Received: 8 April 2021 | Revised: 14 May 2021 | Accepted: 20 May 2021

DOI: 10.1111/cts.13086

ARTICLE

Toward pharmacogenetic *SLCO1B1*-guided dosing of methotrexate in arthritis using a murine *Slco1b2* knockout model

Zachary L. Taylor[1,2,3] | **Lauren E. Thompson**[4] | **Heather Bear**[2] | **Tomoyuki Mizuno**[3,5] | **Alexander A. Vinks**[2,3,5] | **Laura B. Ramsey**[2,3,5]

[1]Department of Pharmacology and Systems Physiology, University of Cincinnati, Cincinnati, Ohio, USA

[2]Division of Research in Patient Services, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

[3]Division of Clinical Pharmacology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

[4]Department of Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

[5]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

Correspondence
Laura B. Ramsey, Division of Research in Patient Services, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA.
Email: laura.ramsey@cchmc.org

Funding information
L.B.R. received internal funding from the Cincinnati Children's Research Foundation.

Abstract

Low-dose methotrexate (MTX) is a first-line therapy for the treatment of arthritis. However, there is considerable interindividual variability in MTX exposure following standard dosing. Polymorphisms in *SLCO1B1* significantly effect MTX clearance, altering therapeutic response. One decreased function variant, rs4149056 (c.521T>C, Val174Ala), slows MTX clearance and in vitro uptake of MTX. This phenotype was recapitulated in a mouse model using a knockout (KO) of the murine orthologue, *Slco1b2*. Our objective was to investigate the impact of this phenotype on the pharmacokinetics and therapeutic outcomes of low-dose MTX in a murine model of collagen-induced arthritis (CIA). We evaluated response to MTX in mice with CIA using wildtype (WT), heterozygous, and KO *Slco1b2* mice on a DBA1/J background. Arthritis was macroscopically evaluated daily to quantify disease progression. Mice received 2 mg/kg or a pharmacogenetically guided MTX dose subcutaneously 3 times a week for 2 weeks. MTX concentrations were collected at the end of the study and exposure (day*μM) was estimated using a two-compartment model. Mice displayed a seven-fold range in MTX exposure and revealed a significant exposure-response relationship ($p = 0.0027$). KO mice receiving the 2 mg/kg dosing regimen had 2.3-fold greater exposure to MTX ($p < 0.0001$) and a 66% reduction in overall disease progression ($p = 0.011$) compared to WT mice. However, exposure and response were equivalent when pharmacogenetically guided dosing was used. These studies demonstrate that an exposure-response relationship exists for MTX and that *Slco1b2* genotype affects MTX exposure and therapeutic response. Such evidence supports the use of *SLCO1B1*-pharmacogenetic dosing of low-dose MTX for patients with arthritis.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

There is large interpatient variability in methotrexate (MTX) pharmacokinetic (PK) parameters and response in patients with arthritis. Pharmacogenetic studies in pediatric patients with arthritis are rare.

WHAT QUESTION DID THIS STUDY ADDRESS?

This study determined whether *Slco1b2* impacts MTX PKs and therapeutic response in mice with collagen-induced arthritis and if a pharmacogenetic dose modification would achieve both equivalent therapeutic exposure and response.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study demonstrates that MTX PK and therapeutic response in this model are dependent upon *Slco1b2* genotype. Knockout mice required a 60% dose reduction in order to achieve equivalent exposure to that of wildtype mice receiving a 2 mg/kg subcutaneous dose of MTX.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study supports the association between *SLCO1B1* and MTX PK and therapeutic response. This study, in conjunction with a large prospective study, has the potential to increase the level of evidence for the *SLCO1B1*-MTX gene-drug pair, and could result in the implementation of pre-emptive pharmacogenetic testing for patients receiving MTX.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) are autoimmune inflammatory disorders characterized by persistent joint swelling caused by an accumulation of synovial fluid and thickening of the synovial lining.[1–3] JIA has an incidence ranging from 2 to 20 cases per 100,000 children, making it the most common childhood rheumatic condition and can lead to disabling complications, like local growth retardation or malformation if left untreated.[1,4–6] RA is a common rheumatic condition that affects 0.5–1% of the population and is associated with increased risks for comorbidities and mortality.[7] Current first-line treatment options for RA and JIA include nonsteroidal anti-inflammatory drugs and intra-articular steroid injections for short-term, rapid induction of disease control. More often, clinicians will initially administer methotrexate (MTX), a folate antagonist and first-choice disease-modifying anti-rheumatic drug (DMARD) instead, in an effort to treat inflammatory arthritis during the early "window of opportunity."[1,8,9] Despite the vast evidence that MTX is effective as a DMARD,[10–14] the clinical response to MTX is highly variable, with response rates ranging from 33 to 100% of patients.[13] Several previous clinical studies suggest that ~30% of patients do not respond to benefit from MTX within the first 6 months of treatment, whereas an additional ~30% of patients discontinue treatment due to intolerable side effects.[15–18] Determining the cause for such variability can be complicated by the heterogeneous nature of clinical populations. Nevertheless, past studies have shown that increasing serum MTX and MTX polyglutamate concentrations can improve clinical response.[14] The challenge with utilizing these data is that MTX displays significant interindividual pharmacokinetic (PK) variability. MTX is primarily renally eliminated, with estimates of 55–80% eliminated as unchanged drug in the urine[19] whereas hepatic elimination only accounts for 10% of MTX elimination[19]; yet large ranges of serum MTX concentrations have been observed following the administration of low-dose MTX.[14] This, in tandem with the complexity of the folate pathway, makes it difficult to establish a targeted serum MTX concentration or exposure-response relationship for clinical use. Thus, identifying predictors of response will help clinicians pre-emptively optimize treatment thereby reducing the burden of disease on both the patient and the patient's family.

One possible predictor of response was identified from a genomewide association study published in 2012. A meta-analysis of 1978 patients receiving high-dose MTX revealed one hit, *SLCO1B1*, at genomewide significance.[20] This transporter expressed primarily in the liver is responsible for the sodium-independent hepatic uptake of MTX. Variants of *SLCO1B1* explained 10.7% of the interpatient variability of high-dose MTX in this study,[20–22] which was more than age, sex, and race combined. Furthermore, this study identified the impact of the decreased function *5 haplotype, rs4149056 (c.521T>C, Val174Ala), which was associated with increased systemic concentration of MTX by slowing MTX clearance. Additional clinical studies have confirmed that the *5 variant significantly reduced the clearance of MTX by more than 18% per dysfunctional C allele, leading to an increase in MTX exposure and risk for severe toxicity.[23] Subsequent studies have also demonstrated the pharmacogenetic (PGx) impact of *SLCO1B1* variants rs2306283 (c.388A>G, Asn130Asp) and rs11045819 (c.463C>A, Pro155Thr) on low-dose MTX and therapeutic response in patients with RA[24] and JIA.[25] However, further studies linking *SLCO1B1* to MTX are required in order to increase the

level of evidence for this gene-drug pair and establish dosing recommendations.

The purpose of this study was to explore associations between the *SLCO1B1* haplotype and MTX response in a preclinical model of JIA. Herein, we describe using a murine model of collagen-induced arthritis (CIA) and DBA1/J mice lacking the murine orthologue of *SLCO1B1*, *Slco1b2*, to quantify the PGx impact of *Slco1b2*-genotype on MTX PK and therapeutic response. We hypothesized that an exposure-response relationship would exist: *Slco1b2*-knockout (KO) mice would have increased MTX exposure compared to wildtype (WT) mice resulting in improved therapeutic response. Then, using PK modeling, demonstrate that equivalent therapeutic response can be achieved by accounting for the PGx differences using an *Slco1b2*-genotype guided dosing regimen to generate equivalent exposure of MTX across genotypes.

METHODS

Mice

The mice used in this study were male *Slco1b2* KO, heterozygous (HET), and WT mice of identical genetic background (DBA1/J) between 7 and 15 weeks of age.[26] A total of 155 mice were used across three experiments with the number of mice in each genotype group per experiment being reported with each figure. Fifty-two mice were used in the 2 mg/kg MTX treated arthritis study. Fifty-seven mice were used in the PGx MTX treated arthritis study. Thirty-eight mice were used in the rifampin analysis. Eight mice were used as healthy controls for PK analysis. Mice were housed and handled in accordance with IACUC guidelines. Mice were housed in veterinary services at Cincinnati Children's Research Foundation (Cincinnati, OH) under specific pathogen free conditions and a temperature-controlled environment with a 12-h light/12-h dark cycle. Mice received a standard chow (Rodent Diet 5010, Test Diet) and water ad libitum. Three weeks prior to the start of treatment through the end of the experiments, mice were fed with a folate deficient chow (Basal 5755 Folic Acid Deficient Diet, Test Diet), including hard pellets and puree, to reduce the interference of folate on therapeutic response and toxicity.[27]

Preparation of collagen

Bovine type II collagen (CII; Elastin) was prepared from nasal cartilage by pepsin digestion and subsequent purification as described previously.[28] CII was solubilized to a concentration of 2 mg/ml in 0.01 M acetic acid at 4°C with constant mixing overnight.[28] Complete Freud's Adjuvant (CFA) was produced using 4 mg of mycobacterium (M. tuberculosis Des. H37 Ra, Becton, Dickinson and Company) and 8 ml of Incomplete Freud's Adjuvant (DIFCO Laboratories). Just prior to the immunization, CII was emulsified in CFA at a 1:1 ratio.

Immunization and evaluation of collagen-induced arthritis

Mice received an initial immunization (day 0) of 200 µl of the CII emulsion by intradermal injection near the base of the tail.[29] Mice received a booster intra-dermal immunization of a freshly made 200 µl of the CII emulsion on day 21 (Figure S1). Mice were weighed and evaluated daily for 2 weeks (day 21 to day 35). Arthritis was quantified by author Z.L.T., who was blinded to genotype and treatment, using a macroscopic scoring system[30] ranging from 0 to 4 (0: no detectable arthritis; 1: swelling of the paw or one digit; 2: two joints involved; 3: three or four joints involved, and 4: severe arthritis of the entire paw and digits). The arthritic index for each mouse was the summation of the four individual paws.[31] Mice were also evaluated based on the degree of paw swelling using an arthritic severity score (range, 0–2).[32,33] Therapeutic response and disease progression were further quantified by analyzing the area under the arthritic index-time curve (AI AUC), which provided estimates for total disease exposure.

Treatment protocols

Mice with arthritis were randomly divided into treated and untreated groups on day 23, ensuring equivalent age and weight across treated and untreated groups. Mice assigned to the treated group received either a uniform dose (2 mg/kg) or a PGx dose of subcutaneous MTX six times, starting on day 23 and thereafter as indicated in Figure S1.

PK studies

For subcutaneous administration of MTX, the stock solution (25 mg/ml, Hospira Inc.) was diluted with saline to make a final drug concentration of 0.2 mg/ml (2 mg/kg). For the uniform dosing of 2 mg/kg, a total of 10 µl/g body weight was administered subcutaneously. When indicated, 5 µl of rifampin (4 mg/ml in saline; Chem-Impex International) per gram of body weight was injected into the tail vein 3 min before MTX administration.[34] PGx guided dosing used the same stock 0.2 mg/ml, but administered less volume per gram of body weight. On day 35,

retro-orbital eye bleeds using heparin-lined capillary tubes and Eppendorf tubes with EDTA were performed at least 30 min following the subcutaneous administration of MTX and animals were euthanized. Separate mouse PK studies were performed using a single subcutaneous dose of 2 mg/kg in nonarthritic mice. Plasma samples from these single dose studies were collected from 15 to 100 min after the dose to determine the standard PK profile of subcutaneous MTX in the mouse (Figure S2). The area under the concentration-time curve (AUC) between nonarthritic and arthritic mice was similar (Figure S3). Blood samples were centrifuged at 5000 rpm for 5 min at 4°C, and plasma was collected and stored at −20°C until analysis. The liver and right kidney were weighed then immediately flash frozen and stored at −80°C until analyzed by high-performance liquid chromatography (HPLC).

Methotrexate measurement

Concentration of MTX in plasma and tissue were determined by HPLC (Agilent 1100 series). Ferulic acid served as the internal standard for both plasma and tissue analyses. Plasma samples were deproteinized using silver nitrate and potassium iodide as previously described.[35] A 100 µl plasma sample was then loaded into the autosampler. A 20 µl injection volume was run in a 10 mM sodium phosphate buffer solution pH 7 and 100% methanol (78:22%, v/v) mobile phase and was pumped through a Zorbax ODS column (4.6 × 250 mm, 5 µM; Agilent) at 0.5 ml/min. A series of standards, ranging from 0.275 to 33 µM, were used to generate a calibration curve and linear regression. Plasma MTX concentrations were then determined. Liver and kidney samples were homogenized by a bead homogenizer (Bead Mill 4, Fisherbrand) in 9-fold (volume by weight) 10 mM sodium phosphate buffer pH 7.[36] Homogenates were heated at 100°C for 5 min then centrifuged at 11,300 *g* for 2 min. Supernatant was collected and stored at −80°C. Supernatant samples underwent identical deproteinized as the plasma samples and were run using identical HPLC conditions. The HPLC demonstrated sound linearity with a lower limit of quantification at 0.11 µM. The intra-day precision was 9.7% and the inter-run precision was between 2.3% and 5.6%.

Pharmacokinetic modeling

Body weight, dose, time of dose, time of bleed, and MTX concentration were entered in the appropriate sections of the MW/Pharm software[37] (version 3.82, Mediware, Prague, Czech Republic). Using the preloaded MTX model parameters in MW/Pharm, a base mouse PK model was estimated using a two-stage iterative design.[38] This design is based on the calculation of the population mean and SD of the model parameters at each iteration. Once the preloaded human MTX model was scaled to the mouse, the plasma concentrations from the separate PK mouse studies were entered into MW/Pharm. The base mouse model then served as prior information for the individual model fits.[38] The model outputs were analyzed and refined, generating our final mouse model. PK data collected from treated mice on day 35 were loaded into the final model and Bayesian estimation was performed. The models provided individualized PK estimates, such as AUC and clearance (CL). PK differences across genotypes were analyzed, which aided in the development of each individual genotype model (WT: CL = 0.063 L/h, V = 0.025 L; HET: CL = 0.027 L/h, V = 0.018 L; KO: CL = 0.021 L/h, V = 0.012 L).

PGx dose modification

MW/Pharm was used to estimate the AUC of the concentration-time curve for each of the PGx PK mouse models.[37] Using the uniform dose of 2 mg/kg, AUCs were estimated for each mouse genotype. Then, PGx dose adjustments for HET and KO mice were estimated using MW/Pharm simulations in order to achieve equivalent exposure to 2 mg/kg WT mice. Estimated exposures from separate single dose mouse PK studies validated the in silico PGx dosing modifications for HET and KO mice. The PGx treatment study used a dose reduction of 60% for KO mice (0.8 mg/kg) and 50% for HET mice (1 mg/kg) while maintaining the 2 mg/kg dose in WT mice.

Histological analysis

Kidneys from WT, HET, and KO mice were fixed in 4% phosphate-buffered formalin for 48 h. The tissue was then processed and embedded in paraffin. Tissue sections (4 µm) were mounted on Superfrost Plus slides (Fisherbrand) and stained using hematoxylin and eosin.[28,33] Semiquantitative histopathology analysis was performed for each kidney using a (0–4) scale for each of the following scoring criteria for acute tubular necrosis (ATN): eosinophilic casting, cell blebbing, cell swelling, dilation of Bowman's space, retraction of glomerular tuft, prominence of juxtaglomerular apparatus, inflammation, loss of basement membrane, and necrosis.[39] Paws and knees were fixed in 4% phosphate-buffered formalin for 48 h then decalcified in a tris hydrochloride solution for 14 days. Histopathological analysis of the kidneys focused on the degree of MTX toxicity whereas the paws and knees were used to visually assess the degree of arthritis. Representative images are

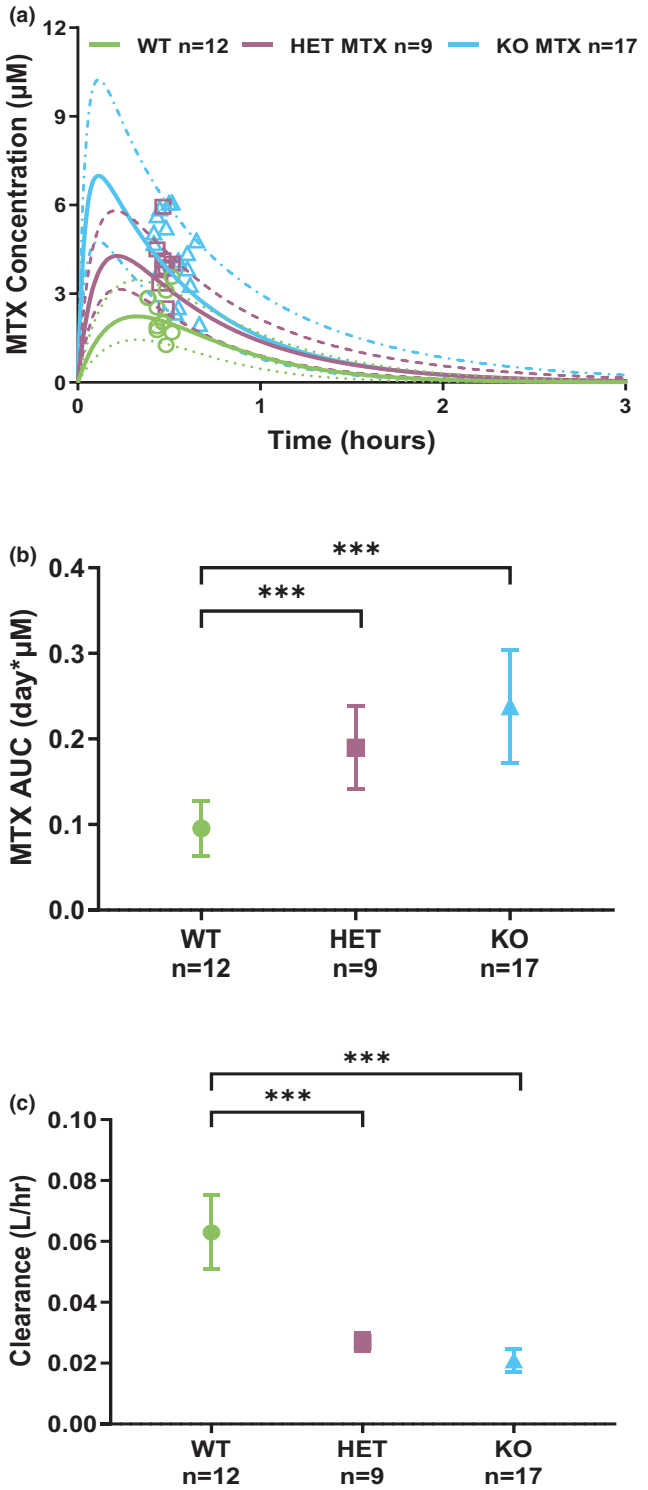

FIGURE 1 Impact of *Slco1b2*-genotype on methotrexate (MTX) pharmacokinetics. The mean model predicted concentration-time profiles for each genotype, with in vivo plasma MTX concentrations, depict elevated plasma MTX in the heterozygous (HET) and knockout (KO) mice following a subcutaneous 2 mg/kg dose of MTX (a). KO mice also demonstrated significantly greater estimated MTX exposure (b) and reduced MTX clearance (c), resulting in a seven-fold range in estimated MTX exposure. In (a), the solid line represents the mean model predicted concentration-time profile for each genotype and the dotted lines represent the ± 2SD for the respective population average. The data points represent measured plasma MTX concentrations. The symbols in (b) and (c) represent the mean. The error bars represent SD. *** Represents a $p < 0.0001$. AUC, area under the curve; WT, wildtype

shown in Figure S4. Images were captured by an Evos XL Core (Invitrogen).

Statistical analysis

Statistical analysis was completed using GraphPad Prism 8.0.1 (GraphPad Software). Comparison of disease progression and therapeutic response used parametric one- and two-way analysis of variances (ANOVAs). The area under the arthritic index-time curve was calculated using the trapezoidal rule in GraphPad Prism. A linear regression was performed to determine the exposure-response relationship. Comparison of clearance values, estimated MTX exposure, and liver MTX used a parametric one-way ANOVA with pairwise comparison and test for trend. A two-way ANOVA was used to determine the interaction between rifampin pretreatment and *Slco1b2* genotype. Semiquantitative histological analysis of the kidneys used a nonparametric one-way ANOVA. A p value less than 0.05 was considered statistically significant.

RESULTS

Impact of *Slco1b2*-genotype on MTX pharmacokinetics in arthritic mice after repeated dosing

PK analysis using MW/Pharm demonstrated that *Slco1b2*-genotype significantly affected MTX exposure (Figure 1a,b; Table S1, $p < 0.0001$) and clearance (Figure 1c, $p < 0.0001$). A 2.3-fold increase in average MTX exposure was observed in treated KO mice compared to treated WT mice (Figure 1b, $p < 0.0001$) and a roughly 3-fold decrease in MTX clearance (Figure 1c, $p < 0.0001$). KO mice have significantly lower amounts of MTX in the liver compared to WT (Figure 2a, $p = 0.0002$) and HET mice ($p = 0.027$) treated with the same 2 mg/kg dose.

PK impact of rifampin on MTX pharmacokinetics in nonarthritic mice

Rifampin is a known inhibitor of the OATP-transporters[34] and pretreatment with rifampin was used to pharmacologically recapitulate the genetic effects of our *Slco1b2*-knockout. Rifampin was intravenously administered into the tail vein 3 min prior to the subcutaneous administration of

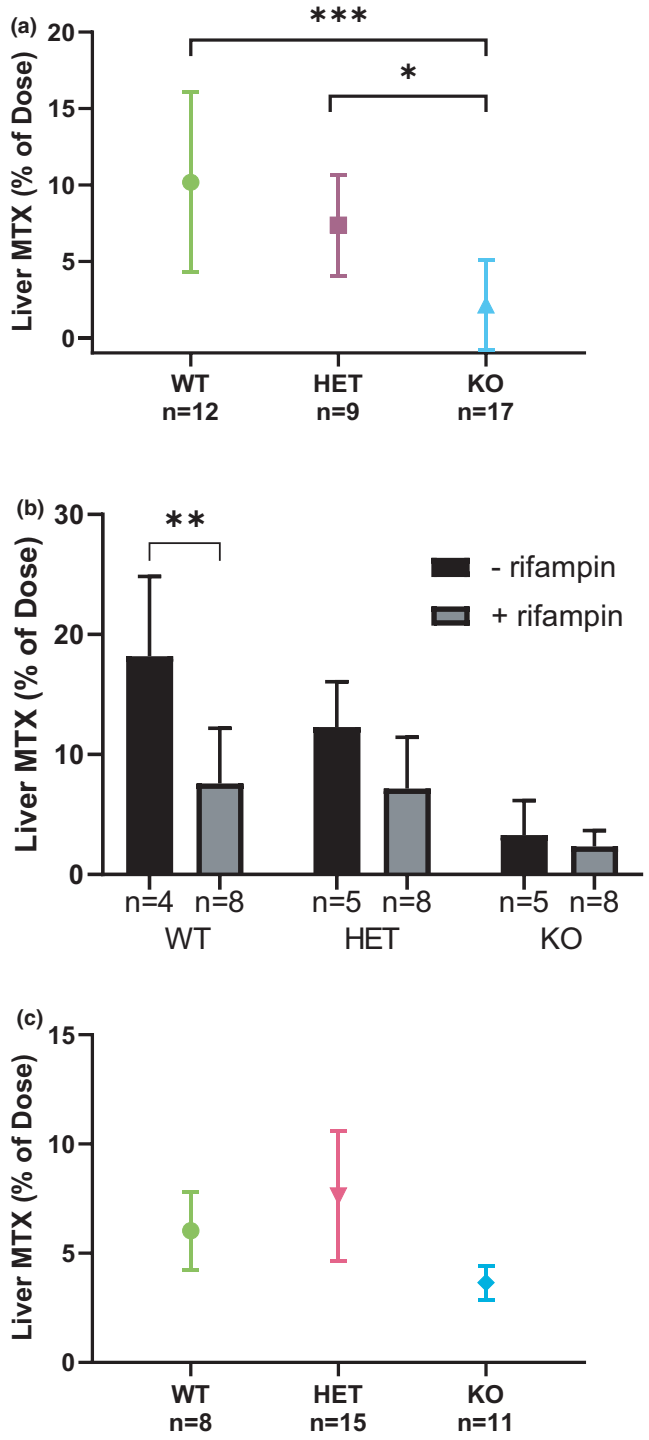

FIGURE 2 The effect of *Slco1b2*-genotype on liver methotrexate (MTX). Knockout (KO) mice (2.14%) had significantly reduced liver MTX (a) compared to wildtype (WT) mice (10.18%, $p = 0.0002$) and heterozygous (HET) mice (7.37%, $p = 0.027$). A two-way analysis of variance (ANOVA) revealed a significant interaction between rifampin pretreatment and *Slco1b2*-genotype ($p = 0.0351$, b). WT mice receiving rifampin had a 58% reduction of liver MTX, HET mice observed a 41.7% reduction of liver MTX, and KO mice observed a 29% reduction in liver MTX. The *Slco1b2*-PGx dose did not generate equivalent liver MTX across genotypes ($p = 0.0007$, c). *** Represents a $p < 0.0001$, ** represents a $p < 0.01$, and * represents a $p < 0.05$

MTX. There was a significant interaction between rifampin pretreatment and *Slco1b2*-genotype on the amount of MTX in the liver, where the transporter is expressed (Figure 2b, $p = 0.035$). Liver MTX is reduced in WT (58%) and HET (41.7%) mice pretreated with rifampin compared to mice not pretreated with rifampin (Figure 2b, $p = 0.012$, $p = 0.09$). Rifampin pretreatment does not significantly affect the amount of MTX in the liver of KO mice (Figure 2b, $p = 0.97$). With rifampin pretreatment, the amount of MTX in the livers was similar across genotypes (Figure 2b, $p = 0.53$).

Response to uniform dosing of MTX

MTX administration significantly reduced the total disease severity (AI AUC) in treated mice by more than threefold in all genotypes (Figure 3a, $p < 0.0001$). An exposure-response analysis revealed a strong, negative correlation between MTX exposure and therapeutic response ($R^2 = 0.242$, $p = 0.0027$, Figure 3c). *Slco1b2*-genotype significantly affected therapeutic response to a uniform dose of MTX (Figure 3b, $p = 0.005$). Histopathological analysis of the forepaw and knee was used to determine if *Slco1b2* genotype caused any observable differences in the articular cartilage of treated mice. KO mice treated with 2 mg/kg MTX visually demonstrate a healthy synovium in both the forepaw and knee. Untreated mice display significant erosion to the articular cartilage and inflammation of the synovium in both the forepaw and knee (Figure S4). Histopathological analysis of the kidneys was used to determine if *Slco1b2* genotype affected the degree of nephrotoxicity, specifically ATN. MTX is a known nephrotoxic agent at high-doses and injury often pathologically presents as ATN.[40] KO mice receiving a uniform dose of 2 mg/kg MTX demonstrated a 66.9% increase in total ATN compared to the WT mice (Figure S5, $p = 0.007$), which was most notably observed as significant dilation of the Bowman's capsule and retraction of the glomerular tuft (Figure S4).

Response to PGx dosing of MTX

PGx-guided MTX dosing reduced disease severity equally across genotypes compared to untreated mice (Figure 4b, $p < 0.0001$). PGx dose modification also generated equivalent therapeutic response across genotypes (Figure 4b, $p = 0.53$). There were no observable differences in the health of articular cartilage in the forepaw or knee of mice treated with an *Slco1b2*-PGx dose (Figure S4). *Slco1b2*-PGx dose modification generated no observable differences in the histopathology (Figure S4) nor statistical differences in the severity of total ATN among treated genotypes (Figure S5b, $p = 0.36$). The PGx dose modification also reduced the observable

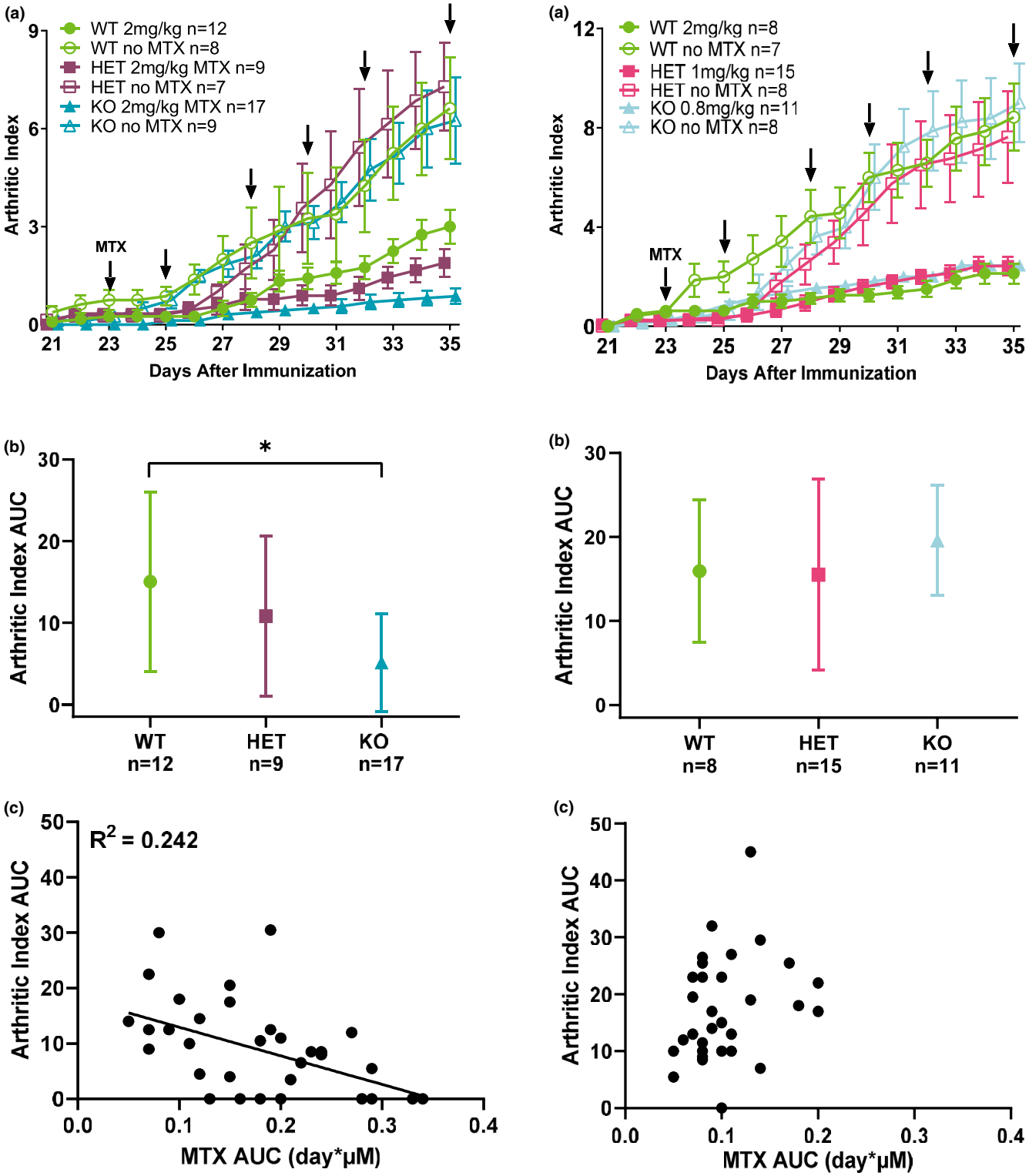

FIGURE 3 *Slco1b2*-genotype effects therapeutic response in mice with collagen-induced arthritis (CIA). Methotrexate (MTX) improved arthritic outcomes (a) and knockout (KO) mice had the greatest therapeutic response (lowest arthritic index area under the curve [AI AUC] b). This resulted in a strong, negative linear relationship between MTX AUC and AI AUC ($p = 0.0027$, c). In (a) and (b), symbols represent the mean; the error bars represent standard error of the mean (a) and SD (b). In (c), the line represents the linear regression and data points represent each mouse. * Represents a $p < 0.05$. WT, wildtype

FIGURE 4 The impact of PGx dosing on therapeutic response to methotrexate (MTX). MTX improved arthritic outcomes (a). Equivalent therapeutic response was achieved using the PGx doses for HET and KO mice ($p = 0.53$, b). The normalized exposure abolished the correlative exposure-response relationship ($R^2 = 0.074$, $p = 0.133$, c). Symbols represent the mean; the error bars represent standard error of the mean (a) and SD (b). In (c), the data points represent each mouse. AUC, area under the curve; HET, heterozygous; KO, knockout; PGx, pharmacogenetic; WT, wildtype

frequency of kidney injury in the KO mice compared with 2 mg/kg treated KO mice (Figure S4).

PGx dose modification and MTX PK

Slco1b2-PGx dose modification generated equivalent concentration-time profiles (Figure 5a; Table S2) and MTX exposure across all genotypes (Figure 5b, $p = 0.60$). Differences in MTX clearance by genotype were similar in the PGx-guided dosing and the uniform dosing study (Figure 5c, Figure 1c). There was no significant correlation between MTX exposure and therapeutic response in the PGx-guided dosing study ($R^2 = 0.074$, $p = 0.133$; Figure 4c). Despite achieving similar plasma MTX using the PGx dose modification, the percentage of the MTX dose that was found in the liver was significantly different across genotypes (Figure 2b, $p = 0.0007$).

DISCUSSION

The goal of our study was to determine the PGx impact of *SLCO1B1* on MTX PK and therapeutic response using a murine model of arthritis. Our study revealed three key findings: (1) there was a significant exposure-response relationship where increasing MTX exposure decreased arthritic severity, (2) *Slco1b2* contributes to the exposure and response variability, and (3) use of an *Slco1b2*-genotype guided dose accounted for this variability and resulted in similar exposure across genotypes.

We were able to demonstrate a significant exposure-response relationship between MTX exposure and arthritic severity. Figure 3c reveals that MTX exposure has a strong, negative correlation with therapeutic response. Therefore, increasing MTX exposure would lower arthritic severity. Previous studies describing MTX and CIA did not investigate pharmacological end points as their primary outcomes and thus, were not able to provide preclinical evidence for an exposure-response relationship for MTX.[41,42] By including PK studies in our experimental design, we were able to collect the information needed to develop a PK model for murine MTX using MW/Pharm. As a result, key PK parameters, such as exposure and clearance, allowed us to further investigate the impact of *Slco1b2* genotype on MTX exposure and response.

With this additional PK data, we were able to determine that *Slco1b2*-genotype contributes to the exposure-response relationship seen in Figure 3 where KO mice displayed significantly lower macroscopic scores of arthritis and a 66% reduction in arthritic index compared to WT mice. Figure 1 illustrates that KO mice had a twofold increase in estimated MTX exposure due to a nearly threefold reduction in MTX

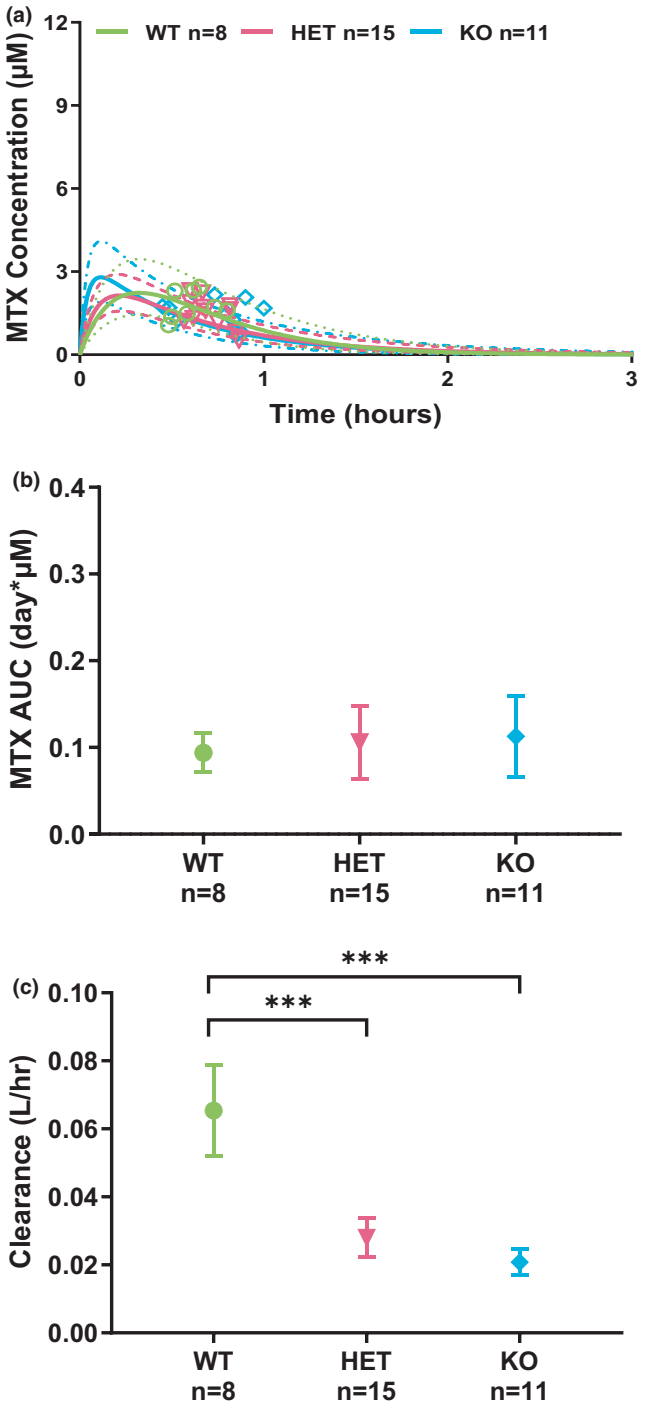

FIGURE 5 Impact of *Slco1b2*-PGx dosing on MTX PK. The mean model predicted concentration-time profiles for each genotype, with in vivo plasma MTX concentrations, depict similar plasma MTX across all genotypes (a). The PGx dose for heterozygous (HET) and knockout (KO) mice generated equivalent MTX area under the curve (AUC, b) while not changing genetic differences in MTX clearance (c). In (a), the solid lines represent the mean model predicted concentration-time profile for each genotype and the dotted lines represent the ± 2SD for the respective population average. The data points represent plasma MTX concentrations. The symbols in (b) and (c) represent the mean. The error bars represent SD. *** Represents a $p < 0.0001$. PGx, pharmacogenetic; PK, pharmacokinetic; MTX, methotrexate; WT, wildtype

clearance compared to WT mice. Visual histopathological analysis of the paws and knees revealed a healthier synovium and less damage to the articular cartilage in KO mice compared to WT mice (Figure S4). Whereas histopathological analysis of the kidneys revealed a statistically significant increase in observed kidney injury in KO mice compared to WT mice (Figure S5), the nephrotoxicity was mild and unlikely to affect patients so long as they are not concurrently taking other nephrotoxic agents. HET mice, who demonstrated intermediate MTX exposure and clearance (Figure 1b,c), demonstrated intermediate therapeutic response in the macroscopic evaluation of arthritis (Figure 3a,b).

These data support our central hypothesis that MTX exposure and therapeutic response are dependent upon *Slco1b2*-genotype. The PK end points of our study are in line with previous PK studies using Slco-gene cluster KO mice[34,43–45] and *Slco1b2*-specific KO mice.[26] When given an intravenous injection of MTX, previous Slco-gene cluster KO mice demonstrated a significant increase in peripheral MTX concentration due to a significant reduction in liver MTX. The genetic impact on liver MTX was then pharmacologically mimicked by administering rifampin prior to the MTX,[34] which was also observed in our study (Figure 2) despite having additional Slco-genes, a different mouse strain, and a different route of administration for MTX.

A major distinction from the previous Slco-gene cluster KO mouse studies is that we were able to contextualize the impact of *Slco1b2*-genotype on MTX PK by analyzing therapeutic response in a murine model of arthritis. Furthermore, we included PK modeling to quantify the effects of *Slco1b2*-genotype, thus providing estimates on MTX exposure and clearance. As a result, we were able to determine an in silico PGx dose that would generate equivalent MTX exposure across all genotypes by accounting for the differences in MTX clearance.

The *Slco1b2*-PGx dose modification was able to generate equivalent therapeutic response (Figure 4a,b) by normalizing MTX exposure across all genotypes (Figure 5a,b). Controlling MTX exposure was achieved using in silico dose modifications that were confirmed in vivo prior to their use. The PGx dose modification was a 50% dose reduction for HET mice and a 60% dose reduction for KO mice. The PK differences between the HET and KO mice were not significantly different, which is why their PGx dose reductions were similar. However, there is a significant difference in pharmaco-equivalent doses for the WT and KO mice due to the threefold difference in estimated MTX clearance. Clinical studies have shown that carriers of the decreased function *5 allele have significantly reduced clearance compared to patients with no decreased function alleles.[21–23] Therefore, we would anticipate a similar dose reduction of MTX in the clinical setting for patients harboring a decreased function allele for *SLCO1B1* as shown in our study. The use of *SLCO1B1*-PGx dose modification could optimize MTX dosing, therefore improve patient outcomes, reduce the burden of disease, minimize drug-induced side effects, and make treatment plans more efficient.

Herein, we provide evidence that *Slco1b2* affects MTX PK and therapeutic response and that *Slco1b2*-PGx dose modification can account for PK variability and optimize therapeutic outcomes. This work provides preclinical evidence for prospective PGx studies in pediatric patients and supports the use of PGx testing of *SLCO1B1* for patients with RA and JIA receiving low-dose MTX. Additional clinical support recently determined that patients with RA and JIA receiving low-dose MTX had poor treatment outcomes if they were harboring an increased function *SLCO1B1* allele.[24,25] Results from our study also support a possible dose reduction for patients carrying decreased function *SLCO1B1* alleles, which may have a more pronounced effect in children than adults.[46] Our genotype-guided dose modification would suggest that patients homozygous for decreased function alleles would benefit from a 50% dose reduction of MTX, reducing their normal starting dose of 15 mg/m^2 to a 7.5 mg/m^2 dose. This dose reduction would allow patients with poor function SLCO1B1 phenotypes to achieve desired therapeutic effects while reducing their possible risk for toxicity. To date, such dosing adjustments are being implemented world-wide for *SLCO1B1* genotype and simvastatin. A Clinical Pharmacogenetics Implementation Consortium guideline recommends a lower dose of simvastatin or alternative therapy for patients harboring the *SLCO1B1*5* allele due to increased risk of simvastatin-induced myopathy.[47]

A current limitation of clinical implementation of *SLCO1B1* testing for low-dose MTX dosing is the lack of an agreed target concentration or target exposure for therapeutic response in arthritis. Studies have shown that higher MTX polyglutamate concentrations are associated with improved therapeutic outcomes in patients with RA and JIA.[9,48] However, results remain conflicting, which highlights both the complexity of and variability in the folate pathway. Genes like *SLC19A1*, encoding RFC1, which is responsible for folate homeostasis and intracellular folate transport,[5,15] and *FPGS*, which is responsible for the polyglutamation of folate analogs, have known polymorphisms that alter kinetic activity.[5,15] These polymorphisms are challenging to study in a murine model given that a universal knockout of *Rfc1* results in embryonic death[49] and DBA/1J mice are deficient in the formation of MTX polyglutamates.[50] Currently, there is an ongoing clinical trial by the CARRA Group (PROMOTE) to investigate PGx variant genomewide associations with MTX polyglutamates, toxicity, and therapeutic outcomes in patients with JIA; this work could provide additional support for examining the effect of variation in *SLCO1B1* and several key PGx genes of interest on MTX response and toxicity in adults with RA or patients with other

autoimmune diseases that are treated with low-dose MTX (e.g., inflammatory bowel disease and psoriasis).

A limitation regarding the studies discussed above involves the treatment protocol. The aforementioned studies used a prophylactic treatment design, which is every other day treatment administered to each mouse regardless of disease progression beginning at day 21, when many mice have no arthritis. Although this treatment protocol is a widely accepted design for CIA studies, patients with arthritis would not receive MTX therapy without showing clinical signs of disease. Additionally, administration of MTX prior to the onset of disease could have delayed the onset of arthritis or reduced the incidence of disease in our mice entirely.

In summary, we have demonstrated an exposure-response relationship for MTX in a murine model of arthritis, mediated by *Slco1b2*, that is translatable to clinical care. We generated a PGx-guided dosing strategy for MTX that normalized exposure across genotypes and showed equivalent efficacy, providing a starting point for dose adjustments in patients. This collective work could provide proof of concept evidence to support pre-emptive PGx testing studies for MTX.

ACKNOWLEDGEMENTS

The authors are grateful to Alex Sparreboom and Joseph Ware for their generosity in providing the *Slco1b2*-knockout mice. Additionally, we are grateful to Sherry Thornton and Matt Flick for their guidance on the implementation of collagen-induced arthritis protocol and use of their equipment. We appreciate the Veterinary Services at CCHMC for providing excellent care of our mice, and Ethan Poweleit for assistance with sample collection. Last, we would like to thank David Hahn for his support with the HPLC.

CONFLICT OF INTEREST

L.B.R. and T.M. received research funding from BTG International. All other authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript. Z.L.T., L.E.T., and L.B.R. designed the research. Z.L.T., L.E.T., and H.B. performed the research. Z.L.T., T.M., and L.B.R. analyzed the data. A.A.V. contributed analytical tools.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Taylor ZL, Thompson LE, Bear H, Mizuno T, Vinks AA, Ramsey LB. Toward pharmacogenetic SLCO1B1-guided dosing of methotrexate in arthritis using a murine Slco1b2 knockout model. *Clin Transl Sci*. 2021;14:2267–2277. https://doi.org/10.1111/cts.13086