Folic acid pathway single nucleotide polymorphisms associated with methotrexate significant adverse events in United States veterans with rheumatoid arthritis

L.A. Davis[1-3], B. Polk[3], A. Mann[2], R.K. Wolff[4], G.S. Kerr[5], A.M. Reimold[6], G.W. Cannon[2,7], T.R. Mikuls[8,9], L. Caplan[2,3]

[1]Denver Health and Hospital Authority, Denver, CO; [2]Department of Veterans Affairs, Denver, CO; [3]University of Colorado School of Medicine, Aurora, CO; [4]University of Utah School of Medicine, Salt Lake City, UT; [5]Department of Veterans Affairs, Washington, DC; [6]Department of Veterans Affairs, Dallas, TX; [7]Department of Veterans Affairs, Salt Lake City, UT; [8]University of Omaha School of Medicine, Omaha, NE; [9]Department of Veterans Affairs in Omaha, NE, USA.

Abstract

Objective

Methotrexate (MTX) is the cornerstone medication in the treatment of rheumatoid arthritis (RA). We examined whether single nucleotide polymorphisms (SNPs) in enzymes of the folic acid pathway (folylpoly-gamma-glutamate synthetase [FPGS], gamma-glutamyl hydrolase [GGH], and methylenetetrahydrofolate reductase [MTHFR]) associate with significant adverse events (SigAE).

Methods

Patients (n=319) enrolled in the Veterans Affairs RA (VARA) registry taking MTX were genotyped for HLA-DRB1-SE and the following SNPs: FPGS (rs7033913, rs10760503, rs10106), GGH (12548933, rs7010484, rs4617146, rs719235, rs11988534), MTHFR (rs1801131, rs1801133). AE were abstracted from the medical record using a structured instrument. SigAE were defined as an AE leading to MTX discontinuation. Covariates included: age, gender, race, RA antibody status, tobacco, RA disease duration between diagnosis and MTX course, Charlson-Deyo comorbidity index, glucocorticoids, use of prior RA medications, and mean 4-variable disease activity score. Cox regression was performed to determine factors associated with time-to-SigAE. A p-value ≤0.005 established significance in the final model.

Results

The presence of ≥1 copy of the minor allele in MTHFR rs1801131 was associated with an increased hazard ratio (HR) of SigAE (HR 3.05, 95% CI 1.48-6.29, p-value 0.003 and HR 3.88, 95% CI 1.62-9.28, p-value 0.002 for heterozygotes and homozygotes for the minor allele, respectively). An interaction term, between FPGS rs7033913 heterozygotes and GGH rs11988534 homozygotes for the minor allele, had a p-value <0.0001.

Conclusion

RA subjects taking MTX may have decreased time-to-SigAE with ≥ 1 copy of the minor allele in MTHFR rs1801131. Further investigation is warranted, as these SNPs may indicate susceptibility to MTX toxicity.

Key words

rheumatoid arthritis, polymorphism, single nucleotide, drug toxicity, methotrexate

Lisa A. Davis, MD, MSCS
Brooke Polk, MD
Alyse Mann, BS
Roger K. Wolff, PhD
Gail S. Kerr, MD
Andreas M. Reimold, MD
Grant W. Cannon, MD
Ted R. Mikuls, MD, MPH
Liron Caplan, MD, PhD

Please address correspondence to:
Lisa Davis, MD MSCS,
Denver Health and Hospital Authority,
777 Bannock St.,
MC 4000, Denver,
Colorado 80204-4507, USA.
E-mail: lisa.davis@ucdenver.edu

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Introduction

Methotrexate (MTX) is the most commonly used disease modifying antirheumatic drug (DMARD) in patients with rheumatoid arthritis (RA) (1). Despite the effectiveness and clinical utility of this medication, many patients experience adverse events (AE) associated with MTX, ranging from 20–60% (2, 3). Over one-third of patients who have chronic illness express strong concerns about long-term effects of their medications (4). If health care providers could better stratify patients according to risk of AE, this may partially allay patients' anxiety and may potentially reduce the cost of screening for AE during MTX use (5), as recommended by the American College of Rheumatology (ACR).

Multiple theories have been put forth to explain why certain patients have AE associated with MTX and others do not. One theory acknowledges differences in MTX absorption, distribution, metabolism, and excretion (ADME) enzymatic activity. Examples of ADME enzymes relevant to MTX include enzymes involved in folic acid/MTX utilisation and the folic acid pathway such as folylpoly-gamma-glutamate synthetase (FPGS), gamma-glutamyl hydrolase (GGH), and methylenetetrahydrofolate reductase (MTHFR). MTX contains one glutamate moiety, and is a structural analog of folic acid (6-8). Once MTX enters the cell, FPGS catalyses the addition of up to four additional glutamates to MTX, creating polyglutamated-MTX (MTX-PG), which allows retention of the active form of MTX in the cell. GGH catalyses the *removal* of glutamates from MTX-PG, thus allowing the efflux of MTX from the cell (8). Certain minor allele substitutions may change the balance between the two enzymes and push the balance either toward greater MTX-PG (retention in the cell) or greater MTX (efflux from the cell) (8, 9). In addition to intracellular retention of MTX, MTX-PG also directly inhibits several enzymes important to purine and pyrimidine synthesis, and may indirectly affect MTHFR. MTHFR catalyses the conversion of 5'10'-methylenetetrahydrofolate to 5'-methyltetrahydrofolate, which is a methyl donor for several important processes, including *de novo* purine synthesis (9). Some specific single nucleotide polymorphisms (SNPs) previously examined in MTHFR are rs1801133 & rs1801131, where minor allele substitutions cause decreased enzymatic activity, and thus may decrease the production of purines (9). Thus, alterations in FPGS, GGH, and MTHFR activity may affect AE.

Minor alleles in the genes encoding these enzymes have all been associated with specific toxicities in RA patients (6). However, reports of the association of ADME genes with MTX AE have been inconsistent in the literature (3, 10-18). One potential explanation for the heterogeneity of findings is that many of these studies do not account for critical potential confounders such as concomitant medications, ethnicity, or comorbidity status, or may not have been of sufficient length to gather AE. Furthermore, prior studies have not leveraged the potential effects of time-to-event analyses, or do not distinguish clinically meaningful AE (those resulting in medication discontinuation) from non-significant AE.

It was therefore our goal to examine whether SNPs in genes encoding for ADME enzymes that participate in the folic acid pathway (*MTHFR, GGH, FPGS*) are associated with time-to-significant adverse events (SigAE) complicating MTX treatment, after controlling for important confounders.

Patients and methods

Setting

This sub-study of the Veterans' Affairs Rheumatoid Arthritis (VARA) prospective registry included data from three participating US Department of Veterans Affairs Medical Centers: Denver, CO; Omaha, NE; and Dallas, TX. VARA is a prospective longitudinal clinical registry and biologic repository based at 11 VA medical centers (Omaha, NE; Salt Lake City, UT; Washington, DC; Dallas, TX; Denver, CO; Jackson, MS; Portland, OR; Brooklyn, NY; and Iowa City, IA; Birmingham, AL; Little Rock, AR), which has been fully described elsewhere (19, 20). Briefly, all enrollees meet the ACR 1987 criteria for RA (21), and patients

who are enrolled allow collection of demographic and longitudinal clinical data and biologic samples (serum, plasma, DNA). As of October 2012, almost 1900 patients have been enrolled into the registry, with over 16,000 observations and 8,100 total patient years of observation. Data available through VARA and its linkage with national VA administrative datasets includes detailed pharmacy dispensing information, disease activity scores, select laboratory values, and detailed administrative (claims) data.

Patient inclusion criteria

To be included in our substudy, patients must have met the following criteria:

1) be enrolled in VARA;

2) have exposure to MTX subsequent to VARA enrolment;

3) attend rheumatology clinics at one of the three selected VARA sites (Denver, CO; Omaha, NE; or Dallas, TX).

These sites were chosen as a convenience sample due to administrative permissions. Patients of at least 18 years of age were eligible to be enrolled.

Data sources/variable definitions

– Clinical and administrative data

Patient characteristics, including age, gender, race (Caucasian *vs*. non-Caucasian), rheumatoid factor (RF) positivity, anti-citrullinated protein antibody (ACPA) positivity, tobacco use (never users *vs*. former users *vs*. current users), RA disease duration between diagnosis and the MTX course in question, and mean 28-joint four variable disease activity score (DAS-28) (22) (utilising the erythrocyte sedimentation rate and including all measurements) since the time of enrolment in VARA, were derived from the VARA Clinical Database. A Charlson-Deyo co-morbidity index was defined using International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) codes (23). These administrative data originated from links to the Veterans Affairs (VA) inpatient patient treatment files (PTF).

– Genotyping

DNA samples were derived from whole blood collected from study subjects at the time of VARA enrolment. Quantitative polymerase chain reaction was used to genotype patients' DNA. SNP selection for *FPGS* and *GGH* utilised a tagSNP approach to detect associations with SNP blocks defined by linkage disequilibrium (LD), utilising the following parameters: 1) LD blocks were defined using a Caucasian LD map TagSNPs with an r^2=0.8; 2) minor allele frequency (MAF) >0.1; 3) range of -1,500 basepairs (bps) from the initiation codon to +1,500 bps from the termination codon; and 4) 1 SNP/LD bin. *MTHFR* SNPs were chosen a priori based upon previously published reports (16, 18).The patients were genotyped for the following SNPs: *FPGS* (rs7033913, rs10760503, rs10106); *GGH* (rs12548933, rs7010484, rs4617146, rs719235, rs11988534) and *MTHFR* (rs1801131, rs1801133). Genotyping was performed using either a BeadExpress platform (Illumina, San Diego, CA) (SNPs for *FPGS, GGH,* and *MTHFR* 1801133) or by TaqMan assay (*MTHFR* 1801131) using a GeneAmp 9700 PCR machine (Applied Biosystems, Foster City, CA) with endpoint analysis on a PRISM 7900HT Sequence Detection System (Applied Biosystems). Additionally, the participants were genotyped for the human leukocyte antigen shared epitope (HLA-DRB1-SE) containing alleles as previously described (24).

– Pharmacy data

We derived MTX and glucocorticoid use data through linkage of VARA with the Pharmacy Benefits Management (PBM), a pharmacy-specific program within the VA. We defined separate MTX courses according to the presence of a 90-day gap in the MTX that would be available to patients based upon VA prescription dispensing data. A 90-day gap was selected because of the preference of 90-day refills within the VA and because previous analyses have demonstrated similar results using 30-, 60-, and 90-day gaps (25). We defined glucocorticoid use based on whether subjects were ever dispensed oral glucocorticoids during the time of the MTX prescription and we used PBM pharmacy data to determine whether MTX was the first disease-modifying anti-rheumatic drug (DMARD).

– Adverse events

We systematically reviewed cohort participants' medical records for MTX-associated AE from the time of MTX initiation, as defined by the first dispensed prescription of MTX in the VA pharmacy benefits management (PBM), until 120 days following the end of their last MTX prescription. We selected 120 days due to the usual follow up time in the VA (typically every 3–4 months), and due to the pharmacology of MTX. Serum levels of MTX are reduced by 80-95% within 24 hours (26), however, various MTX-PG persist an average 4-10 weeks until they are undetectable after cessation of MTX (27). We used a structured abstraction instrument and standard definitions to define AE, and we included only those AE thought to be "likely" or "probably" related to MTX by the provider, and excluded those characterised as "possibly" or "unlikely." We classified the AE within the following categories and subcategories: 1) *dermatologic*: alopecia, rash, photosensitivity, or nodulosis; 2) *gastrointestinal*: oral ulcers/stomatitis, nausea/vomiting/anorexia/dyspepsia, or diarrhoea; 3) *haematologic*: leukopenia (peripheral white blood cell count <3,500), thrombocytopenia (platelets <100,000), or new-onset anaemia (haemoglobin <13.5g/dL in men and <10.0 g/dL in women); 4) *hepatic*: transaminitis greater than the upper limit of normal for the local clinical laboratory, fibrosis, or cirrhosis; 5) *infectious*: infections that are recurrent/persistent defined by the treating health care provider; 6) *central nervous system*: headache, dizziness/vertigo, fatigue/malaise within 48 hours of MTX, depression/mood alteration, or memory impairment; 7) *respiratory*: dry cough, dyspnea, or interstitial lung disease (ILD); and 8) *other*: any AE defined by treating physician or patient as associated with MTX (3, 28, 29).

– Significant adverse events

We defined a significant adverse event (SigAE) as an AE attributed to MTX and preceding MTX cessation by ≤120 days; thus, a SigAE is thought to be related to MTX discontinuation. For the time-to-event analysis, we defined the time-to-SigAE as the number of days from the beginning of the course of

Table I. Cohort demographics, divided by those with and without significant adverse events (SigAE) within 7 years' time.

	Cohort			no SigAE			SigAE			
	n=319	%, mean	SD*	n=236	%, mean	SD	n=83	%, mean	SD	*p*- value
Age, mean	319	68.75	10.89	236	68.59	10.68	83	69.19	11.52	0.670
Gender, male, %	314	92.04		83	92.77		231	91.77		0.774
Caucasian, %	314	80.89		231	79.65		83	84.34		0.352
RF positive, %	314	84.71		231	84.42		83	85.54		0.807
ACPA positive, %	265	81.89		193	81.87		72	81.94		0.988
Tobacco Use	314			231			83			0.334
Never	62	19.75		50	21.65		12	14.46		
Former	158	50.32		115	49.78		43	51.81		
Current	94	29.94		66	28.57		28	33.73		
Yrs RA dx and MTX, mean	314	9.37	10.84	231	9.48	10.83	83	9.07	10.91	0.767
MTX first DMARD	152	52.63		62	40.79		18	11.84		0.347
Deyo Index, mean	238	1.78	2.17	175	1.66	2.18	63	2.10	2.15	0.176
Glucocorticoid Use, %	200	63.50		151	60.93		49	71.43		0.185
DAS-28, mean	311	3.86	1.10	229	3.85	1.14	82	3.87	0.99	0.863
HLA-DRB1-SE	308			227			81			**0.018**
0 copies	88	28.57		73	32.16		15	18.52		
1 copy	154	50.00		103	45.37		51	62.96		
2 copies	66	21.43		51	22.47		15	18.52		
MTHFR, rs1801131	250			185			65			**0.001**
Homo major (AA)	109	43.60		93	50.27		16	24.62		
Het (AC)	105	42.00		71	38.38		34	52.31		
Homo minor (CC)	36	14.40		21	11.35		15	23.08		
MTHFR, rs1801133	251			186			65			0.335
Homo major (CC)	131	52.19		92	49.46		39	60.00		
Het (CT)	95	37.85		74	39.78		21	32.31		
Homo minor (TT)	25	9.96		20	10.75		5	7.69		
FPGS, rs7033913	250			186			64			0.173
Homo major (TT)	84	33.60		66	35.48		18	28.13		
Het (TC)	122	48.80		92	49.46		30	46.88		
Homo minor (CC)	44	17.60		28	15.05		16	25.00		
FPGS, rs10760503	250			185			65			0.027
Homo major (AA)	79	31.60		53	28.65		26	40.00		
Het (AG)	128	51.20		104	56.22		24	92.31		
Homo minor (GG)	43	17.20		28	15.14		15	23.08		
FPGS, rs10106	250			185			65			0.046
Homo major (AA)	82	32.80		55	29.73		27	41.54		
Het (AG)	133	53.20		107	57.84		26	40.00		
Homo minor (GG)	35	14.00		23	12.43		12	18.46		
GGH, rs12548933	251			186			65			**<0.001**
Homo major (AA)	175	69.72		138	74.19		37	56.92		
Het (AC)	65	25.90		37	19.89		28	43.08		
Homo minor (CC)	11	4.38		11	5.91		0	0.00		
GGH, rs7010484	242			179			63			0.283
Homo major (TT)	103	42.56		80	44.69		23	36.51		
Het (TC)	110	45.45		76	42.46		34	53.97		
Homo minor (CC)	29	11.98		23	12.85		6	9.52		
GGH, rs4617146	249			184			65			0.865
Homo major (CC)	168	67.47		124	67.39		44	67.69		
Het (CT)	72	28.92		54	43.55		18	27.69		
Homo minor (TT)	9	3.61		6	3.26		3	4.62		
GGH, rs719235	251			186			65			0.331
Homo major (GG)	138	54.98		99	53.23		39	60.00		
Het (GT)	96	38.25		72	38.71		24	36.92		
Homo minor (TT)	17	6.77		15	8.06		2	3.08		
GGH, rs11988534	251			186			65			0.276
Homo major (CC)	141	0.56		110	59.14		31	47.69		
Het (CT)	90	35.86		62	33.33		28	43.08		
Homo minor (TT)	20	7.97		14	7.53		6	9.23		

*SD: standard deviation; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibody; Yrs RA dx and MTX: years between rheumatoid arthritis diagnosis and methotrexate course; MTX: methotrexate; DMARD: disease-modifying anti-rheumatic drug; DAS-28: 28 joint 4-variable disease activity score; HLA-DRB1-SE: major histocompatibility complex, class II, DR beta 1 shared epitope, high-risk for rheumatoid arthritis; MTHFR: methylenetetrahydrofolate reductase; FPGS: folypoly-gamma-glutamate synthetase; GGH: gamma-glutamyl hydrolase; ACPA: anti-citrullinated protein antibody; homo: homozygous; het: heterozygous

MTX to the first SigAE. If no SigAE was identified, subjects were censored one day after the end of their MTX course.

Statistical methods

Hardy-Weinberg *p*-values, stratified by race (30), were calculated using chi squared tests. We examined whether the assumptions of the Cox proportional hazards were satisfied in our multivariable models by graphing the hazards. SNPs were not presumed to behave in any particular manner (dominant, codominant, recessive) and thus were modeled separately in the models. Characteristics for subjects with a SigAE and those without a SigAE were compared using Student's *t*-test or chi squared test, as appropriate. We utilised Cox proportional-hazards regression to determine factors associated with time-to-SigAE, and we clustered on individual patients when they contributed more than one course of MTX use to the analysis. Analyses were limited to seven years due to attrition of participants' MTX courses. Variables with an alpha level ≤0.2 in the univariate analysis were included in an intermediate model, which was then evaluated for interaction terms, which might indicate the presence of epistasis. Variables and interaction terms from the intermediate model with a *p*-value ≤0.05 were retained in the final model. In order to account for multiple SNPs being tested, a conservative alpha level ≤0.005 (Bonferroni correction) was considered significant in the final model. All analyses were performed using STATA version 12 (College Station, TX).

Ethics

Each participating VARA site obtained Institutional Review Board approval, and all participants were enrolled with informed consent. The Colorado Multiple Institutional Review Board granted approval for this VARA sub-study, as did the VARA Scientific Ethics Advisory Committee. No vulnerable populations are enrolled in VARA, including children, neonates, prisoners, or pregnant women, and this study is in accordance to the Declaration of Helsinki (31).

Table II. Adverse events and significant adverse events.

	All AE*		SigAE	
Adverse event	n=642	%	n=93	%
Dermatologic	16	2.49	0	0.00
Alopecia	3	0.47	0	0.00
Rash	6	0.93	0	0.00
Photosensitivity	1	0.16	0	0.00
Nodulosis	7	1.09	0	0.00
Gastrointestinal	79	12.31	11	11.83
Oral Ulcers/Stomatitis	19	2.96	1	1.08
N/ V/ Anorexia/ Dyspepsia	54	8.41	7	7.53
Diarrhea	11	1.71	3	3.23
Haematologic	195	30.37	20	21.51
Leukopenia	13	2.02	0	0.00
Thrombocytopenia	6	0.93	1	1.08
Anaemia	179	27.88	19	20.43
Hepatic	168	26.17	17	18.28
Transaminitis	168	26.17	17	18.28
Fibrosis	0	0.00	0	0.00
Cirrhosis	0	0.00	0	0.00
Infectious Disease	81	12.62	12	12.90
Central Nervous System	15	2.34	1	1.08
Headache	4	0.62	0	0.00
Dizziness/Vertigo	1	0.16	0	0.00
Fatigue/Malaise	9	1.40	1	1.08
Depression/Mood	1	0.16	0	0.00
Memory Impairment	1	0.16	0	0.00
Respiratory	45	7.01	21	22.58
Dry Cough	15	2.34	5	5.38
Dyspnea	21	3.27	8	8.60
Interstitial Lung Disease	26	4.05	14	15.05
Other	46	7.17	11	11.83

*AE: adverse events; SigAE: significant AE; N: nausea; V: vomiting.

Results

Of the approximately 1400 subjects enrolled in VARA at the time the genetic analysis was performed, 1011 had genetic samples available for analysis. Of these, 319 individuals met the inclusion criteria and were abstracted for AE, resulting in 1196 total patient-years of observation. The average individual had 2.0 courses of MTX (95% confidence interval [CI] 1.9–2.1). The mean length of the MTX course for the entire cohort was 719 days (95% CI 655–783 days), while the mean duration of MTX course was 511 days (95% CI 428–595 days) for those with a SigAE and 804 days (95% CI 722–887 days) for those without a SigAE (*p*-value <0.0001).

The demographics of the cohort are shown in Table I. Overall, our cohort was elderly (mean age 69 years), male (92%), Caucasian (81%), seropositive (85% RF positive, 82% ACPA positive), former smokers (50%), using concomitant glucocorticoids (64%), and with an average DAS-28 of 3.86, consistent with moderate disease activity. Significant differences between those who had a SigAE and those who did not were observed in the detection of HLA-DRB1-SE in addition to *MTHFR* rs1801131, *FPGS* rs10760503 and *GGH* rs12548933. The cohort, stratified by race, was in Hardy-Weinberg equilibrium.

Totals of the AE and SigAE can be found in Table II. These 642 AE were recorded in 270 individuals. First AE (n=268) occurred over 497 person years (PY) of observation, yielding an incidence rate of first AE of 0.54 per PY (95% CI 0.48–0.61). SigAE (n=93) had an incidence rate of 0.08 per PY (95% CI 0.06–0.10). Of the AE, haematologic abnormalities were the most common, with 28% of all AE being new-onset anaemia. The most common single SigAE was new-onset anaemia. The most common SigAE category was respiratory, with ILD comprising 15% of all SigAE. However, ILD contributed only 4% of all AE. There were no cases of hepatic fibrosis or cirrhosis in our cohort.

Table III. Initial and final models for the Cox regression of predictors of time-to-significant adverse event (SigAE).

		Initial Model				Final Model			
Variable		HR*	95% CI		*p*-value	HR	95% CI		*p*-value
Age		1.00	0.98	1.01	0.750				
Gender		0.96	0.42	2.20	0.919				
Caucasian		0.88	0.49	1.56	0.655				
RF positive		0.98	0.53	1.82	0.955				
ACPA positive		0.96	0.53	1.74	0.890				
Tobacco use		1.25	0.90	1.72	0.185				
Yrs RA dx and MTX		1.00	1.00	1.00	0.571				
MTX first DMARD		0.72	0.38	1.38	0.328				
Deyo Index		1.06	0.97	1.16	0.221				
Glucocorticoid use		2.07	1.16	3.69	0.014	2.28	1.16	4.49	0.017
Avg. DAS-28		1.09	0.90	1.32	0.389				
HLA-DRB1-SE		1.16	0.90	1.49	0.253				
MTHFR, rs1801131	Homo major (AA)	1.72	1.25	2.38	0.001	ref.			
	Het (AC)					3.05	1.48	6.29	0.003
	Homo minor (CC)					3.88	1.62	9.28	0.002
MTHFR, rs1801133		0.80	0.54	1.19	0.272				
FPGS, rs7033913	Homo major (TT)	1.35	0.93	1.94	0.112	ref.			
	Het (TC)					0.99	0.39	2.48	0.976
	Homo minor (CC)					0.38	0.04	3.54	0.393
FPGS, rs10760503		0.87	0.61	1.24	0.444				
FPGS, rs10106		0.80	0.56	1.15	0.232				
GGH, rs12548933		1.30	0.91	1.85	0.149				
GGH, rs7010484		1.07	0.74	1.56	0.708				
GGH, rs4617146		0.95	0.60	1.50	0.834				
GGH, rs719235		0.84	0.56	1.27	0.409				
GGH, rs11988534	Homo major (CC)	1.30	0.97	1.74	0.083	ref.			
	Het (CT)					0.74	0.15	3.61	0.712
	Homo minor (TT)					1.78	0.42	7.51	0.430
rs7033913* rs11988534	Homo major/ Homo major					ref.			
	Het/Het					3.22	0.54	19.33	0.201
	Het/Homo minor					$1.29\ e^{-17}$	$1.88\ e^{-18}$	$8.81\ e^{-17}$	<0.001
	Homo minor/ Het					22.21	1.47	336.80	0.025
	Homo minor/ Homo minor					4.74	0.32	70.76	0.259

*HR: hazard ratio; Std. Err.: standard error; 95% CI: 95% confidence interval; RF: rheumatoid factor; ACPA: anti-cittrulinated protein antibody; RA: rheumatoid arthritis; Yrs RA dx and MTX: years between rheumatoid arthritis diagnosis and methotrexate course; MTX: methotrexate; DMARD: disease-modifying anti-rheumatic drug; DAS-28: 28 joined 4-variable disease activity score; HLA-DRB1-SE: major histocompatibility complex, class II, DR beta 1 shared epitope; ref: referent; MTHFR: methylenetetrahydrofolate reductase; FPGS: folypoly-gamma-glutamate synthetase; GGH: gamma-glutamyl hydrolase; ACPA: anti-citrullinated protein antibody; Homo: homozygous; Het: heterozygous; rs7033913 *rs11988534: interaction term between FPGS rs7033913 & GGH rs11988534.

The results of the initial and final models of the Cox regressions are shown in Table III. The final model had a resulting Harrell's C statistic of 0.731. One SNP and one interaction term were significant predictors of time-to-SigAE in the final multivariable models. *MTHFR* rs1801131 heterozygotes for the minor allele (AC) had a hazard ratio (HR) of 3.05, 95% confidence interval (CI) of 1.48–6.29 with a *p*-value of 0.003, while homozygotes for the minor allele (CC) had a HR of 3.88, 95% CI 1.62–9.28, *p*-value 0.002. Figure 1 shows the Nelson-Aalen cumulative hazard curves for time-to-discontinuation of MTX for *MTHFR* rs1801131 (logrank *p*-value=0.002). One interaction term, representing the epistasis of *FPGS* rs7033913 heterozygotes on *GGH* rs11988534 homozygotes for the minor allele, was significant with a *p*-value <0.0001.

Discussion

In our study, we examined genetic polymorphisms in enzymes that participate in the folic acid pathway in order to determine their association with SigAE, or adverse events linked with the cessation of MTX. We found that RA subjects taking MTX were at increased risk of SigAE if they have at least one copy of the minor allele in *MTHFR* rs1801131. In a post hoc analysis examining the attributable risk for SigAE from the minor allele, we found that *MTHFR* rs1801131 minor allele accounts for 50.6% of the risk of the SigAE in those with the *MTHFR* rs1801131 minor allele, and *MTHFR* rs1801131 accounts for 37.2% of the risk of SigAE in the entire population. The *MTHFR* rs1801131 minor allele is known to result in reduced enzymatic activity (9), and our findings are consistent with this reduced activity. However, our work is in contrast to that in several other studies, including that of Fisher and Constein(6) and Ranganathan *et al*. (3). Fisher and Cronstein (6) performed a metaanalysis of MTHFR polymorphisms and toxicity, and found that *MTHFR* rs1801133, rather than rs1801131, was associated with increased toxicity. Ranganathan *et al*. (3) evaluated six genes in the MTX path-

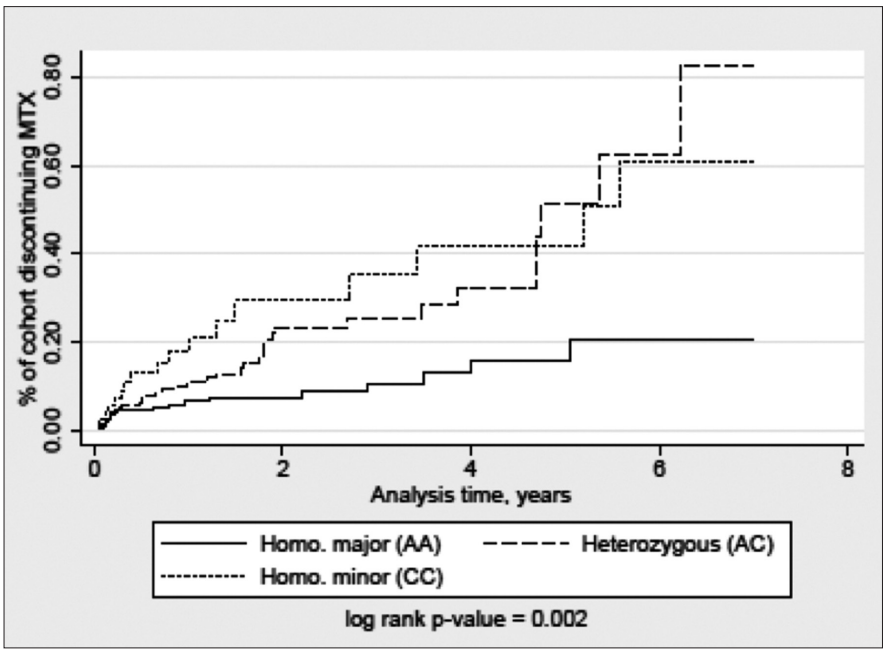

Fig. 1. Discontinuation of methotrexate, by *MTHFR* rs1801131 status.

way, including *MTHFR*, in a retrospective cohort study utilising a cross-validation design; they also found *MTHFR* rs1801133, rather than rs1801131 was associated with alopecia. While the reason for the discrepancy between our findings and those of prior investigators remains uncertain, we suspect that the difference might be attributed to the definitions of toxicity used in our individual studies (our definition incorporated an explicit definition and required discontinuation of the medication), the statistical methods (time-to-event in our study versus logistic regression), and/or the populations (though the Ranganathan *et al*. study was also performed in a VA population, the population studied was only 65% Caucasian). As our work is hypothesis-generating, we believe that further investigation is warranted, as this SNP may indicate a susceptibility to MTX toxicity.

Additionally, we found that an interaction between *FPGS* rs7033913 and *GGH* rs11988534 was significant for the heterozygote/homozygous minor individuals (HR 1.29 e^{-17}, 95% CI 1.88e^{-18}-8.81e^{-17}, *p*-value <0.0001). While this interaction is statistically significant, this significance may be due to small numbers, as only 6 individuals were in this category.

We found that, on average, a patient will experience one AE over a typical two-year period of treatment with MTX. However, the rate of SigAE was much lower – approximately one SigAE per six people on a typical two-year course of MTX. Investigators have previously suggested that such a rate of SigAE may be sufficient to frustrate prevailing treat-to-target strategies (32).

We found that the single most common AE in the general cohort was haematologic (30% of all AE), with new-onset anaemia the leading subcategory. For the SigAE, respiratory was the most common category (23% of all SigAE), with 15% of SigAE related to ILD. However, the incidence of ILD was low in the total cohort, at 4%. This prevalence of ILD-related SigAE may reflect health care providers' heightened sensitivity for MTX-associated ILD, rather than true incident ILD. Our inability to access confirmatory tests for ILD prevents us from conclusively distinguishing these alternatives. However, the rate of SigAE related to ILD was greater than elevated hepatic enzyme SigAE, despite the prevailing practice of systematic testing for hepatic AE through routine laboratory assays.

Low-dose MTX (10–25mg/week) is considered the first-choice therapy among DMARDs for the treatment of RA (28) and is recommended as either monotherapy or in combination therapy in those with markers of poor prognosis in RA (33). Once the decision has been made to start therapy with MTX, the ACR recommends obtaining baseline complete blood count, liver transaminase levels, serum creatinine, and performing screening for hepatitis B and C in those at higher risk for these conditions. Following the initiation of MTX, these guidelines recommend that complete blood count, liver transaminase levels, and serum creatinine be followed every 2–4 weeks for 3 months, and then every 8–12 weeks thereafter (5). Based upon our retrospective observations, the average SigAE occurs approximately 1.4 years into a course of MTX, indicating that the recommended continual monitoring for MTX AE may be justified. Due to the high prevalence of respiratory SigAE, it may be beneficial to perform a baseline chest x-ray or computed tomography of the chest to differentiate between RA-associated ILD and MTX-associated ILD, though cost-benefit analyses should be performed prior to such a recommendation. Additionally, if the *MTHFR* rs1801131 minor allele proves to be associated with increased risk of SigAE, testing for this particular polymorphism may be advantageous in guiding drug therapy and drug monitoring.

Limitations

The AE/SigAE were derived from retrospective analysis of medical records. While we used a structured instrument to abstract these events, it is likely that some minor AE were not recorded in the medical records, resulting in underestimates for these AE. Additionally, since it is a retrospective analysis of data, we cannot prove that MTX was directly responsible for the AE recorded in the medical record. Though folic acid supplementation is thought to decrease AE, it cannot be accurately tracked in the VA system as many veterans receive it over-the-counter. However, as many US products are widely supplemented with folic acid, (34) tracking over-the-counter supplementation is theoretically less important. Another limitation is that we cannot definitively link the diagnosis of ILD to MTX use rather than the un-

derlying RA. For the ILD to be counted as MTX-associated, the ILD must have been described by the health care provider to be "likely" or "probably" associated with MTX. However, not all patients had a baseline chest x-ray exam. Furthermore, our study cohort is largely older Caucasian males with long-standing RA. Hence, our results may not generalise to other RA populations. Finally, we did not study all SNPs that have previously been identified as potentially impacting MTX metabolism (7, 35), including the ATP-binding cassette transporters (ABC transporters).

Strengths

As part of a structured and comprehensive health care system, VARA is significantly strengthened by the fact that a large majority of included patients receive medical care solely through the VA network, and thereby the vast majority of each patient's medical records were available for abstraction. VARA has been acquiring data since 2003 and is an established registry with 1196 patient-years abstracted for this study. This study was performed in a relatively large cohort (n=319), and AE were abstracted using a structured instrument. Moreover, we differentiated between AE and SigAE, which is useful in a clinical setting to help determine the factors associated with actual MTX cessation, and is unique among published studies of MTX AE. We incorporated time-to-event analysis, which increases the power of the study. Finally, this study involves a population that is uniquely vulnerable to MTX AE given their advanced age, and does represent a "real-world" population of elderly Caucasian patients.

In summary, our study has shown the association of *MTHFR* rs1801131 with time-to-significant adverse event in those taking methotrexate for treatment of rheumatoid arthritis. While our work is hypothesis-generating, we believe that the next step in the pursuit of pharmacogenomics related to MTX toxicity is to expand the SNPs evaluated, while applying the time-to-event modeling methods. Utilising these methods may uncover additional SNPs associated with MTX toxicity.

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