Biomolecules and Biomedicine
ISSN: 2831-0896 (Print) | ISSN: 2831-090X (Online)
Journal Impact Factor® (2024): 2.2
CiteScore® (2024): 5.2
www.biomolbiomed.com | blog.bjbms.org

SUPPLEMENTAL DATA

MTX pathway gene variants, erythrocyte methotrexate polyglutamates, and treatment outcomes in rheumatoid arthritis

Peihong Wang[1#], Cuilv Liang[1#], Limei Lin[1], Jieli Lan[2], Yin Zhang[1*], Weiping Xie[3*]

[1]Department of Pharmacy, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, China;

[2]Division of Science and Technology, The Second Affiliated Hospital, Fujian Medical University, Quanzhou, China;

[3]The Affiliated Quanzhou Center for Disease Control and Prevention of Fujian Medical University, Quanzhou, China.

[#]Equally contributed to this work and share first authorship: Peihong Wang and Cuilv Liang.

*****Correspondence to Yin Zhang: zyin1973@163.com; Weiping Xie: xwp@fjmu.edu.cn

Full article is available at the following link: MTX pathway gene variants, erythrocyte methotrexate polyglutamates, and treatment outcomes in rheumatoid arthritis

Table S1. Results of entries in the Bioanalytical Method Validation Guidance for Industry

Entries	
Reference Standards and Critical Reagents	MTXPG1-6 standards were purchased from Schircks Laboratories (Jona, Switzerland). The LOT number of 6 MTXPG1-6 standard: MTXPG1(16.411 lot 014), MTXPG2(16.412 lot105), MTXPG3(16.413 lot 17), 7 MTXPG4(16.414 lot 12), MTXPG5(16.415 lot 15), MTXPG6 (16.416 lot 31). Critical Reagents： LC-MS-grade methanol, Milli-Q water, OAsis MAX Solid-phase extraction columns, perchloric acid, et al.
Calibration Curve	We have determined the calibration curve and show a good linear range (Supplement Table 3).
Quality Control Samples	Since we were unable to obtain commercial quality control samples, we controlled the process using spiked standards instead.
Selectivity and Specificity	The guidance states that when using liquid chromatography/mass spectrometry (LC/MS) methods, the sponsor or applicant should evaluate the effects of the matrix on ion suppression, ion enhancement, or extraction efficiency. Therefore, we tested the matrix effect as required. The results showed that the matrix effects for MTXPG 1-6 were 62.2%, 77.3%, 81.2%, 67.5%, 80.1%, and 84.6%, respectively. Additionally, liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers advantages such as high selectivity, high specificity, and high sensitivity.
Sensitivity	We have determined the LLOQ for MTXPG1-6.

Accuracy, Precision, and Recovery	We have performed the determination of the minimum limit of quantification, spike recovery and precision test for MTXPG1-6.
Stability	We did not perform the stability test at that time, but reference [1] reported the stability experiment results of MTXPGs. MTXPGs were stable at -80℃ and room temperature (25℃) over 2 months, indicating that MTXPGs were stable during the detection process.
Matrix Effects	Standard series of 5–250 nmol/L were prepared using blank blood matrix sample solution and pure water, respectively, and the slopes of the MTXPG1-6 calibration curves in blood matrix and pure water were compared. The slope ratio was used as the matrix effect; the matrix effects for MTXPG1-6 were: 62.2%, 77.3%, 81.2%, 67.5%, 80.1%, and 84.6%, respectively. The results indicate that MTXPG1-6 all exhibit a certain degree of matrix suppression; therefore, in practical testing, it is necessary to use calibration curves prepared in blank matrix-matched samples for quantification.
Dilution Effects	The linear range of the standard curve encompassed our test results, and our samples did not require dilution. Therefore, the dilution effect was not evaluated
Partial and Cross Validations	Partial validations evaluate modifications of already validated bioanalytical methods while cross validation is a comparison of validation parameters of two or more bioanalytical methods or techniques that are used to generate data within the same study or across different studies. Therefore, neither type of validation is applicable to our study [33].

Abbreviations: MTXPG: Methotrexate polyglutamate; MTX: Methotrexate; LLOQ: Lower limit of quantification.

Table S2. Recoveries and precision results of individual MTXPGn compounds

MTXPGn	Spiked Concentration 5nmol/L		Spiked Concentration 25nmol/L		Spiked Concentration 50nmol/L	
	Average Recovery Rate	RSD (%)	Average Recovery Rate	RSD (%)	Average Recovery Rate	RSD (%)
MTXPG1	90.3	5.38	90.0	3.75	90.9	4.98
MTXPG2	90.2	4.95	89.6	4.26	90.5	3.78
MTXPG3	89.9	6.10	86.3	6.22	91.4	4.09
MTXPG4	82.8	6.78	86.4	4.51	85.3	3.75
MTXPG5	80.4	7.53	86.8	4.49	89.1	4.10
MTXPG6	85.6	6.13	81.7	7.58	83.4	6.32

Abbreviations: MTXPGn: Methotrexate polyglutamate; RSD: Relative standard deviation.

Table S3. Linear regression equation and detection limits of individual MTXPGn

MTXPGn	Equation of Linear Regression*	Correlation Coefficent（r)	LOD (nmol/L)	LOQ [b] (nmol/L)	Linearity Range(nmol/L)
MTXPG1	$y=2.26*10^4x-7.92*10^4$	0.9998	0.07	0.22	0.22-250
MTXPG2	$y=7.52*10^3x-3.85*10^4$	0.9993	0.12	0.40	0.40-250
MTXPG3	$y=1.68*10^3x-9.80*10^3$	0.9988	0.14	0.48	0.48-250
MTXPG4	$y=2.73*10^2x-2.04*10^2$	0.9993	0.88	2.93	2.93-250
MTXPG5	$y=5.16*10^2x-2.16*10^3$	0.9990	1.40	4.65	4.65-250
MTXPG6	$y=1.12*10^3x-6.29*10^2$	0.9995	0.46	1.52	1.52-250

*: y: Peak area; x: MTXPGn concentration; b: Values of patient samples below the LOQ were reported as “<LOQ>”; LOD: Limit of detection; LOQ: Limit of quantification.

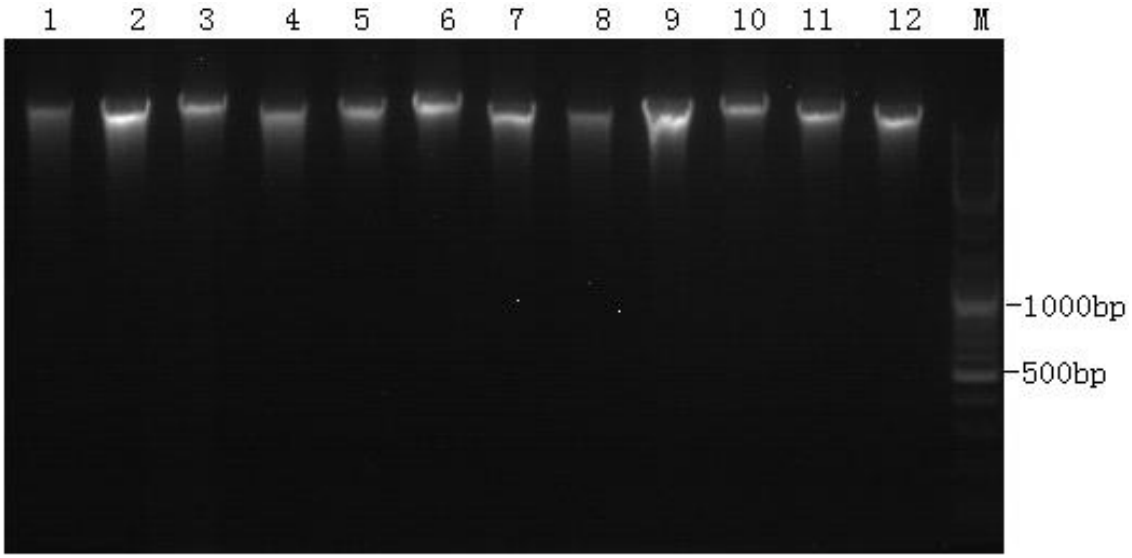

Figure S1. Quality assessment of genomic DNA prior to SNP genotyping. Genomic DNA was extracted from EDTA-anticoagulated peripheral venous blood and assessed by agarose gel electrophoresis (5 μL per sample on 1% agarose in 1×TAE buffer, 120–180 V for 15 min). Lanes 1–12 show representative patient DNA samples; lane M shows the DNA size marker (SM0331). The presence of a single high-molecular-weight band with minimal smearing indicates intact, non-degraded genomic DNA suitable for downstream PCR and MassARRAY genotyping. **Abbreviations**: DNA: Deoxyribonucleic acid; EDTA: Ethylenediaminetetraacetic acid; PCR: Polymerase chain reaction; SNP: Single nucleotide polymorphism; TAE: Tris–acetate–EDTA.

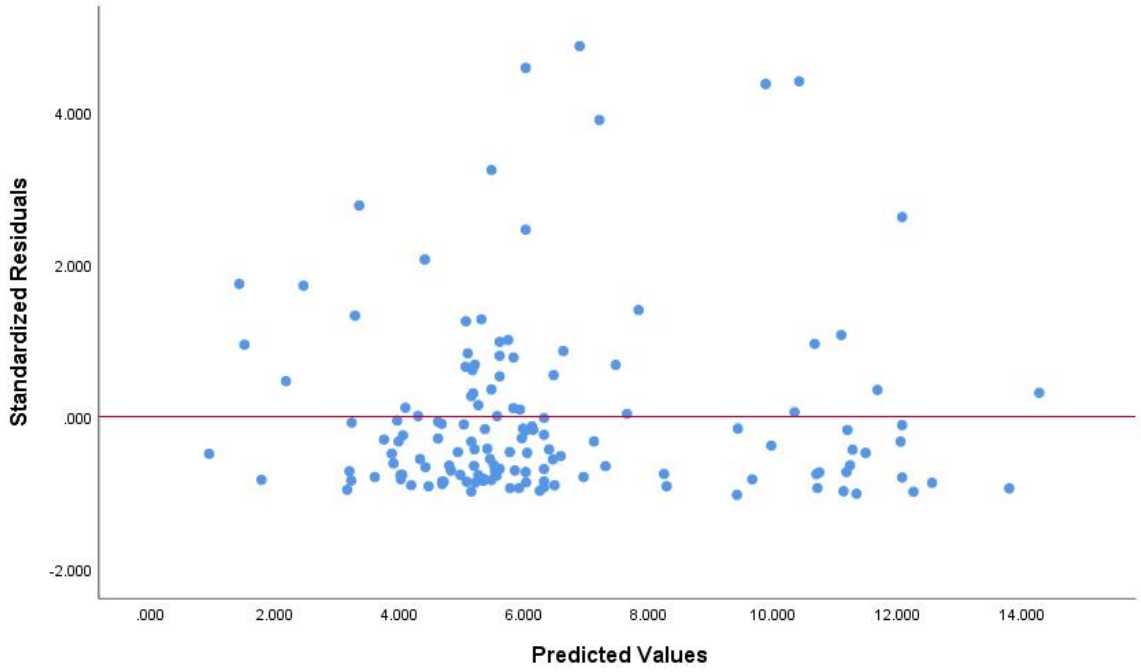

Figure S2. Residual–fitted diagnostic for the MTXPG1 generalized linear model. Standardized residuals are plotted against fitted (predicted) erythrocyte MTXPG1 concentrations from the multivariable model including MTX-pathway SNPs and clinical covariates. The horizontal reference line (red) indicates zero residuals; the scatter around this line is inspected for systematic structure (e.g., curvature or funneling) to evaluate model specification and homogeneity of variance. **Abbreviations**: MTX; Methotrexate; MTXPG: Methotrexate polyglutamate.

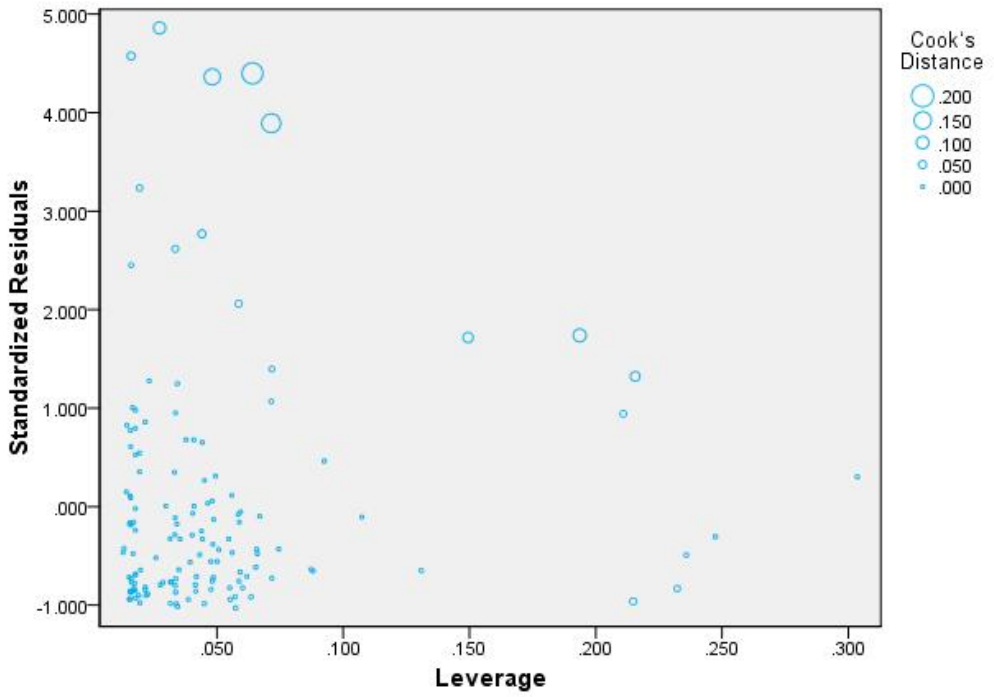

Figure S3. Leverage–Cook's distance influence diagnostics for the MTXPG1 generalized linear model. Standardized residuals are plotted against leverage (hat values) for the multivariable model including MTX-pathway genotypes and clinical covariates; marker size is proportional to Cook's distance (right), highlighting observations with the greatest potential influence on regression coefficients. No data point approaches commonly used thresholds for strong influence. **Abbreviations**: MTX: Methotrexate; MTXPG: Methotrexate polyglutamate.

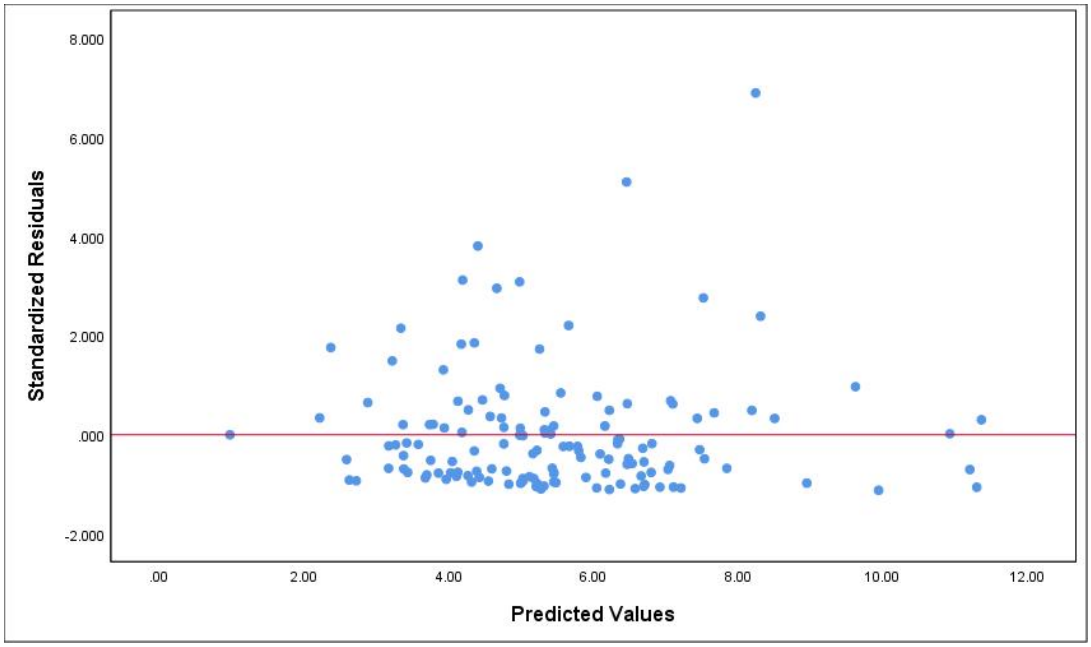

Figure S4. Residual–fitted diagnostic for the MTXPG2 generalized linear model. Standardized residuals are plotted against fitted (predicted) erythrocyte MTXPG2 concentrations from the multivariable model including MTX-pathway SNPs and clinical covariates. The horizontal reference line (red) denotes zero residuals and facilitates visual evaluation of systematic structure (e.g., curvature) and non-constant variance across the range of fitted values. **Abbreviations**: MTX: Methotrexate; MTXPG: Methotrexate polyglutamate.

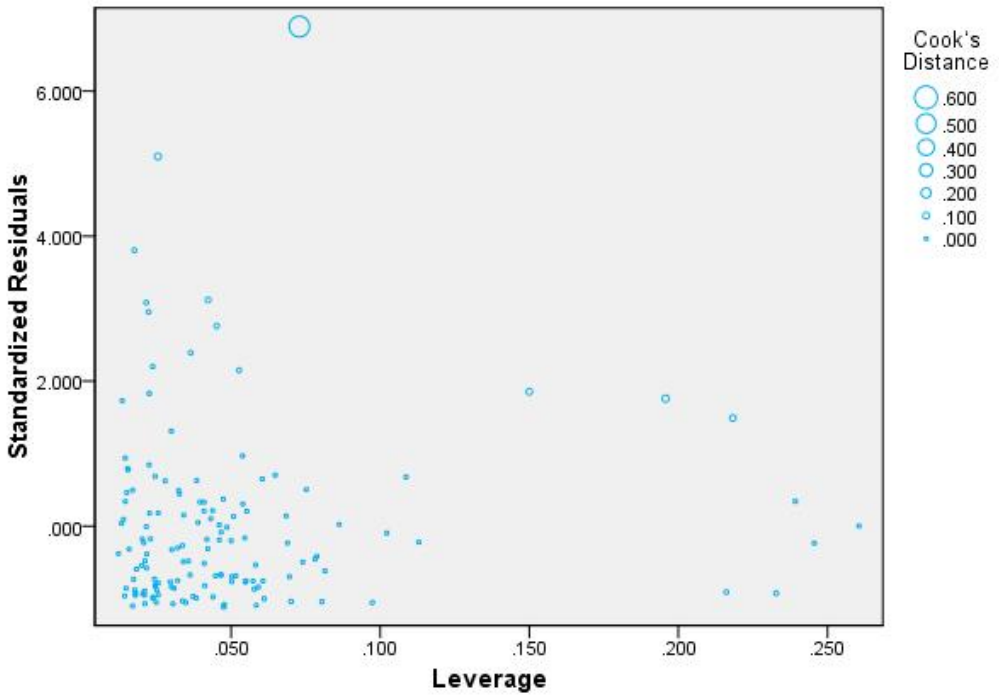

Figure S5. Leverage–Cook's distance influence diagnostics for the MTXPG2 generalized linear model. Standardized residuals are plotted against leverage (hat values), with point size proportional to Cook's distance to highlight observations with high leverage and/or large residuals that may disproportionately affect model coefficients. **Abbreviation**: MTXPG, methotrexate polyglutamate.

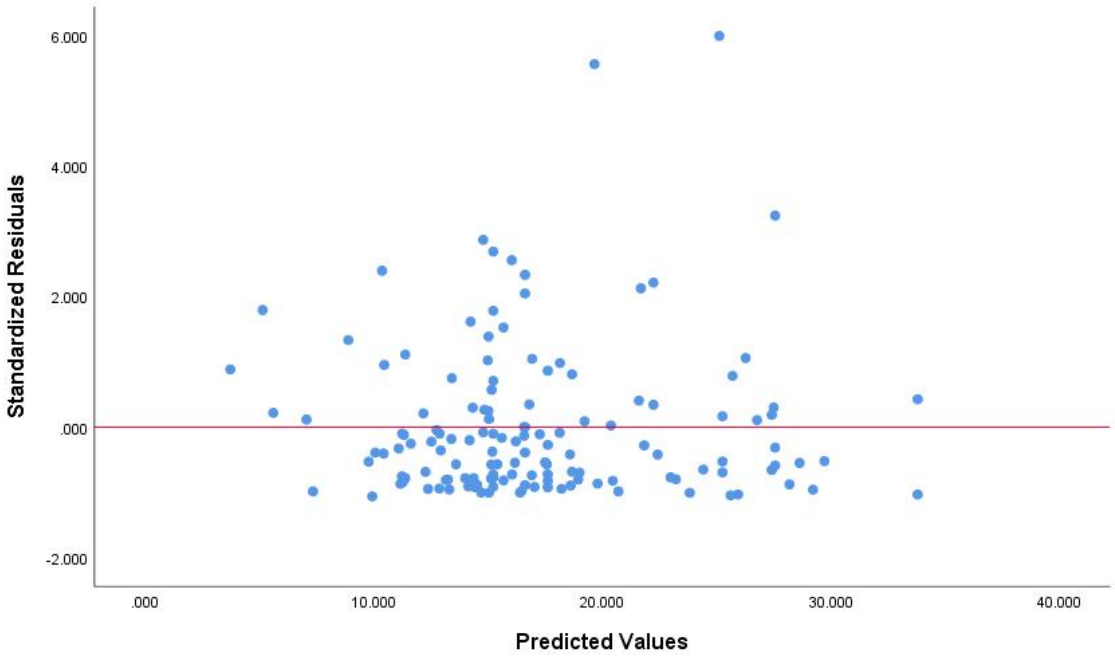

Figure S6. Residual–fitted diagnostic for the total MTXPGs generalized linear model. Standardized residuals are plotted against fitted (predicted) total erythrocyte MTXPGs concentrations from the multivariable model including MTX-pathway genotypes and clinical covariates. The horizontal reference line (red) indicates zero residuals and is used to screen for systematic structure (e.g., curvature) and non-constant variance across the fitted range. **Abbreviations**: MTX: Methotrexate; MTXPGs: Total methotrexate polyglutamates.

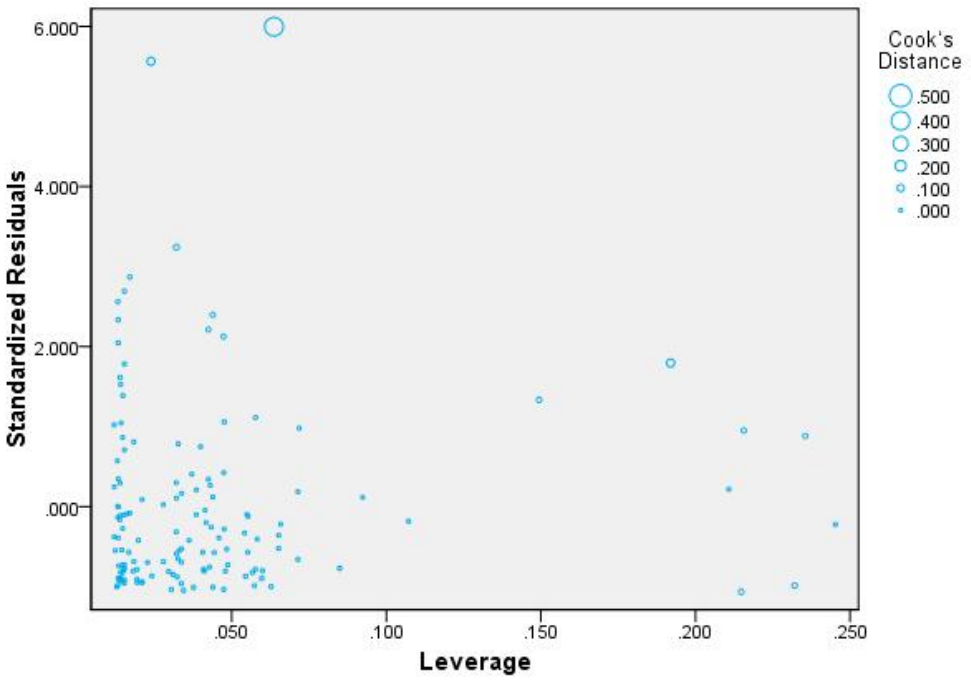

Figure S7. Leverage–Cook's distance influence diagnostics for the total MTXPGs generalized linear model. Standardized residuals are plotted against leverage (hat values); point size is proportional to Cook's distance, highlighting observations with the greatest potential influence on regression coefficients. All Cook's distances remain below commonly used thresholds for strong influence (i.e., <1). **Abbreviation**: MTXPGs: Total methotrexate polyglutamates.