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ORIGINAL ARTICLE

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Methotrexate accumulation in target intestinal mucosa and white blood cells differs from non-target red blood cells of patients with Crohn's disease

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Abstract

Background: Intracellular methotrexate polyglutamates (MTX-PGs) concentrations are measurable in red blood cells (RBCs) during MTX treatment. MTX-PG_3 concentrations correlate with efficacy in patients with Crohn's disease (CD). Since RBCs are not involved in pathogenesis of CD and lack extended MTX metabolism, we determined MTX-PGs accumulation in peripheral blood mononuclear cells (PBMCs: effector cells) and intestinal mucosa (target cells) and compared those with RBCs as a potential more precise biomarker.

Methods: In a multicentre prospective cohort study, blood samples of patients with CD were collected during the first year of MTX therapy. Mucosal biopsies were obtained from non-inflamed rectum and/or inflamed intestine. MTX-PGs concentrations in mucosa, PBMCs and RBCs were measured by liquid chromatography–tandem mass spectrometry.

Results: From 80 patients with CD, a total of 27 mucosal biopsies, 9 PBMC and 212 RBC samples were collected. From 12 weeks of MTX therapy onwards, MTX-PG_3 was the most predominant species (33%) in RBCs. In PBMCs, the distribution

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was skewed towards MTX-PG_1 (48%), which accounted for an 18 times higher concentration than in RBCs. Long-chain MTX-PGs were highly present in mucosa: 21% of MTX-PG_{total} was MTX-PG_5. MTX-PG_6 was measurable in all biopsies.

Conclusions: MTX-PG patterns differ between mucosa, PBMCs and RBCs of patients with CD.

KEYWORDS
Crohn's disease, methotrexate, pharmacokinetics, therapeutic drug monitoring

1 | INTRODUCTION

Methotrexate (MTX) is an anti-folate drug, which at low dosages (≤25 mg/week) elicits anti-inflammatory effects against immune-mediated inflammatory diseases (IMIDs), including rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA) and Crohn's disease (CD).[1–3] Patients with CD present with abdominal pain and diarrhoea due to segmental transmural inflammation of the gastrointestinal tract.[4] MTX is prescribed in patients with mild CD either as steroid sparing monotherapy or as co-medication to infliximab.[2] MTX monotherapy reduces and/or prevents disease activity in approximately 65% of patients; however, >40% of patients experience side effects, which limits its durable use as merely 26% of patients continue MTX after one year of treatment.[5] Therapeutic drug monitoring (TDM) has been proposed to guide individualized therapy with the aim to improve MTX efficacy and reduce toxicity.[6] Since stable plasma concentrations are not reached in low-dose MTX treatment, MTX-polyglutamates (MTX-PGs) are generally measured in red blood cells (RBCs) for TDM purposes, especially in patients with RA.[7–10] These studies revealed that higher RBC MTX-PG concentrations were associated with a lower disease activity.[10] Recently, we demonstrated that RBC MTX-PG_3 accumulation may serve as a potential tool for TDM in patients with CD as well, since RBC MTX-PG_3 concentration correlated with therapeutic efficacy (lower biochemical disease activity) and a higher MTX drug-survival rate.[5]

The PG-moieties are formed intracellularly. Within 24 h after subcutaneous (SC) administration cellular uptake of the circulating monoglutamate MTX form (MTX-PG_1) via the reduced folate carrier (RFC) has occurred.[11,12] Within the cell, the enzyme folylpolyglutamate synthetase (FPGS) catalyses the addition of glutamate moieties (up to $n = 6$) to MTX, resulting in MTX-PG_n (Figure 1).[13] MTX-$PG_{3–6}$ and in lesser extent MTX-PG_2 are no longer substrates for selected members of the ATP-Binding Cassette (ABC) drug efflux transporters, resulting in intracellular retention of MTX. MTX-PGs are the pharmacologically active metabolites and exert potent inhibition of target enzymes in folate metabolism, resulting in its therapeutic effect.[13,14] MTX-PGs can be deconjugated by γ-glutamyl hydrolase (GGH), localized in lysosomes.[15] The chain length of the MTX-PGs within the cell is determined by the balance of import, export, polyglutamylation, deconjugation, cellular folate status and the lifespan of the cell.[16]

Over the past decades, MTX-PGs have been measured in RBCs for reasons of convenience: RBCs are abundantly available, easily accessible, as well as routinely manageable within laboratory settings. From a TDM perspective,

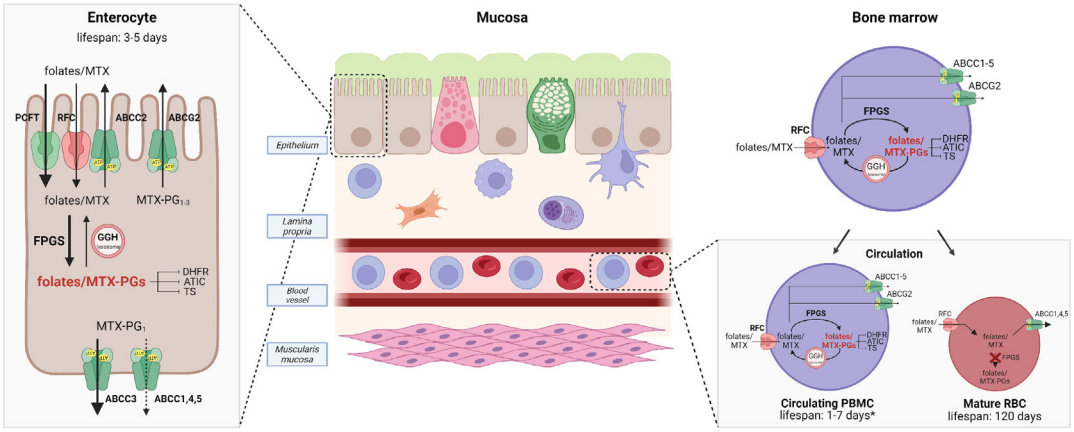

FIGURE 1 Schematic illustration of intracellular methotrexate metabolism in enterocytes, PBMCs and RBCs. **Middle**: intestinal mucosal biopsy consisting of the following layers and cells: (1) *epithelium*, a single cell layer of enterocytes (dominant), goblet cells, Paneth cells and entero-endocrine cells (last one not shown). (2) *Lamina propria*, containing blood capillaries, lymph vessels, inflammatory cells and mesenchymal cells. (3) *Muscularis mucosa*, containing smooth muscle fibres. **Left:** enterocyte in detail. Intracellular absorption of MTX can proceed via two folate transporters: PCFT and RFC. PCFT has a low pH optimum (i.e. pH 5.5) and mediates uptake after oral administration of MTX. RFC, with a pH optimum of pH 7.4, is primarily involved in MTX uptake after subcutaneous administration and to a lesser extent after oral MTX administration. Enhanced *FPGS* enzymatic activity in enterocytes facilitates addition of multiple glutamate groups to the MTX (i.e. MTX-polyglutamates, MTX-PGs) or folate molecule to enhance their intracellular retention. The enzyme *GGH* deconjugates polyglutamates from MTX or folates. Efflux of MTX-PG_{1-3} and MTX-PG_1 at the apical membrane is facilitated by the ABCG2 and ABCC2 drug efflux transporter respectively. Efflux of MTX-PG_1 at the basolateral membrane is mediated by ABCC3 drug efflux transporter predominantly and by ABCC1, ABCC4, and ABCC5 in lesser extent. The thickness of the arrow indicates preferred transporter. **Right:** circulating PBMCs, mature RBCs and their precursor cells. Intracellular absorption of MTX is mediated by RFC. Efflux of MTX-PG_1 (and in lesser extent MTX-PG_2) can proceed via ABCC1–5 drug efflux transporters and efflux of MTX-PG_{1-3} by ABCG2 (the latter being absent in RBCs). Mature RBCs lack FPGS and GGH activity in contrast to PBMCs and precursor cells. *: PBMCs represent a collection of T-cells, B-cells, NK cells, and monocytes, each with a different life-span. Abbreviations: PCFT: proton-coupled folate transporter; RFC: reduced folate carrier; ABC: ATP binding cassette (transporter family); FPGS: Folylpolyglutamate Synthetase; GGH: gamma-glutamyl hydrolase; MTX-PG: methotrexate Polyglutamate; RBC: red blood cell; PBMC: peripheral blood mononuclear cell. Target enzymes: DHFR: Dihydrofolate reductase; ATIC: 5-Aminoimidazole-4-Carboxamide ribonucleotide Formyltransferase; TS: thymidylate synthase. Figure created with BioRender.com

it is important to consider that RBC MTX-PGs originate from MTX polyglutamylation in bone marrow myeloid precursor cells, which harbour proficient FPGS activity.[17] During erythroid cell differentiation, FPGS activity is rapidly lost, with some residual activity in reticulocytes but no detectable activity in mature RBCs (Figure 1).[17] The MTX-PGs observed in RBCs are theorized to be a reflection and retention of MTX-PGs formed during the precursor stages.[18] Hence, MTX-PGs formed in the early stage of erythrocyte cell differentiation are retained during their entire 120 days lifespan. Another consideration is that RBCs are neither immune effector cells in the pathophysiology of CD nor are they the primary target in the treatment of patients with CD on MTX. Accordingly, information regarding the dynamics and variability of MTX-PG accumulation in immune effector cells (e.g. peripheral blood mononuclear cells, PBMCs) or in the primary target (intestinal mucosa) would be of additional pharmacological relevance.

In this explorative study, we aimed to define possibly improved MTX-TDM options for patients with CD. We therefore investigated MTX-PG concentrations and distribution profiles in mucosal tissue, PBMCs compared with RBC of patients with CD during the first year of MTX therapy. Secondly, we assessed the influence of MTX dose and treatment duration on MTX-PG accumulation.

2 | MATERIALS AND METHODS

2.1 | Study design and patients

Patients included in this study were enrolled in the prospective longitudinal 'MTX-PG//CD cohort' study, which has been described in detail before.[5] In short, 80 adults with CD starting SC MTX treatment in routine care, without concomitant biological therapy, were included in 10 medical centres (of which two university hospitals and eight general hospitals) in The Netherlands. All patients, except for two patients who deviated from the study protocol, started with a dose of 25 mg MTX SC once a week with the option to taper after eight weeks to 15 mg SC

once a week. All patients received folic acid supplementation (5 mg once a week, 48 h after MTX administration, orally). Patients were followed for 12 months or upon the endpoint (i.e. MTX discontinuation, switch to oral MTX or initiation of concomitant step-up therapy which was defined as addition of systemic corticosteroids, start of a biologic drug and/or CD-related surgery) was reached.

The study has been approved by the medical ethics committees of the participating hospitals. The study was conducted in accordance with good clinical practice guidelines and the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[19] All patients gave their informed consent before inclusion.

2.2 | Data and sample collection

Data collection and blood withdrawal was scheduled prior to the start of MTX (baseline), at weeks 8, 12, 24, 52 and, if applicable, when the endpoint was reached. Clinical data included drug adherence (patients' self-reported by use of a Visual Analogue Scale [VAS])[20] and MTX dose. For mucosal biopsies, the biopsy site(s) and local inflammation status were recorded.

RBCs and whole blood (WB) samples were collected in 4 ml EDTA tubes from all study participants. Packed RBCs were obtained from EDTA WB tubes by centrifugation at 1700 $\times g$ for 10 min at 4°C and the cell pellet fraction (500 µl) was stored at −80°C until analysis.

PBMCs were collected from study participants at one participating centre in 10 ml lithium heparin whole blood tubes. Isolation of PBMCs was performed by Ficoll gradient centrifugation. The isolated PBMCs were subsequently washed with sterile Phosphate Buffered Saline +1% Fetal Calf Serum (at room temperature).[9] PBMCs were counted, aliquoted, snap-frozen and stored as dry pellets at −80°C until analysis.

Mucosal biopsies were obtained by two options: 1) During an ileocolonoscopy that was indicated (colorectal surveillance, disease activity) and scheduled by the treating physician. Biopsies were taken from the rectum and from an inflamed site (if present); 2) By rectoscopy, performed in six participating centres from week 24, after the patient gave additional informed consent. Rectoscopies were performed without bowel preparation with the only aim to take study biopsies from the rectal mucosa. Biopsies were snap-frozen in liquid nitrogen and stored at −80°C.

2.3 | MTX-PG measurements

RBC MTX-PGs were analysed from the cell pellet samples with an ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometer (UPLC-ESI-MS/MS; Triple-Quadrupole TSQ Quantiva, Thermo Scientific) using stable-isotope-labelled internal standards MTX-PG_{1-6}, as described by Den Boer et al.[21] Under circumstances that RBC cell-pellet samples were unavailable, MTX-PGs were instead measured in the WB sample and their concentrations were corrected for haematocrit. RBC and haematocrit-corrected WB MTX-PG concentrations were analysed together and are reported either in nmol/L packed RBCs or as fmol/1 $\times$ 10^6 cells for direct comparison with PBMCs.

PBMC MTX-PG_{1-4} were measured by UPLC-ESI-MS/MS, as described by Hebing et al.,[9] MTX-$PG_{5,6}$ could not be assessed for this matrix, likely due to interference of PBMC matrix components such as phospholipids and the low concentrations of MTX-$PG_{5,6}$.[9] Individual MTX-PG accumulation in PBMCs is expressed as fmol/1 $\times$ 10^6 cells.

Mucosal biopsies were first weighed, followed by the addition of 300 µl cold demineralized water (dH_2O) and homogenized in the Fisherbrand bead mill 24 (2 $\times$ 15 s at 6 m/s with a 30 s interval at room temperature). The homogenates were centrifuged for 10 min at 14 000 $\times g$ at 4°C. Afterwards, 20 µl supernatant was pipetted in a 1.5 ml microtube, followed by the addition of 20 µl of 6 nmol/l stable-isotope labelled MTX-PG_{1-6} internal standard mixture and 32 µl 16% perchloric acid. Upon 30 min incubation on ice, another centrifugation step (15 min at 14 000 $\times g$, 4°C) was applied. The supernatant was transferred to a 0.22 µm spin column and centrifuged for 15 min at 21 000 $\times g$ (4°C). MTX-PGs in the resulting eluate were measured with a UPLC-ESI-MS/MS, based on a previously described method by Hebing et al.[9] Protein concentrations were measured in the homogenate obtained by the bead mill procedure using the Bradford assay according to the manufacturer's manual (Bio-Rad Laboratories, Veenendaal, The Netherlands). Quantities of MTX-PGs in mucosal tissue were expressed as fmol/mg protein.

MTX-PG_{total} represents the sum of all individual MTX-PGs measured in one sample.

2.4 | Statistical analysis

Descriptive statistics for demographic data were reported using means (± standard deviation [SD]), medians (with IQR: first quartile–third quartile) or percentages and analysed with the Mann–Whitney-U or t-test, depending on the (non-) parametric distribution. A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed using R (R foundation for statistical computing. Vienna, Austria. Version 4.2.1).

A distinction was made between samples of patients using MTX ('on' MTX therapy) versus those samples

collected from patients who had discontinued MTX for more than one week ('off' MTX therapy). All analyses were performed on samples of patients on MTX therapy, with the exception of analyses on the effect of MTX discontinuation, which were performed on patients off MTX therapy.

As this was a real world study, clinical and biochemical data was not always collected at the exact pre-defined time point. Therefore, time periods used for analyses were defined as the median week of actual data collection along with the minimum and maximum week of data collection; e.g. week 8 (weeks 4–10), week 12 (10–14), week 25 (14–28), and week 51 (28–61). For analyses in an indicated time period, only one sample per patient per period was included.

MTX-PGs accumulation and distribution profiles in mucosal tissues and PBMCs of patients using MTX were compared with those of matched RBCs. Matching RBC samples were defined as the samples of the same patient with (for mucosa) the shortest time interval between biopsy retrieval and/or (for PBMCs) obtained during the same blood withdrawal. Variability of MTX-PG concentrations between patients was presented as the percentage coefficient of variation (CV [%]; i.e. ratio SD over the mean, multiplied by 100%).

Differences in MTX-PG accumulation over time on MTX therapy, off MTX therapy, in relation to MTX dose, biopsy site and inflammation at the biopsy site were assessed with a (multivariable) linear mixed model analysis and reported as regression coefficient (unstandardized β) with standard error (SE)).

3 | RESULTS

3.1 | Cohort

The MTX-PG//CD cohort consisted of 80 patients at baseline, of whom 73 patients continued until week

TABLE 1 Patients demographics and characteristics of patients matrices.

	Mucosa	PBMC	RBC
Number of all patients/samples	18/27	6/9	77/212
Number of patients/samples on MTX	16/24	5/6	74/193†
Moment of measurements, n^			
< week 10	2	2	67
Week 10–15	1	2	61
Week 15–28	9	2	61
> week 28	12	0	26
Age in years, mean ± SD	58 ± 11	54 ± 13	55 ± 13
Male gender, n (%)	5 (31)	1 (20)	28 (38)
BMI in kg/m^2, mean ± SD	24.5 ± 4.6	27.5 ± 4.7	26.1 ± 4.9
eGFR in ml/min/1.73m^2, mean ± SD	102 ± 13	97 ± 12	96 ± 14
Disease location, n (%)			
Ileum	6 (38)	2 (40	36 (49)
Colon	5 (31)	0 (0)	15 (20)
Ileum and colon	5 (31)	3 (60)	21 (28)
Upper gastrointestinal involvement	0(0)	0	2 (3)
Disease behaviour, n (%)			
Inflammatory	10 (63)	2 (40)	50 (68)
Stricturing	6 (38)	3 (60)	24 (32)
Penetrating	1 (6)	0 (0)	3 (4)
MTX dose, n^ (%)			
25 mg/week	2 (8)	1 (17)	81 (42)
15 mg/week	22 (92)	5 (83)	112 (58)

Note: Characteristics of patients *on* MTX only. All patients with mucosa and PBMC samples had concomitant RBC samples. Only one patient had a RBC, PBMC and mucosa sample.

† = 13 samples (6.7%) were haematocrit-corrected WB measurements.

^ = number of samples (instead of number of patients).

8, 64 patients until week 12 and eventually 21 patients after the last visit (median week 51). A total of 213 RBC, 11 PBMC and 28 mucosal samples were collected during follow-up. Two PBMCs samples were excluded because of technical shortcomings in sample storage. One mucosal and one WB outlier with disproportionally high MTX-PG concentrations, probably due to pre-analytical technical issues, were excluded as well. Patients' demographic characteristics and sample sizes of mucosal tissue, PBMCs and RBCs of patients on MTX therapy are shown in Table 1.

During follow-up, MTX dose in 56 patients were tapered from 25 mg/week to 15 mg/week and one patient switched back from 15 mg/week to 25 mg/week (at week 26). A 100% adherence to MTX was recorded in at least 75% of the patients, whereas poorer adherence (VAS < 80%) was noted in one to three patients during each measurement.

Mucosal biopsies were taken at ileum (n = 5; of which one of a patient who discontinued MTX), colon (n = 6, all on MTX) and rectum (n = 16; of which two of patients who discontinued MTX). Upon endoscopic examination, all biopsies taken from the ileum were from inflamed tissue, whereas all biopsies taken from the rectum were from non-inflamed tissue. Colon biopsies consisted of four inflamed and two non-inflamed samples. The median duration of MTX usage during the collection of the biopsies was 28 weeks (min 5 – max 54 weeks). Aside from two patients using 25 mg/week, of whom one discontinued MTX before endoscopy, all other patients were on 15 mg MTX/week.

3.2 | MTX-PG concentrations and distribution profiles

MTX-PG profile in RBCs of patients with CD on MTX therapy revealed the characteristic distribution: at week 12, RBC MTX-PG_3 was the predominant polyglutamate (33% of MTX-PG_{total}), followed by MTX-PG_1 (26%), MTX-PG_4 (20%), MTX-PG_2 (13%) and finally the least prevalent MTX-PG_5 (9%) (Figure 2, Supplementary Table 1). The accumulation of RBC MTX-PGs during follow-up showed disparate patterns. Notably, RBC MTX-PG_3 concentrations remained stable after 12 weeks, whereas the concentration of MTX-$PG_{4,5}$ decreased gradually over time, which coincided with higher MTX-PG_2 concentrations (MTX-PG_4: $\beta = -5.7$, SE = 2.7, $p = 0.04$; MTX-PG_5:

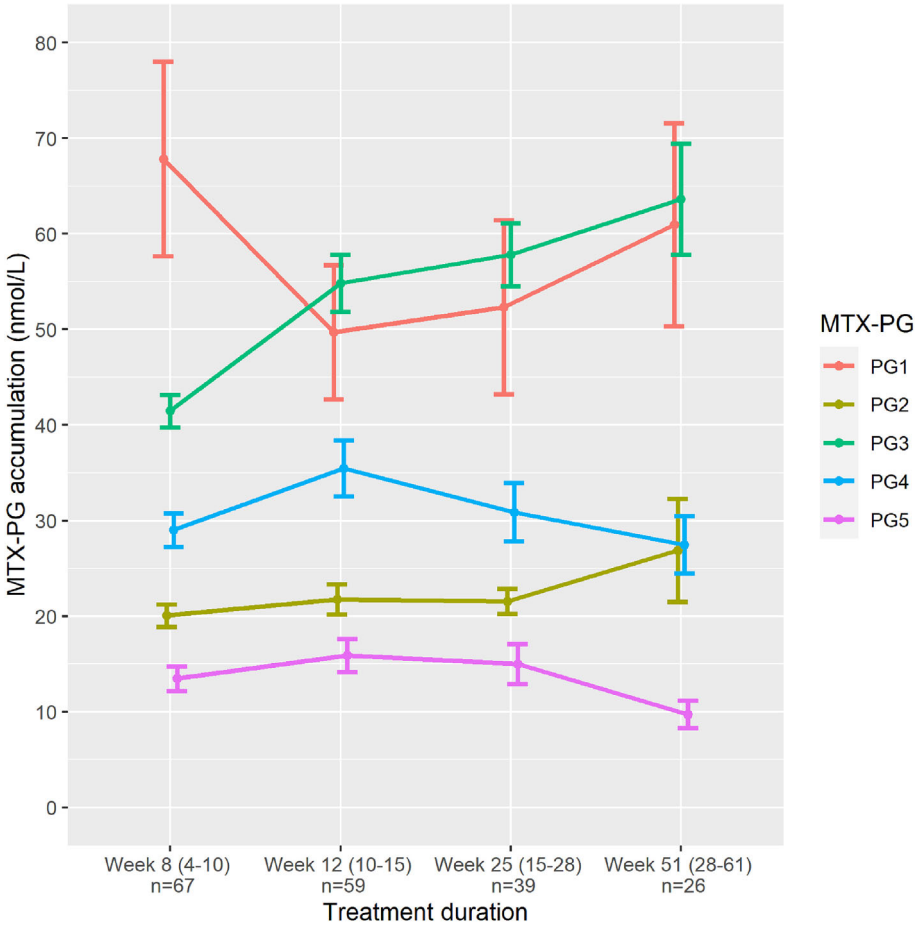

FIGURE 2 Longitudinal effect of MTX-PG distribution in RBCs. Data are presented as mean ± standard deviation.

$\beta = -5.6$, SE = 1.4, $p < 0.01$; MTX-PG_2: $\beta = 4.5$, SE = 1.8, $p = 0.01$).

Analysis of MTX-PG accumulation and distribution in PBMCs revealed MTX-PG_1 as the most abundant species (48%), followed by the other species in decreasing concentrations (Figure 3, Supplementary Table 1). In a direct comparison of MTX-PG accumulation expressed as fmol/10^6 cells, PBMCs accumulated markedly higher concentrations than matched RBCs; 18-fold for MTX-PG_1, 12-fold for MTX-PG_2, 3-fold for MTX-PG_3 and 1.5-fold for MTX-PG_4 (Figure 4A).

MTX-$PG_{1–6}$ concentrations were measurable in all intestinal mucosal biopsies: there was marked accumulation with interpatient variability (Figure 4B, Supplementary Table 1). No correlation was observed between treatment duration and the concentrations of mucosal MTX-PGs ($p > 0.1$). In the mucosa, MTX-PG_1 was the predominant species (29%) (Figure 3) but long chain MTX-PGs (i.e. MTX-$PG_{4–6}$) were also highly prevalent (42%, versus 26% in RBCs and 9% in PBMCs). The proportion of MTX-PG_3 in the intestinal mucosa was smaller than in matched RBCs (PG_3: mucosa 18% vs. RBC 36%), whereas the proportions of MTX-PG_1 and MTX-PG_5 were higher (PG_1: mucosa 30% vs. RBC 23%, PG_5: mucosa 22% vs. RBC 9%) and of MTX-PG_2 and MTX-PG_4 similar (PG_2: mucosa 12% vs. RBC 13%, PG_4: mucosa 19% vs. RBC 18%).

3.3 | MTX-PG accumulation after MTX discontinuation

We next analysed a total of 19 RBC, three PBMC and three mucosal samples from patients with CD who discontinued their MTX treatment. Mucosal samples were retrieved two to three weeks, PBMCs three to four weeks and RBCs four weeks (range 2–10 weeks) after MTX discontinuation. Results presented in Table 2 showed that particularly MTX-PG_1 concentrations dropped significantly in all three matrices. In mucosa and RBCs, MTX-PG_2 was also poorly retained after MTX discontinuation, while long chain MTX-$PG_{4–5}$ (and MTX-PG_6 for mucosa as well) were largely retained. In PBMCs, however, MTX-PG concentrations of all species declined rapidly: short chain MTX-$PG_{1–3}$ declined by 93–98% and MTX-PG_4 by 75% in the first month.

3.4 | Determinants of MTX-PG concentrations

The effect of MTX dosing on MTX-PG accumulation in RBCs was examined for the 15 mg/week vs. 25 mg/week SC administrations. The latter dose resulted in a significantly higher accumulation of RBC MTX-PG_4 ($\beta_{25mg/week} = 6.9$, SE = 2.9, $p = 0.02$) and RBC MTX-PG_5 ($\beta_{25mg/}$

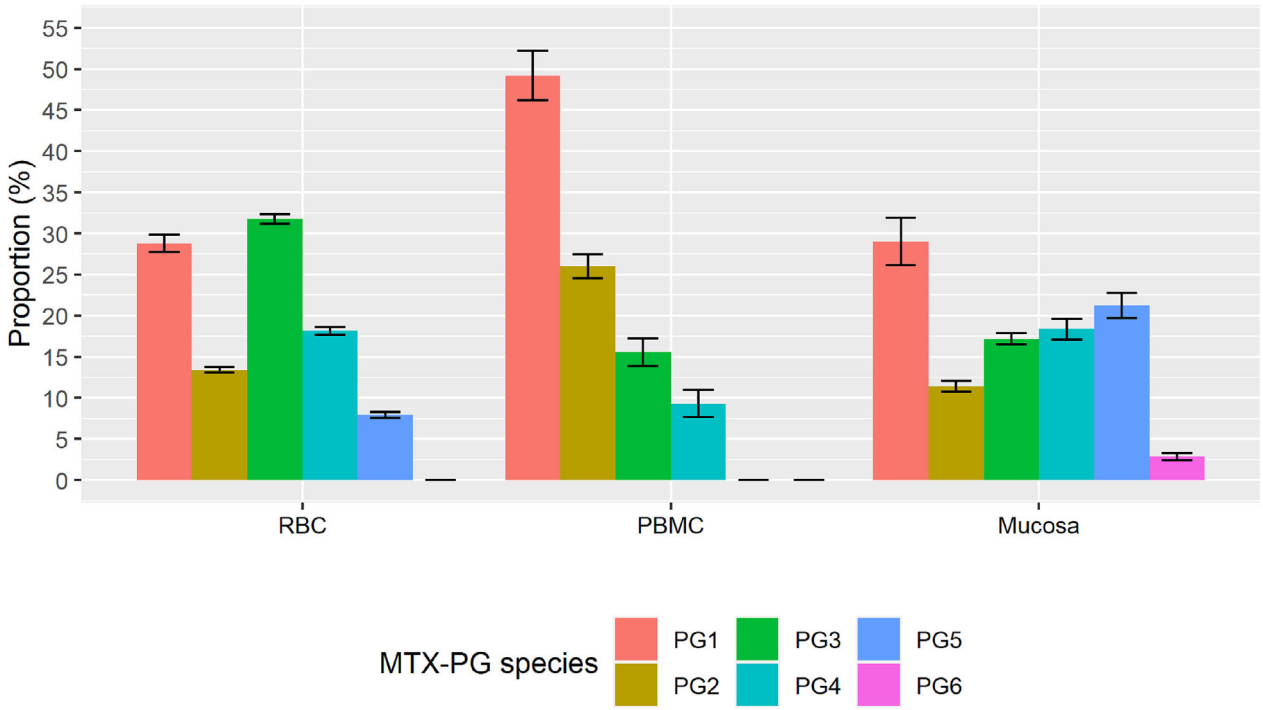

FIGURE 3 MTX-PG distribution (%) in RBCs, PBMCs and intestinal mucosa. All patients were on MTX. Results depict the mean ± standard deviation of 193 RBC, 6 PBMC and 24 mucosal samples. PBMC MTX-$PG_{5,6:}$ not measurable. RBC MTX-PG_6: not detectable.

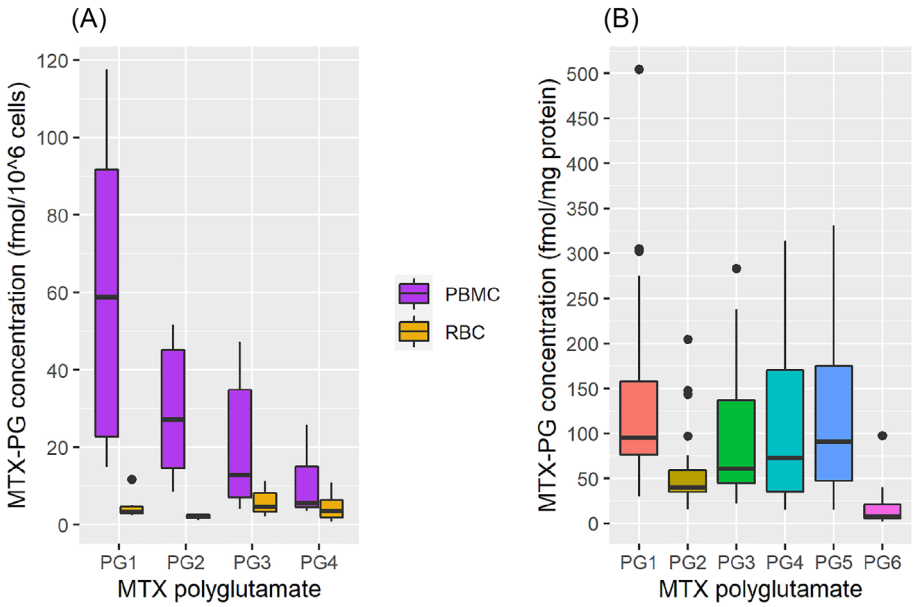

FIGURE 4 MTX-PG accumulation in (A) matched PBMCs and RBCs (B) intestinal mucosa. All patients were on MTX.

$_{week}$ = 4.3, SE = 1.5, p = 0.01) both after correction for the duration of MTX use. RBC MTX-PG_3 showed the same trend however not significant ($\beta_{25mg/wk}$ = 6.4, SE = 3.6, p = 0.07). Analysis of MTX dose in mucosal and PBMC samples was not feasible because of insufficient sample sizes.

Except for MTX-PG_1, no relation between location (biopsy sites) and mucosal MTX-PG accumulation was observed. Mucosal MTX-PG_1 was significantly higher in ileum (n = 4) compared with colon (n = 6; β = 117.7, SE = 47.8, p = 0.049) and rectum (n = 14; β = 106.9, SE = 40.8, p = 0.040). However, this effect was lost after correcting for the inflammation status at the biopsy site. Median MTX-PG_{2-6} concentrations in inflamed biopsies (n = 16) were higher, but not statistically significant, compared with non-inflamed biopsies (n = 8). Median MTX-PG_1 concentrations in inflamed biopsies were equal to median concentrations of non-inflamed biopsies (96 fmol/mg protein).

4 | DISCUSSION

We demonstrated marked differences in the MTX-PG_n distribution and accumulation profiles between intestinal mucosa, PBMCs and RBCs. MTX-PG accumulation was approximately 10 times higher in PBMCs than in RBCs. The accumulation of long-chain MTX-PGs was the highest in intestinal mucosa. After MTX discontinuation, a rapid decrease was observed of short-chain MTX-PGs in PBMCs. These results reveal the dynamics of MTX-PGs accumulation and varying retention in different cell types and tissues, which could be relevant for future MTX-TDM options for patients with CD.

Several studies in RA and JIA, and one preliminary study in CD patients, examined MTX-PG accumulation in RBCs of patients during MTX therapy, revealing dose-dependent kinetic profiles reaching a steady state after three months, with MTX-PG_3 as the dominant form.[7–9,22,23] The kinetics of MTX-PG accumulation in RBCs in our study was similar to our previous studies in RA.[8,9] Our recent cross-sectional cohort of patients with CD showed comparable RBC MTX-PG distribution profiles (with MTX-PG_3 as the dominant form), but lower MTX-PG_4 (not detectable in one patient) and MTX-PG_5 (not detectable in six patients) concentrations than in the current study.[24] The higher MTX-$PG_{4,5}$ concentration in the current cohort could be due to the SC route of administration and relatively high MTX dosage, compared with 37% oral users and a mean dose of 15.5 mg/week in the cross-sectional cohort. The current study corroborated these findings over a longer treatment duration. One remarkable observation was that after one year of treatment, RBCs had higher MTX-PG_2 and lower MTX-$PG_{4,5}$ concentrations compared with three months. It is conceivable that this could be due to down-regulation of FPGS activity during prolonged MTX therapy. Previous studies in RA have indicated a reduction in FPGS mRNA expression and catalytic activity in patients who were treated with MTX long-term.[14,25] This results in a relative increase in short-chain MTX-PGs due to lower conversion to longer chain MTX-PGs. Additionally, we observed a marked inter-patient variability (CV) in RBC MTX-PG accumulation, comparable with previously published variability in patients with RA.[8,9] This variability

TABLE 2 MTX-PG accumulation in intestinal mucosa, PBMCs and RBCs during MTX therapy and after MTX discontinuation.

	Mucosa (fmol/mg protein)			PBMC (fmol/10^6 cells)			RBC (nmol/L)		
MTX-PG	**On MTX (n=24)**	**Off MTX (n=3)**	***p***	**On MTX (n=6)**	**Off MTX (n=3)**	***p***	**On MTX (n=193)**	**Off MTX (n=19)**	***p***
PG 1	95.6 (76.2–157.8)	31.6 (24.2–33.4)	< 0.01	58.8 (22.8–91.7)	1.6 (1.3–1.7)	0.02	37.1 (26.4–54.8)	3.5 (1.9–7.3)	<0.01
PG 2	40.0 (35.0–58.5)	21.1 (20.9–25.0)	0.02	27.3 (14.6–45.1)	0.8 (0.8–0.9)	0.02	20.6 (15.2–25.5)	11.7 (8.0–14.5)	<0.01
PG 3	60.9 (44.4–136.8)	43.0 (38.2–79.5)	0.44	12.8 (7.1–34.9)	0.8 (0.5–1.0)	0.02	49.5 (34.9–62.4)	35.8 (22.2–49.9)	0.01
PG 4	75.2 (35.1–170.2)	67.0 (53.9–113.3)	0.97	5.7 (4.6–15.0)	1.4 (1.3–1.4)	0.02	27.4 (17.4–41.7)	21.3 (10.4–29.8)	0.04
PG 5	90.8 (47.1–175.3)	61.9 (58.5–106.5)	0.80	NM	NM		10.5 (6.2–17.4)	9.9 (4.0–15.8)	0.30
PG 6	8.2 (5.4–20.9)	8.1 (6.9–11.4)	1.00	NM	NM		ND	ND	

Note: Duration of MTX discontinuation ('off MTX'): 2–3 weeks for mucosa, 3–4 weeks for PBMCs, mean of 4 weeks for RBCs (min 1.5 – max 10 weeks).
The conversion of the unit of RBCs to (fmol/10^6) = (1 nmol/L) / 10.
Abbreviations: ND: not detectable. NM: not measurable.

underscores both the option to use these MTX-PGs for TDM as well as the need to identify determinants of MTX-PG accumulation.[26]

To our knowledge, we are the first to examine MTX-PG accumulation in PBMCs of patients with CD. Accumulation of total MTX-PGs in PBMCs was roughly 10-fold higher than RBCs (concentrations expressed as fmol/10^6 cells). This is explained by the fact that PBMCs are nucleated cells (in contrast to RBCs) with strict regulation of active folate transport and metabolism to fuel biosynthetic processes.[14,27,28] Nevertheless, the MTX-PG distribution profile in PBMCs of patients with CD largely consisted of short-chain MTX-PG. This profile and level of accumulation is identical to that of PBMCs of patients with RA.[9,29] PBMCs accumulated less long-chain MTX-PGs, which is likely attributable to their short lifespan in circulation (for monocytes 1–3 days)[30,31] compared with RBCs, which in turn may not provide enough time for the conversion of monoglutamate MTX to longer chain MTX-PGs. Another contributing factor for the low concentrations of long-chain MTX-PGs in PBMCs could, theoretically, be a high GGH enzymatic activity[32] (Figure 1), but this was not assessed in the present study. Lastly, unfavourable FPGS kinetics (high affinity for natural folates, low affinity for MTX) with low-dose MTX leads to unfavourable conditions for extensive MTX-polyglutamylation in PBMCs.[25] Myeloid precursor cells have high FPGS activity, explaining the more long-chain MTX-PGs in RBCs compared with PBMCs.[33] In a pilot setting, we determined FPGS activity in seven baseline PBMC samples of our cohort with the method of Muller et al, and found comparable FPGS activity as in PBMCs of healthy donors (being 100-fold lower than in highly proliferative leukaemia cells).[25]

The primary target tissue in CD treatment, intestinal mucosa, revealed substantial long-chain MTX-PGs concentrations in contrast to RBC and PBMC. Egan et al. reported MTX accumulation ($MTX\text{-}PG_{total}$ in fmol/mg wet weight per biopsy by a competitive dihydrofolate reductase binding assay) but MTX-PG distribution profiles were not investigated.[23] Formation of long-chain MTX-PGs implies that mucosae harbour abundant FPGS activity (Figure 1), which is in line with proliferative tissues having a high folate need.[17] Reports on human mucosa organoids, colon cancer mucosae and rodent proliferative intestinal epithelium demonstrated relatively high levels of FPGS activity as well.[34–36]

Patients with CD discontinuing their MTX treatment in our longitudinal cohort provided a unique opportunity to investigate how this impacted MTX-PGs retention in the three matrices. The most dramatic decrease of MTX-PGs was observed in PBMCs, particularly for short-chain MTX-PGs (Table 2). This may be explained by, first, the

relatively low polyglutamylation rate in PBMCs. Second, by the short lifespan of PBMCs in the circulation, leading to PBMCs unexposed to MTX (as plasma MTX is undetectable 24 hours after administration).[12] Third, PBMCs express drug efflux transporters of the ABC protein family that can efflux MTX-PG_{1-3}, compared with RBCs which predominantly efflux MTX-PG_1 and to a lesser extent MTX-PG_2 (Figure 1).[37,38] In RBCs, longer chain MTX-PGs decline to some extent one month after MTX discontinuation due to newly formed RBCs that are not exposed to MTX, therefore diluting circulating RBC MTX-PGs until the latter fraction reaches the end of their final lifespan of 120 days (ranging 84–147 days).[18,39] The most striking observation is that long-chain MTX-PGs were well retained in intestinal mucosa after MTX discontinuation. Clinically, this may have implications for providing recommendations for subsequent missing doses of MTX.[40] Although the concentration-effect relationship of MTX-PGs in PBMCs and intestinal mucosa has not been determined yet, we could hypothesize that MTX efficacy would be low after three to four weeks of MTX discontinuation based on low concentrations in the effector cell (PBMCs). However, we could also expect prolonged mucosal effects by remaining intestinal MTX-PGs after MTX treatment discontinuation by patients with CD.

The strength of our study is that MTX-PG accumulation in patients with CD was analysed in unexplored matrices, side-by-side with RBCs, which has historically served as the preferred matrix for its convenience and known concentration-effect relation. Measurements of MTX-PGs in target tissues in other IMIDs (synovium for RA and skin biopsies for psoriasis) are still lacking. We employed a sensitive and quantitative LC-MS/MS method with stable isotope-labelled internal standards for each individual MTX-PG to assess the distribution of intracellular intestinal MTX-PG subspecies. Lastly, our study was performed longitudinally over one year of MTX treatment and was extended to patients who discontinued MTX treatment when possible, creating a unique dataset.

Our study had some limitations as well. First, the analysis of mucosal biopsies did not include sorting into different cells that make up the mucosa, i.e. epithelial cells, goblet cells, lymphocytes, and fibroblasts.[41] The cellular composition varies not only throughout the ileum, left and right colon and rectum (distal more goblet cells and fewer lymphocytes), but also per biopsy within one patient (lymphocytes are distributed in a patchy way), and during inflammation (the proportion of epithelial cells in a colonic biopsy being reduced from 50% to 10–20% because of the increase of immune cells).[41] Various cell types differ in lifespan, proliferation and activation status with accompanying folate metabolic activity, including FPGS catalytic activity, which may lead to variations in total MTX-PG accumulation concentrations and PG distribution profiles. To gain a more detailed understanding of the regulation of MTX-PG accumulation in mucosal effector cells, cell sorting by flow cytometric analyses would be necessary.[42] Second, we lacked sampling of RBCs and PBMCs at earlier time points, for example at four weeks after start of MTX therapy, which could have provided an even better insight into the profile of MTX-PG accumulation. However, based on similar analysis of these cells of patients with RA,[9,29] it is anticipated that for patients with CD as well, MTX-PG_1 would still be the dominant PG form in both RBCs and PBMCs at week four. Third, as we studied pharmacokinetics and pharmacodynamics in a real-life clinical setting, there was a wide amount of variability in MTX administration (dosing, duration of use when measuring the concentration) and in patients (CD subtype; presence, severity and location of inflammation), as well as a low yield of collecting PBMC and mucosa samples, making it challenging to draw firm conclusion for clinical practice yet. However, this study has provided new insights into the distribution and accumulation profile of MTX in patients with CD, enabling further research.

4.1 | Conclusions

MTX-PGs are present and readily detectable in intestinal mucosa and PBMCs of CD patients with CD administered MTX. Intestinal mucosa showed high concentrations of long-chain MTX-PGs, while PBMCs showed higher short-chain MTX-PGs. MTX-PG accumulation in PBMCs is approximately 2-(MTX-PG4) to 18-(MTX-PG1) fold higher than in RBCs. In RBCs, MTX-PG3 became the most abundant PG-form after 12 weeks of MTX therapy and continued to be so for the entire period of 1 year therapy. Since MTX-PG accumulation profiles markedly differ between immune effector cells (PBMCs), target cells (mucosa), and RBCs, future TDM studies could focus on cell and/or tissue-specific analyses.

AUTHOR CONTRIBUTIONS

Maartje M. van de Meeberg: Formal analysis; investigation; methodology; writing—original draft. **Janani Sundaresan:** Formal analysis; resources; Writing—review and editing. **Marry Lin:** Formal analysis; resources; writing—review and editing. **Gerrit Jansen:** Conceptualization; writing—review and editing. **Eduard A. Struys:** Formal analysis; resources; writing—review and editing. **Herma H. Fidder:**

Supervision; resources; writing—review and editing. **Bas Oldenburg:** Resources; writing—review and editing. **Wout G. N. Mares:** Resources; writing—review and editing. **Nofel Mahmmod:** Resources; writing—review and editing. **Dirk P. van Asseldonk:** Resources; writing—review and editing. **Svend T. Rietdijk:** Resources; writing—review and editing. **Loes H. C. Nissen:** Resources; writing—review and editing. **Nanne K. H. de Boer:** Resources; writing—review and editing. **Gerd Bouma:** Conceptualization; funding acquisition; methodology; resources; supervision; writing—review and editing. **Maja Bulatović Ćalasan:** Conceptualization; funding acquisition; methodology; supervision; writing—review and editing. **Robert de Jonge:** Conceptualization; funding acquisition; methodology; supervision; writing—review and editing.

CONFLICT OF INTEREST STATEMENT

All conflicts are outside the submitted work: *H.H.F.* has served as a speaker for both Janssen and Takeda. She has served as a consultant for Takeda, Galapagos, and Ferring. She has received a research grant from Takeda. *B.O.* has served as a speaker for Takeda, Galapagos, and MSD. He has received grants from Pfizer, Takeda, and Ferring. He has served as a consultant/on the advisory board for Janssen, Pfizer, Takeda, and Cablon. *G.B.* has served as the speaker for Janssen and Takeda. He has served as a consultant for Roche, Takeda, and Calyps Biotech. *N.K.H.d.B.* has served as a speaker for AbbVie and MSD. He has also served as a consultant and/or principal investigator for TEVA PharmaBV and Takeda. He has received a (unrestricted) research grant from DrFalk, TEVA PharmaBV, MLDS and Takeda. *L.H.C.N.* has served on the advisory board for AbbVie and Ferring. *M.B.C* has received an unrestricted grant from Takeda, CSL Behring and Pharming. The other authors have nothing to declare.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

PATIENT CONSENT STATEMENT

All patients gave their informed consent before inclusion in the study.

AUTHORSHIP STATEMENT

All authors approved the final version of the manuscript. Guarantor: R.d.J.

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