glu$_{3–5}$) were associated with lower disease activity at 3 months and 1 year of MTX therapy. [6] The cross-sectional nature of our pediatric study does not allow for a longitudinal assessment. However, despite the significant positive correlation of long-chain MTX-Glu$_n$ with MTX dose in our study (ϱ = 0.56; p = 0.01), we did not observe a relationship with disease response, suggesting that the relationship between long-chain MTX-Glu$_n$ and treatment response may be disease-specific and could differ between JIA and pediatric IBD.

Although this is the largest study of MTX-Glu$_n$ in IBD to date, the limitations of our study reside with its small sample size and cross-sectional design. The present study design does not allow us to comment on the relationship between maintenance MTX-Glu$_n$ concentrations and long-term disease outcomes in IBD. The decision to study patients receiving combination therapy with MTX and IFX may be viewed as another study limitation; however, at our institution, MTX is most often used in combination with biologics, rather than as monotherapy, for the treatment of IBD. Thus, the selected study population is more generalizable to the patient population at large, which is important for translating study results into clinical practice. To account for potential confounders from concomitant IFX treatment, IFX troughs were measured at the time of MTX-Glu$_n$ assessment, using a single CLIA-approved assay, and incorporated into our statistical analyses. Future investigations should be aimed at longitudinal, combination, and monotherapy MTX studies, to build on our observations of the potential role of long-chain MTX-Glu$_n$ in the therapeutic drug monitoring of systemic exposure to MTX, and short-chain MTX-Glu$_n$ as potential biomarkers of IBD responsiveness to MTX treatment. Prospective, multicenter, dose-controlled trials of MTX could help to identify therapeutic target ranges for MTX-Glu$_n$ in IBD.

4. Materials and Methods

Patients: Children enrolled in a cross-sectional study of IFX monotherapy vs. combination IFX therapy with concurrent immunomodulators, and receiving medication doses in accordance with prescriber preference for clinical care of IBD [20], were included in this secondary analysis. All participants were enrolled at a single outpatient infusion center at the Children's Mercy Hospital (Kansas City, MO, USA). Only patients receiving therapy with both IFX (Remicade[®]) and MTX were included in this secondary analysis (Figure 3), with maintenance dosing defined as no changes in dose or interval of either drug for at least two IFX infusion cycles.
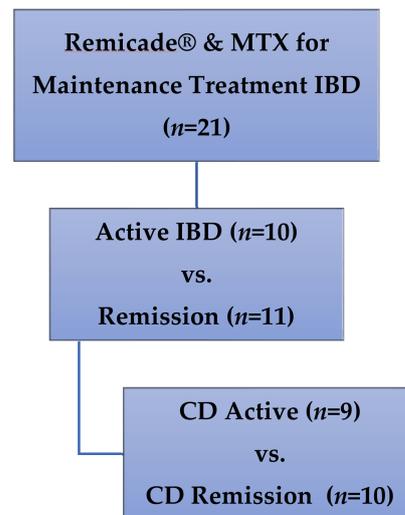
Figure 3. Patients included in this secondary analysis of methotrexate polyglutamates in pediatric IBD. MTX: methotrexate, IBD: inflammatory bowel disease, CD: Crohn's disease.

Clinical parameters: Clinical parameters were assessed using Physician Global Assessment (PGA) scores via agreement by two independent pediatric gastroenterologists. Due to limitations in sample size, PGA mild, moderate, and severe were grouped together as active disease (active) and compared to quiescent disease (remission).

Analytical techniques and validation: MTX-Glu$_{1-6}$ concentrations were measured in erythrocytes, using high-performance liquid chromatography/tandem mass spectrometry utilizing a previously established assay [20]. IFX troughs and anti-IFX antibodies (anti-IFX) were measured using a CLIA-approved NF-kB luciferase gene-reporter assay, with lower and upper limits of quantification at 0.65 and 40 μg/mL, respectively (ARUP Laboratories, Salt Lake City, Utah, UT, USA).

Statistical analysis: Nonparametric tests (e.g., Wilcoxon Rank Sum and Fisher's Exact tests) were used to compare MTX-Glu, IFX, anti-IFX, laboratory, and demographic data in children with remission vs. active disease. Spearman's correlation (ϱ) was used to look for relationships between continuous variables, and multivariable logistic regression models were used to look for differences between study groups, while controlling for covariates. Previously reported cut-off values in rheumatology of MTX-Glu$_{Total}$ > 74 nmol/L and MTX-Glu$_2$ > 22 nmol/L were probed to discriminate remission from active disease in IBD [5]. A significance level of 0.05 in SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA), was used for all analyses. Unless otherwise specified, data are reported as median (IQR).

Author Contributions: R.M.: Performed data collection, and data analysis and interpretation. Reviewed current literature in both rheumatology and gastroenterology with regard to use of methotrexate polyglutamates for clinical purposes. Wrote the manuscript and participated in manuscript edits and revisions. R.F.: Participated in study design and provided expertise on methotrexate polyglutamates and IFX analytics. Participated in data analysis and interpretation. Revised and edited manuscript. M.B.: Provided expertise on methotrexate polyglutamates and IFX analytics. Participated in data analysis and interpretation. Revised and edited manuscript. A.S.: Provided expertise in statistical methods and analyses. Participated in data analysis and interpretation. Provided revisions and edits for manuscript. L.V.H.: Provided expertise on analytical techniques of measuring methotrexate polyglutamates in erythrocytes with HPLC/MS. Participated in data analysis and interpretation. Provided revisions and edits for manuscript. T.H.: Independently reviewed clinical data, assigned Paris classifications, and PGA scores. Provided revisions and edits for manuscript. R.C.: Participated in study design, data collection, and data analysis and interpretation. Provided revisions and edits for manuscript. V.S.: Participated in study design, data collection, and data analysis and interpretation. Provided expertise with regard to pharmacotherapy in patients with IBD. Participated in and provided oversight for manuscript preparation. Provided revisions and edits for the manuscript. All authors have read and agreed to the published version of the manuscript.

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