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Tese de Doutoramento em Ciências Médicas

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

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METHOTREXATE PHARMACOGENOMICS AND PREDICTORS OF THERAPEUTIC OUTCOME IN RHEUMATOID ARTHRITIS

Tese de Candidatura ao grau de Doutor em Ciências Médicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Aos "Vs da minha Vida" dedico, com amor, o poema "O Aprendiz" da autoria de Carolina Represas.

Ao Vítor, por todos os dias me fazer acreditar nesta mensagem e, à Victória, para que a leia durante toda a sua Vida e acredite nas suas palavras.

Quantos pores-do-sol fizeram uma alma cantar
Quantos sorrisos criaram outros em redor
Quantos corações falaram sem se controlar
Não contei

Quantas crianças gritaram o que é melhor
Quantas caras que eram frias ganharam sabor
Quantas mãos se deram, e quantos lábios se tocaram
Só porque sim

Oiço o que o céu não diz
Sinto o que vento não toca
Aprendo com o aprendiz
E aceito com peito a derrota
Lembro o que eu não fiz mas vou
Ainda vou fazer por mim

Tanta gente que quer, tanta história pela frente
Tanta luta no desejo e o agir indiferente
Tenta marcar o teu passo e amanhã estar presente
Eu nunca falto
Entre dias mais cinzentos e outros de cor
Há que procurar as tintas e pintar calor
E se o hoje se confunde lembra-te do ontem
E sorri

Podes ter a lua e o sol
Pegadas no teu caminho
Só não rasgues páginas em branco
O livro não se escreve sozinho

NOTA PRELIMINAR

Declara-se, para informação do papel do candidato na execução desta Tese, que o autor participou da conceção e execução do trabalho experimental que deu origem aos resultados, bem como na análise, interpretação e elaboração dos artigos científicos integrantes desta dissertação. O autor escreveu ainda a introdução, a discussão e as conclusões, salvaguardando as correções e recomendações providenciadas pelos seus orientadores.

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O trabalho de investigação foi realizado no Grupo de Oncologia Molecular do Instituto Português de Oncologia do Porto Francisco Gentil, Entidade Pública Empresarial, em colaboração com o Instituto Superior de Ciências da Saúde - Norte e o Centro Hospitalar de São João, Entidade Pública Empresarial.

Porto, 30 de julho de 2014

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Áurea Rosa Nunes Pereira Lima

RESULTADOS CIENTÍFICOS

De acordo com o disposto no n.º 1 do artigo 34.º do Decreto-Lei n.º 74/2006, publicado em Diário da República, 1.ª série, n.º 60 de 24 de Março de 2006, e republicado pelo Decreto-Lei n.º 115/2013, publicado em Diário da República, 1.ª série, n.º 151 de 7 de Agosto de 2013, que procede à terceira alteração ao Decreto-Lei n.º 74/2006, de 24 de Março de 2006, desta dissertação resultaram várias contribuições científicas, apresentadas sob a forma de artigos que se encontram publicados, aceites ou submetidos, a revistas científicas internacionais sujeitas a arbitragem:

Artigos Originais

Aurea Lima, Miguel Bernardes, Hugo Sousa, Rita Azevedo, Lúcia Costa, Francisco Ventura, Vítor Seabra, Rui Medeiros. *SLC19A1* 80G allele as a biomarker of methotrexate-related gastrointestinal toxicity in Portuguese rheumatoid arthritis patients. *Pharmacogenomics* (Fator de Impacto em 2012: 3.86). 2013. doi: 10.2217/pgs.13.244. [Publicado].

Aurea Lima, Joaquim Monteiro, Miguel Bernardes, Hugo Sousa, Rita Azevedo, Vitor Seabra, Rui Medeiros. Prediction of methotrexate clinical response in Portuguese rheumatoid arthritis patients: implication of *MTHFR* rs1801133 and *ATIC* rs4673993 polymorphisms. *BioMed Research International* (Fator de Impacto em 2012: 2.88). 2014. doi: http://dx.doi.org/10.1155/2014/368681. [Publicado].

Aurea Lima, Vítor Seabra, Miguel Bernardes, Rita Azevedo, Hugo Sousa, Rui Medeiros. Role of key *TYMS* polymorphisms on Methotrexate therapeutic outcome in Rheumatoid Arthritis Portuguese patients. [Submetido].

Aurea Lima, Miguel Bernardes, Rita Azevedo, Joaquim Monteiro, Hugo Sousa, Rui Medeiros, Vitor Seabra. *SLC19A1*, *SLC46A1* and *SLCO1B1* polymorphisms as predictors of Methotrexate-related Toxicity in Portuguese Rheumatoid Arthritis patients. *Toxicological Sciences* (Fator de Impacto em 2012: 4.33). [Aceite].

Artigos de Revisão

Aurea Lima, Rita Azevedo, Hugo Sousa, Vitor Seabra, Rui Medeiros. Current approaches for *TYMS* polymorphisms and their importance in molecular epidemiology and pharmacogenetics. *Pharmacogenomics* (Fator de Impacto em 2012: 3.86). 2013; 14(11):1337-1351. doi: 10.2217/pgs.13.118. [Publicado].

Aurea Lima, Hugo Sousa*, Joaquim Monteiro*, Rita Azevedo*, Rui Medeiros, Vitor Seabra. Genetic polymorphisms in low-dose methotrexate transporters: the current relevance as methotrexate therapeutic outcome biomarkers. *Pharmacogenomics* (Fator de Impacto em 2012: 3.86). [Aceite].

*** Igual Contribuição.**

PREFÁCIO DO AUTOR

Sempre acreditei que, "ser Farmacêutico é bem mais do que ser o técnico do medicamento… e que tal implicava encarar a profissão com garra e sentimento". Hoje, fruto dos conhecimentos apreendidos e das experiências vividas, percebo que o Farmacêutico está para o medicamento como o Médico está para a doença, o que me apazigua mas também me inquieta, pois não sei quem está realmente para o doente?. No meio desta inquietude, percebo que da integração do medicamento na doença resulta a dimensão holística do doente e que, nesta se enquadra a razão de existir da Farmacogenómica.

A perceção de que cada um de nós é diferente dos demais é assustadora mas igualmente motivante. E pensar que a individualidade começa tão precocemente, teoricamente desde logo após o melhor espermatozóide fecundar o melhor óvulo. E, desde então, há uma série de múltiplos fatores que influenciam o património genético, aquilo que somos, em fases tão peculiares da Vida e até ao momento em que a Vida nos devolve à Terra.

Fruto dos avanços técnico-científicos, a longevidade é uma realidade! Vivemos claramente mais anos mas, e infelizmente, tal não tem uma relação linear com o viver bem e/ou melhor. Talvez o facto de o Homem não ter sido concebido para viver no ambiente complexo que caracteriza o século XXI possa constituir uma explicação. Já Darwin havia percebido que só os mais aptos sobreviveriam, pelo que, por vezes reflicto sobre as doenças como manifestação dessa inadaptabilidade, e que o Homem tanto tenta vencer.

Assim, acredito na Farmacogenómica como contributo indispensável à Medicina Personalizada, e creio que o futuro da Saúde e a Qualidade de Vida dos doentes passará por "olhar cada doente" como um Ser único, integrando a Farmacogenómica na sua dimensão biopsicossociocultural, de forma a colmatar as suas imperfeições de adaptação às características do Mundo em que Vive.

REFLEXÃO DO AUTOR

Este é mais um marco da minha Vida
Pautado por esforço, trabalho, dedicação e sacrifício
Marcado por quatro anos de lágrimas e gargalhadas,
Por leitura, escrita, discussão de ideias e aquisição de saber.

Tantas coisas aprendi e vivi.
Como cresci…

Muitos me perguntam como consegui?.
Nem eu sei…

No meio de tantas outras coisas, foi árduo
Mas, hoje estou aqui!
Estou certa de que algum sorriso eu perdi,
Na esperança de um dia o resgatar.

Mas só as lembranças que doem ou fazem sorrir deixam saudade…

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A ti, Avô, que mesmo tendo partido vives todos os dias no meu coração, obrigada por me acompanhares no pensamento.

Ao meu irmão e à minha cunhada, que julgo não perceberem muito bem o porquê de tudo isto, agradeço os momentos de lazer que me proporcionaram, as suas atitudes que me permitiram refletir se tanto trabalho faz sentido, se viver é isto…

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À Lu, o meu coração salta de cada vez que te vejo a correr… como és fofinha. Obrigada pelos teus miminhos.

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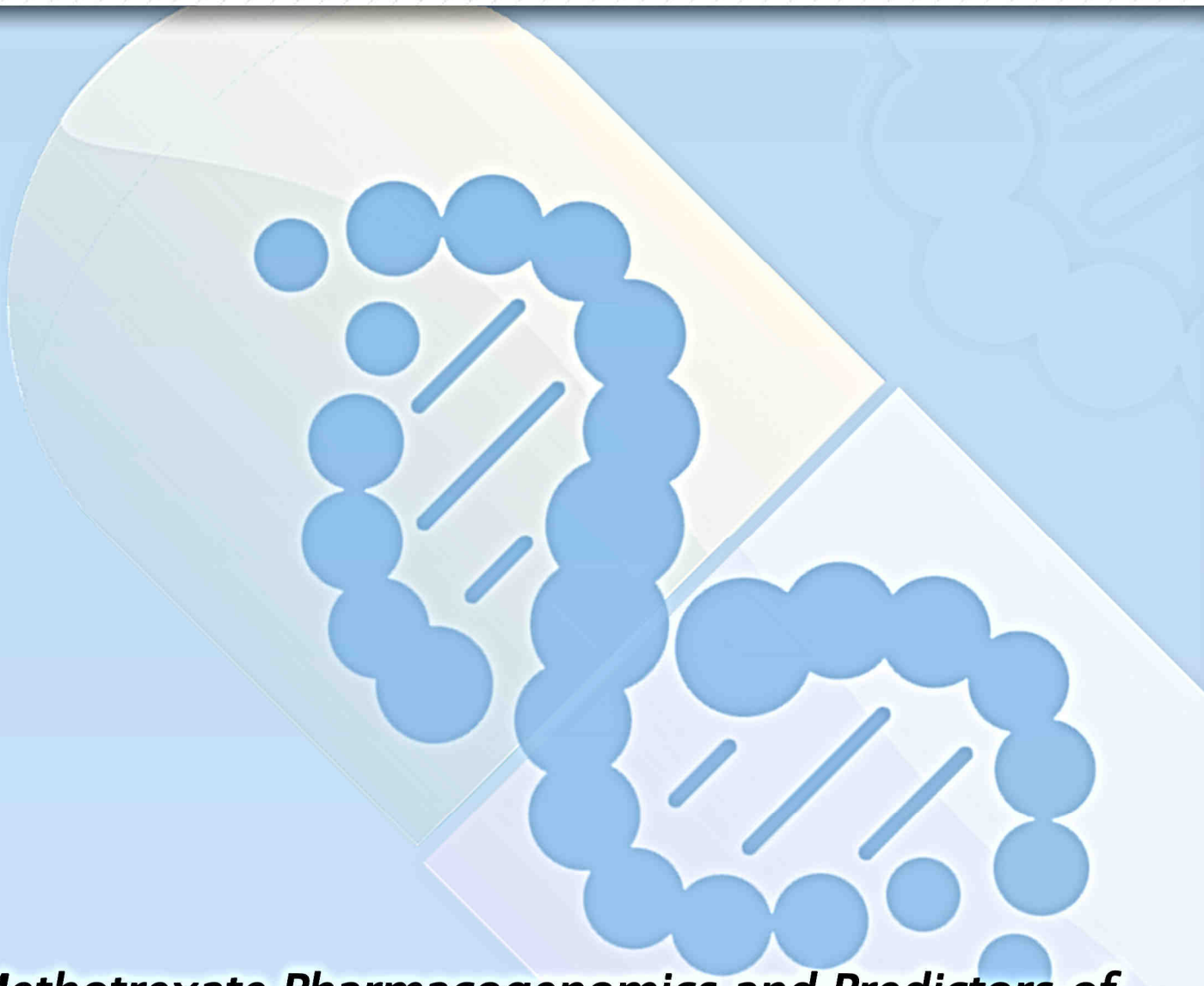
RESUMO



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

RESUMO

INTRODUÇÃO. A artrite reumatoide (AR) é uma doença autoimune, caracterizada pela inflamação crónica de múltiplas articulações, condicionando considerável incapacidade. O metotrexato (MTX) é o fármaco antirreumático modificador da doença mais utilizado no tratamento da AR. Embora o mecanismo responsável pelo seu efeito terapêutico não esteja completamente esclarecido, proteínas responsáveis pelo seu transporte membranar, bem como proteínas envolvidas nas suas vias intracelulares, podem ser determinantes na farmacocinética, da ocorrência de reações adversas (RAs) ao fármaco e de diferentes perfis de resposta clínica. Variáveis clinico-patológicas, bem como polimorfismos genéticos em genes codificantes para proteínas envolvidas no mecanismo de ação do MTX, têm sido descritos como contributivos para a variabilidade da resposta ao tratamento observada entre os doentes. Com o progresso da Farmacogenómica, a previsão da resposta ao tratamento dos doentes com AR sob terapêutica com MTX, oferecerá uma ferramenta preponderante para a tradução do papel dos polimorfismos genéticos na prática clínica, e será essencial para sustentar um avanço na área da Medicina Personalizada.

OBJETIVO. Avaliar a influência de vinte variáveis clinico-patológicas e de vinte e oito polimorfismos genéticos, em treze genes codificantes para proteínas envolvidas no mecanismo de ação do MTX, como potenciais preditores da efetividade e/ou da toxicidade do MTX, em doentes Portugueses com AR.

MATERIAS E MÉTODOS. Foi realizado um estudo retrospetivo num coorte de 233 doentes com AR e sob terapêutica com MTX, recrutados consecutivamente entre janeiro de 2009 e dezembro de 2012. Os dados clínico-patológicos dos doentes foram colhidos dos registos clínicos individuais dos doentes, obtidos no decorrer das visitas hospitalares. A todos os doentes foram colhidas amostras de sangue total para posterior genotipagem. Os resultados terapêuticos, nomeadamente a inefetividade do MTX e a toxicidade decorrente do MTX, foram definidos atendendo a critérios

descritos na literatura. A análise estatística realizada incluiu análises univariada e multivariada, atendendo a genótipos, a haplótipos e ao índice de risco toxicogenético (IRT).

RESULTADOS E DISCUSSÃO. Variáveis clinico-patológicas. Atendendo à efetividade do MTX, os resultados demonstraram que, não fumadores, anticorpo antipeptídeo citrulinado positivo, anticorpos antinucleares positivos, pontuação alta no questionário de avaliação da saúde e, uso de fármacos anti-inflamatórios não esteróides, estavam associados à inefetividade; enquanto a via de administração subcutânea demonstrou-se associada a efetividade. Relativamente à ocorrência de RAs ao MTX, a análise multivariada demonstrou uma associação entre a ocorrência de perturbações gastrointestinais (GI) e uma menor duração do tratamento, bem como com a não suplementação com ácido fólico. Mais ainda, a análise univariada revelou haver associação estatisticamente significativa entre a ocorrência de toxicidade global decorrente do MTX e valores mais elevados na velocidade de sedimentação eritrocitária. Polimorfismos genéticos. No que concerne ao transporte membranar do MTX, vinte e três polimorfismos, em dez genes codificantes para "solute carriers" (SLCs) e transportadores "adenosine triphosphate (ATP)-binding cassette" (ABCs), foram estudados relativamente à sua influência na ocorrência de RAs ao MTX. Dos resultados obtidos, um risco acrescido para a ocorrência de toxicidade global demonstrou-se associado aos portadores do alelo G para o *SLC19A1* rs7499, aos homozigóticos GG para o *SLC46A1* rs2239907 e, aos portadores do alelo T e homozigóticos TT para o *SLCO1B1* rs4149056. Por outro lado, da análise do IRT constatou-se que os doentes com Índice 3 apresentavam um risco para toxicidade global em cerca de 18 vezes superior àqueles com Índice 1. Um risco aumentado para perturbações GI foi observado nos portadores do alelo G e nos homozigóticos GG para o *SLC19A1* rs7499, nos portadores do alelo G para o *SLC19A1* rs1051266, nos portadores do alelo A para o *SLC19A1* rs2838956 e, nos portadores do alelo T e nos homozigóticos TT para o *SLCO1B1* rs4149056. Mais ainda, o modelo de regressão logística demonstrou que os portadores do alelo G para o *SLC19A1* rs1051266

apresentavam um risco acrescido de gastrotoxicidade nos primeiros meses de tratamento. Adicionalmente, o haplótipo GGAG para o *SLC19A1* rs7499, rs1051266, rs2838956 e rs3788200 demonstrou-se associado a um risco acrescido para gastrotoxicidade; e, da análise do IRT verificou-se que os doentes com Índice 4 apresentavam um risco para gastrotoxicidade em cerca de 9 vezes superior àqueles com Índice 1. Considerando a via dos folatos e a influência do *metilenotetrahidrofolato redutase* (*MTHFR*) rs1801133 na efetividade do MTX, os resultados demonstraram uma associação estatisticamente significativa entre os homozigóticos TT e a inefetividade. Atendendo à via da síntese *de novo* de purinas e à influência do *5-aminoimidazol-4-carboxamida ribonucleótido transformilase* (*ATIC*) rs4673993 na efetividade do MTX, os portadores do alelo T demonstraram-se associados a um risco acrescido para inefetividade. Quanto à via da síntese *de novo* de pirimidinas, mais precisamente de três dos polimorfismos genéticos no *timidilato sintetase* (*TYMS*) (rs34743033, rs2853542 e rs34489327), os resultados demonstraram não haver diferenças estatisticamente significativas em relação à ocorrência de RAs mas, e atendendo à efetividade do MTX, os genótipos 3R3R, 3RC3RG e os portadores do alelo 6bp-, demonstraram-se associados à inefetividade do MTX. A análise multivariada confirmou a associação entre um risco acrescido para inefetividade e os portadores do alelo 6bp-; e, da análise haplotípica verificou-se que os haplótipos portadores simultaneamente dos alelos 3R e 6bp- estavam associados a inefetividade do MTX.

CONCLUSÃO. Este trabalho sugere que polimorfismos genéticos, combinados com variáveis clínico-patológicas poderão auxiliar na identificação de doentes que não beneficiem do tratamento com MTX. Deste modo, poderão ser úteis como biomarcadores da resposta ao tratamento e, consequentemente, ajudar os médicos a personalizar o tratamento dos doentes com Artrite Reumatoide.

ABSTRACT

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

ABSTRACT

INTRODUCTION. Rheumatoid Arthritis (RA) is autoimmune disease, characterized by a chronic inflammation of multiple peripheral joints, leading to serious disability. Methotrexate (MTX) is the most widely used disease-modifying antirheumatic drug for RA treatment. Although the mechanism responsible for its therapeutic action is not thoroughly understood, MTX membrane transport proteins and proteins in intracellular pathways can be major determinants of pharmacokinetics, adverse drug reactions (ADRs) and clinical response profiles. Patients' clinicopathological variables, as also as genetic polymorphisms in genes encoding for proteins involved in MTX action mechanism, have been described as contributors to the observed therapeutic outcome variability among the patients. With Pharmacogenomics progress, the improvement of the prediction of RA patients' outcome to MTX treatment will offer a powerful tool for the translational role of genetic polymorphisms into clinical practice and will be essential to sustain a breakthrough in the field of Personalized Medicine.

OBJECTIVE. Evaluate the influence of twenty clinicopathological variables and of twenty-eight genetic polymorphisms, in thirteen genes encoding for proteins involved in MTX action mechanism, as potential predictors of clinical response to MTX and/or MTX-related toxicity occurrence in Portuguese RA patients.

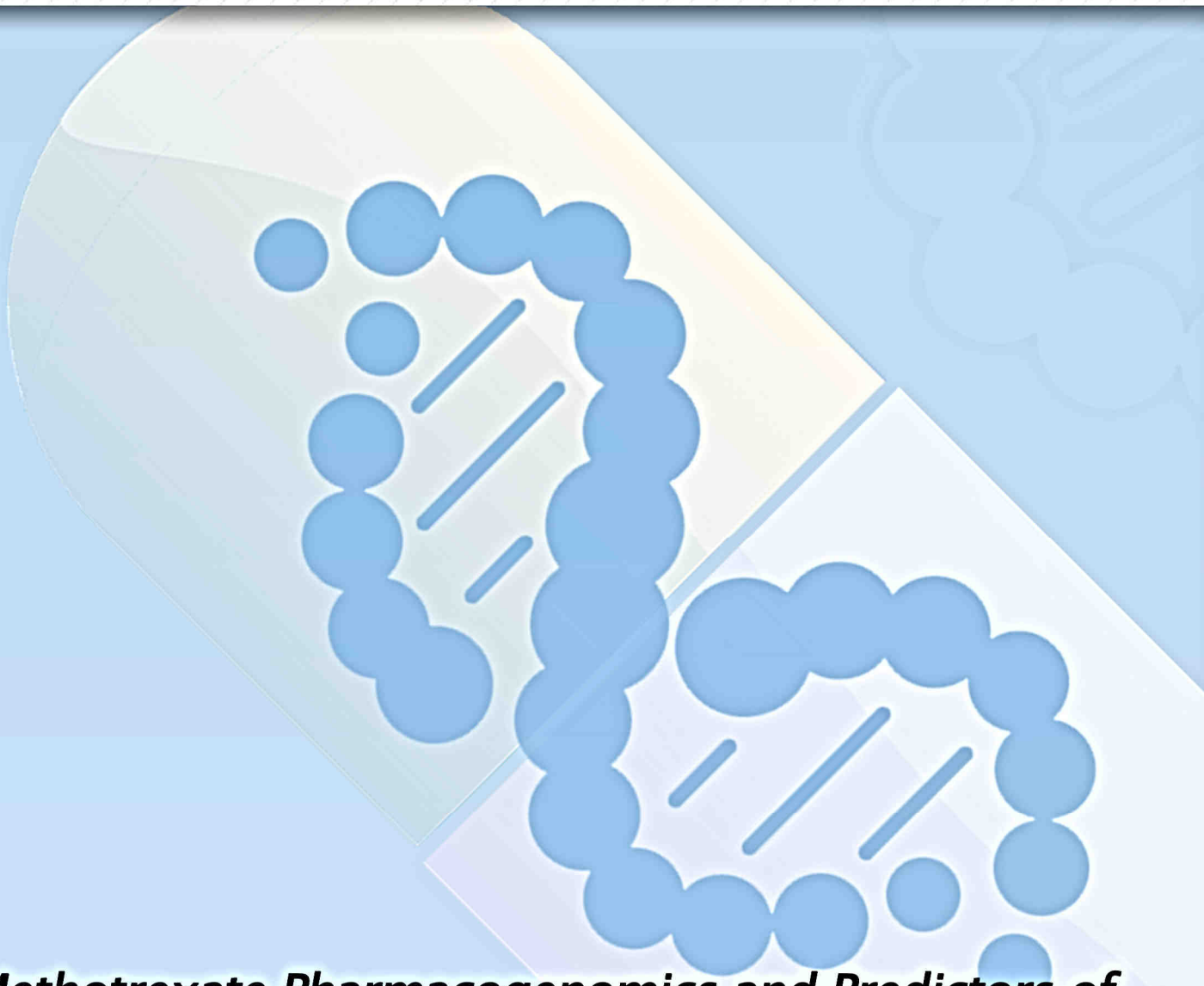
MATERIAL AND METHODS. A retrospective study in a cohort of 233 RA patients treated with MTX recruited consecutively between January 2009 and December 2012, was performed. Clinicopathological data were collected from individual clinical records during patients' regular visits to hospital and whole blood samples were used for genotyping. Therapeutic outcome endpoints, non-response to MTX and MTX-related toxicity occurrence, were defined attending literature described criteria. Statistical analyses were performed including univariate and multivariate analyses, using genotypes, haplotypes and toxicogenetic risk index (TRI).

RESULTS AND DISCUSSION. Clinicopathological variables. Regarding to clinical response to MTX, results demonstrated that non-current smokers, positivity to anti-cyclic citrullinated peptide and to antinuclear antibodies, increased score in higher health assessment questionnaire, and, non-steroidal anti-inflammatory drugs users, were associated with non-response; while subcutaneous administration route was associated with response. In relation to MTX-related toxicity occurrence, multivariate analysis demonstrated that lower MTX treatment duration and non-folic acid supplementation were associated with MTX-related gastrointestinal (GI) toxicity occurrence and, from univariate analysis, higher erythrocyte sedimentation rate levels were associated with MTX-related overall toxicity. Genetic polymorphisms. Regarding to the MTX-carrier mediated transport pathway, twenty-three polymorphisms in ten genes encoding to solute carriers (SLCs) and adenosine triphosphate (ATP)-binding cassette (ABC) transporters were studied and evaluated their influence in the occurrence of MTX-related toxicity. An increased risk for MTX-related overall toxicity was observed for *SLC19A1* rs7499 G carriers, *SLC46A1* rs2239907 GG and *SLCO1B1* rs4149056 T carriers and TT. Moreover, TRI analysis revealed that patients with Index 3 were 18-fold more likely to present an ADR when compared to those with Index 1. An increased risk for MTX-related GI toxicity was associated with *SLC19A1* rs7499 G carriers and GG, *SLC19A1* rs1051266 G carriers, *SLC19A1* rs2838956 A carriers and, *SLCO1B1* rs4149056 T carriers and TT. Furthermore, multivariate Cox regression model demonstrated a higher risk of earlier MTX-related GI toxicity for *SLC19A1* rs1051266 G carriers. Additionally, the haplotype GGAG for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200 has shown to be associated with increased GI toxicity; and, TRI analysis revealed that patients with Index 4 were 9-fold more likely to present a GI disorder when compared to those with Index 1. Considering the folate pathway and the influence of *methylenetetrahydrofolate reductase* (*MTHFR*) rs1801133 in MTX clinical response, analyses demonstrated that T homozygotes were associated with over 4-fold increased risk for non-response. Attending to the *de novo* purine

synthesis pathway and the influence of *5-aminoimidazole-4-carboxamide ribonucleotide transformylase* (*ATIC*) rs4673993 in clinical response to MTX, T carriers were associated with over 4-fold increased risk for non-response. For *de novo* pyrimidine synthesis pathway, more precisely of three polymorphisms in *thymidylate synthase (TYMS)* (rs34743033, rs2853542 and rs34489327), the obtained results demonstrated no statistically significant differences regarding MTX-related toxicity but, and considering the clinical response to MTX, genotypes 3R3R, 3RC3RG and 6bp- carriers were associated with non-response. Multivariate analysis confirmed the increased risk for non-response to MTX in 6bp- carriers; and, haplotype multivariate analysis revealed that haplotypes harboring both 3R and 6bp- alleles were associated with non-response to MTX.

CONCLUSION. This work suggests that genetic polymorphisms combined with clinicopathological data may help to identify patients whom will not benefit from MTX treatment and, therefore, could be helpful as biomarkers of MTX therapeutic outcome and, thereby, assist clinicians to personalize RA treatment.

THESIS OUTLINE



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

THESIS OUTLINE

This thesis is structured in eleven main chapters as follow:

Chapter I. Introduction

This introductory chapter provides a description of Rheumatoid Arthritis and Methotrexate, and exploits the applicability of Pharmacogenomics in the field of Personalized Medicine in Rheumatoid Arthritis patients.

Chapter II. Objectives

This chapter presents the main and specific objectives of the thesis.

Chapter III. Materials and Methods

This chapter includes all the information about the search strategy, study design and participants' selection, and provides the description of the data and sample collection, genotyping procedures and statistical analysis.

Chapter IV. Genetic polymorphisms in low-dose methotrexate transporters: the current relevance as methotrexate therapeutic outcome biomarkers

This chapter presents a comprehensive review about Methotrexate pharmacokinetics processes and the current data on genetic polymorphisms in genes encoding for low-dose Methotrexate membrane transporters as also their influence in Methotrexate therapeutic outcome.

Chapter V. *SLC19A1* 80G allele as a biomarker of methotrexate-related gastrointestinal toxicity in Portuguese rheumatoid arthritis patients

This chapter presents an original work that elucidates the clinical relevance of the *solute carrier 19 family 1 G80A* polymorphism and a set of clinicopathological variables as putative biomarkers of Methotrexate-related toxicity in Portuguese Rheumatoid Arthritis patients.

Chapter VI. *SLC19A1*, *SLC46A1* and *SLCO1B1* polymorphisms as predictors of methotrexate-related toxicity in Portuguese rheumatoid arthritis patients

This chapter elucidates the influence of single nucleotide polymorphisms in genes encoding for Methotrexate membrane transport proteins on the occurrence of Methotrexate-related toxicity in Portuguese Rheumatoid Arthritis patients.

Chapter VII. Prediction of methotrexate clinical response in Portuguese rheumatoid arthritis patients: implication of *MTHFR* rs1801133 and *ATIC* rs4673993 polymorphisms

This chapter elucidates the association of clinical response to Methotrexate of several clinicopathological variables as also of *methylenetetrahydrofolate reductase* C677T and *aminoimidazole carboxamide adenosine ribonucleotide transformylase* T675C polymorphisms in Portuguese Rheumatoid Arthritis patients.

Chapter VIII. Current approaches on *thymidylate synthase* polymorphisms and its importance on molecular epidemiology and pharmacogenetics

This chapter presents a review about thymidylate synthase biological and pharmacological role and its genetic polymorphisms association with diseases susceptibility, Methotrexate therapeutic outcome (clinical response and toxicity occurrence) and patients' survival.

Chapter IX. Role of key *TYMS* polymorphisms on methotrexate therapeutic outcome in rheumatoid arthritis Portuguese patients

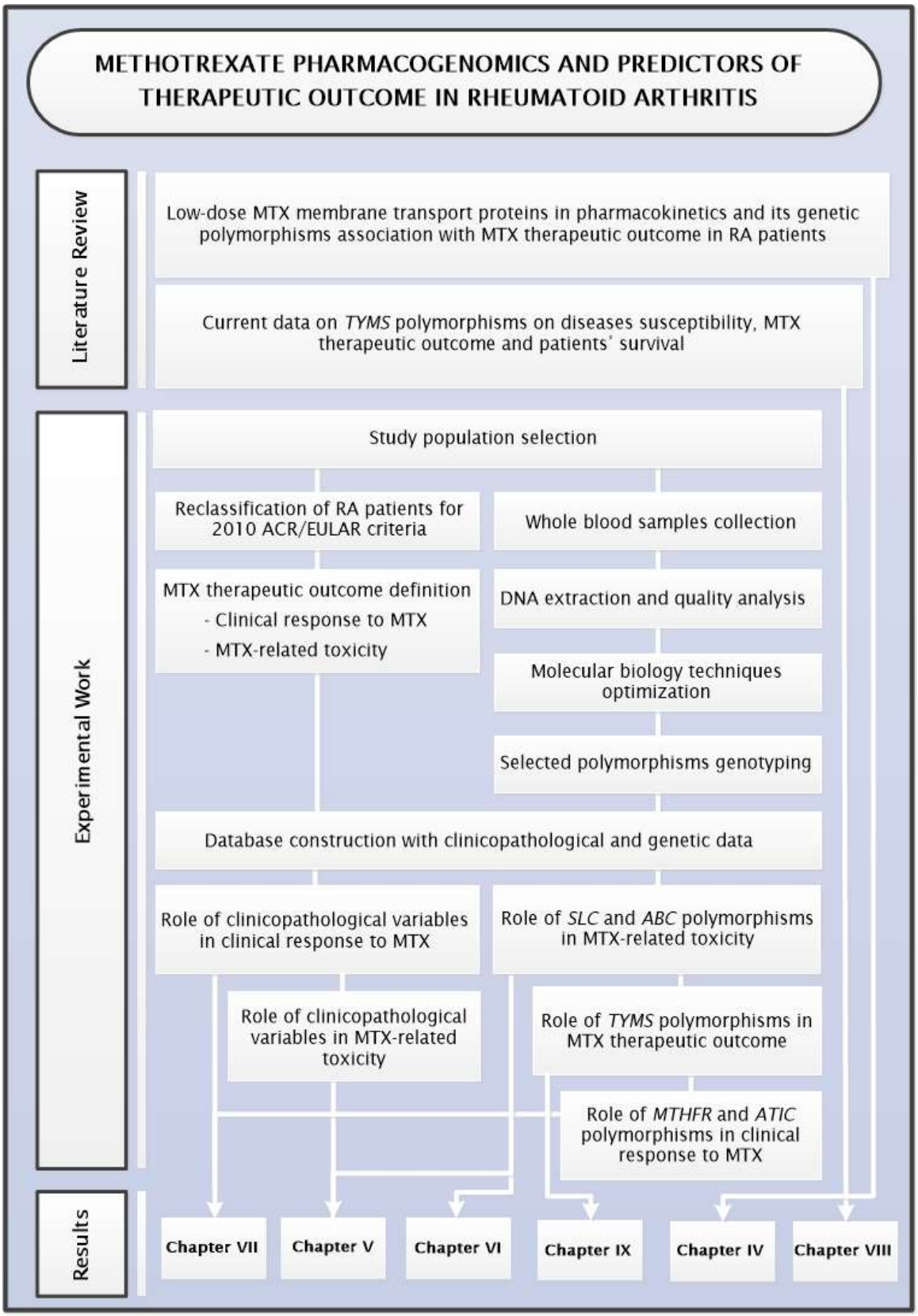
This chapter elucidates the clinical relevance of the most studied *thymidylate synthase* polymorphisms (rs34743033, rs2853542 and rs34489327), by genotype and haplotype-based approaches, in Methotrexate therapeutic outcome of Portuguese Rheumatoid Arthritis patients.

Chapter X. General Discussion

This chapter discusses several aspects of this work' results including clinicopathological and genetic variables association with therapeutic outcome to Methotrexate and explores its limitations.

Chapter XI. Concluding Remarks and Future Perspectives

This chapter presents the main conclusions and highlights future work that is needed to be done and its importance.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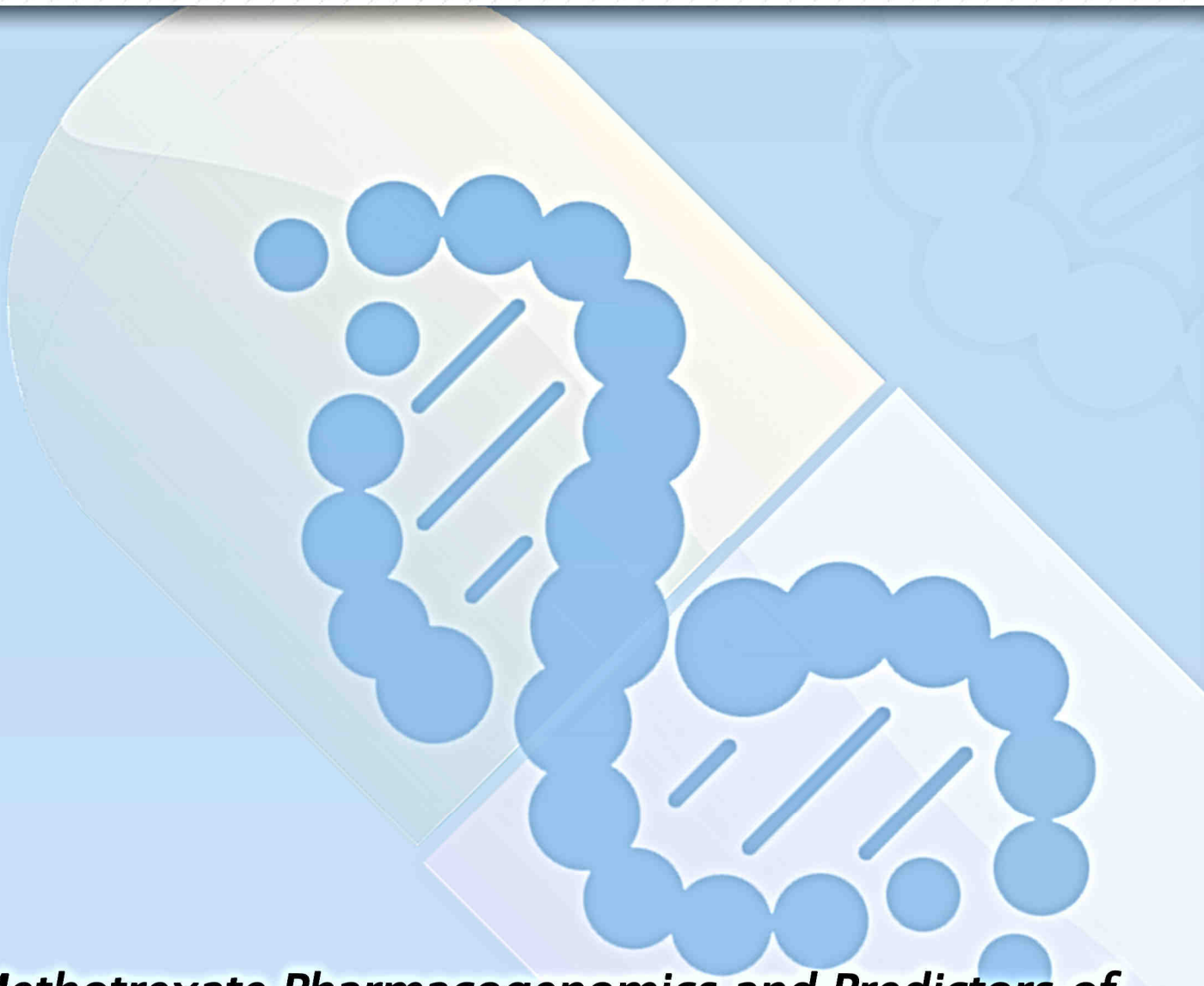

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

ACRONYMS & SYMBOLS

Letters

A

A: Adenine

aa: Amino acid

Ab: Antibody

ABC: Adenosine triphosphate (ATP)-binding cassette

ABCB1: Adenosine triphosphate (ATP)-binding cassette sub-family B member 1

ABCC1: Adenosine triphosphate (ATP)-binding cassette sub-family C member 1

ABCC2: Adenosine triphosphate (ATP)-binding cassette sub-family C member 2

ABCC3: Adenosine triphosphate (ATP)-binding cassette sub-family C member 3

ABCC4: Adenosine triphosphate (ATP)-binding cassette sub-family C member 4

ABCG2: Adenosine triphosphate (ATP)-binding cassette sub-family G member 2

ACPA: Anti-citrullinated protein antibody

ACR: American College of Rheumatology

ACRped70: American College of Rheumatology 70 pediatric criteria

ADA: Adenosine deaminase

ADORA: Adenosine receptor

ADORA1: Adenosine receptor subtype A1

ADORA2a: Adenosine receptor subtype A2a

ADORA2b: Adenosine receptor subtype A2b

ADR: Adverse drug reaction

AHCY: S-Adenosylhomocysteinase

Ag: Antigen

AI: Autoimmune

AICAR: Aminoimidazole carboxamide adenosine ribonucleotide

AMP: Adenosine monophosphate

AMPD1: Adenosine monophosphate deaminase 1

ANA: Antinuclear antibody

ANCA: Antineutrophil cytoplasmic antibody

Anti-CCP: Anti-cyclic citrullinated peptide

Ala: Alanine

ALT: Alanine aminotransferase

APC: Antigen-presenting cell

ARE: Adenylate uridylate-rich element

Arg: Arginine

AS: Ankylosing spondylitis

Asp: Aspartate

AST: Aspartate aminotransferase

ATIC: 5-Aminoimidazole-4-carboxamide ribonucleotide transformylase

ATP: Adenosine triphosphate

AUC: Area under the curve

AUF1: Adenine-uracil-rich factor 1

Auto-Ab: Autoantibody

Auto-Ag: Autoantigen

B

B19V: Parvovirus B19

BC: Before Christ

BCRP: Breast cancer resistance protein

bDMARD: Biological disease-modifying antirheumatic drug

BMI: Body mass index

boDMARD: Biological originator disease-modifying antirheumatic drug

bp: Base pairs

bsDMARD: Biosimilar disease-modifying antirheumatic drug

C

C: Cytosine

cAMP: Cyclic-adenosine monophosphate

CCP: Cyclosporine

CD3: Cluster of differentiation 3

CDAI: Clinical disease activity index

Chr: Chromosome

CI: Confidence interval

CMV: Cytomegalovirus

CNS: Central nervous system

COX-2: Cyclooxygenase-2

CRP: C-reactive protein

csDMARD: Conventional synthetic disease-modifying antirheumatic drug

CSF: Cerebrospinal fluid

CTCAE: Common Terminology Criteria for Adverse Events

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

CV: Cardiovascular

D

DAS: Disease activity score

DAS28: Disease activity score in twenty-eight joints

DC: Dendritic cell

Del: Deletion

DHEA: Dehydroepiandrosterone

DHF: Dihydrofolate

DHFR: Dihydrofolate reductase

DIP: Deletion-insertion polymorphism

DMARD: Disease-modifying antirheumatic drug

DNA: Deoxyribonucleic acid

dTMP: Deoxythymidine monophosphate

dTTP: Deoxythymidine triphosphate

dUMP: Deoxyuridine monophosphate

E

E: Extend primer

E-box: Enhancer box

EBV: Epstein-Barr virus

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

e.g.: For example (Latim origin: *exempli gratia*)

eGFR: Estimated glomerular filtration rate

ESR: Erythrocyte sedimentation rate

EULAR: European League Against Rheumatism

F

F: Forward primer

FAICAR: Formyl-aminoimidazole carboxamide adenosine ribonucleotide

FBP: Folate binding protein

Fc: Fragment, crystallizable

FDA: Food and Drug Administration

FGAR: Formyl glycineamide ribonucleotide

FLS: Fibroblast-like synoviocytes

FOLR1: Folate receptor alpha

FOLT: Folate transporter

FPGS: Folylpolyglutamate synthetase

G

G: Guanine

GAR: Glycineamide ribonucleotide

GARS: Glycineamide ribonucleotide synthase

GART: Glycineamide ribonucleotide formyl transferase

GCA: Giant cell arthritis

GGH: Gamma-glutamyl hydrolase

GI: Gastrointestinal

GIT: Gastrointestinal tract

Gln: Glutamine

Gly: Glycine

H

HAQ: Health assessment questionnaire

HCP1: Heme carrier protein 1

HCQ: Hydroxychloroquine

His: Histidine

HLA: Human leukocyte antigen

HLA-DRB1: Human leukocyte antigen, class II, DR beta 1

hnRNPD: Heterogeneous nuclear ribonucleoprotein D

HWE: Hardy-Weinberg equilibrium

I

ID: Identification

IFN-γ: Interferon-gamma

Ig: Immunoglobulin

IL: Interleukin

Ile: Isoleucine

IMP: Inosine monophosphate

IP: Interphalangeal

IQR: Interquartile range

IU: International units

J

JIA: Juvenile idiopathic arthritis

JSN: Joint space narrowing

L

LD: Linkage disequilibrium

LEF: Leflunomide

LST1: Liver-specific transporter 1

Lys: Lysine

M

MAT: Methionine adenosyltransferase

MCP: Metacarpophalangeal

MCT2: Monocarboxylic acid – transporter 2

MDR: Multidrug resistance protein

MDR1: Multidrug resistance protein 1

MMP: Matrix metalloproteinase

MMP-3: Matrix metallopeptidase 3

mRNA: Messenger ribonucleic acid

MRP: Multidrug resistance-associated protein

MRP1: Multidrug resistance-associated protein 1

MRP2: Multidrug resistance-associated protein 2

MS: Methionine synthase

MTHFD1: Methylenetetrahydrofolate dehydrogenase 1

MTHFR: Methylenetetrahydrofolate reductase

MTP: Metatarsophalangeal

MTR: Methyltransferase

MTRR: 5-Methyltetrahydrofolate-homocysteine methyltransferase reductase

MTX: Methotrexate

MTXPG: Methotrexate polyglutamate

N

n.a.: Not assigned

NA: Not analyzed

NCBI: National Center for Biotechnology Information

NPY: Number pack years

NS: Not-statistically significant

NSAID: Non-steroidal anti-inflammatory drug

O

OA: Osteoarthritis

OAT: Organic anion transporter

OATP-2: Organic anion-transporting polypeptide 2

OR: Odds ratio

P

P: Prospective

PADI4: Peptidyl arginine deiminase type IV

PAS: Patient activity score

PASII: Patient activity score-II

PCFT: Proton-coupled folate transporter

PCR: Polymerase chain reaction

PD: Pharmacodynamics

P-GP1: P-glycoprotein1

PGx: Pharmacogenomics

PK: Pharmacokinetics

PO: *Per os*

PPAT: Phosphoribosyl pyrophosphate amidotransferase

PRA: 5-Phosphoribosyl amine

PRPP: 5-Phosphoribosyl-1-pyrophosphate

PTPN22: Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)

R

R: Retrospective

RA: Rheumatoid arthritis

RANKL: Receptor activator of nuclear factor kappa B ligand

RAPID: Routine assessment of patient index data

Ref: Reference

RF: Rheumatoid factor

RFC1: Reduced folate carrier 1

RFLP: Restriction fragment length polymorphism

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

S

S: Statistically significant

SAH: S-Adenosyl homocysteine

SAM: S-Adenosyl methionine

SC: Subcutaneous

SCr: Serum creatinine

SD: Standard deviation

SDAI: Simplified disease activity index

sDMARD: Synthetic disease-modifying antirheumatic drug

SE: Shared epitope

Ser: Serine

SHMT1: Serine hydroxymethyltransferase 1

SJC: Swollen joints count

SLC: Solute carrier

SLC16A7: Solute carrier family 16 member 7

SLC19A1: Solute carrier family 19 member 1

SLC22A11: Solute carrier family 22 member 11

SLC22A6: Solute carrier family 22 member 6

SLC22A8: Solute carrier family 22 member 8

SLC46A1: Solute carrier family 46 member 1

SLCO1A2: Solute carrier organic anion transporter family member 1A2

SLCO1B1: Solute carrier organic anion transporter family member 1B1

SLCO1B3: Solute carrier organic anion transporter family member 1B3

SLE: Systemic lupus erythematosus

SNP: Single nucleotide polymorphism

SOC: System organ class

SSZ: Sulfasalazine

STAT4: Signal transducer and activator of transcription 4

SvH: Sharp-van der Heijde score

T

T: Thymine

TCR: T-cell receptor

Th0: Type-0 T helper

Th1: Type-1 T helper

Th17: Type-17 T helper

THF: Tetrahydrofolate

Thr: Threonine

TJC: Tender joints count

TNF-α: Tumor necrosis factor-alpha

TRI: Toxicogenetic risk index

Trp: Tryptophan

TS: Thymidylate synthase (protein)

tsDMARD: Targeted synthetic disease-modifying antirheumatic drug

TSER: Thymidylate synthase enhancer region

TYMS: Thymidylate synthase (gene)

U

UK: United Kingdom

USA: United States of America

USF: Upstream stimulating factor

UTR: Untranslated region

V

Val: Valine

VAS: Visual analog scale

VNTR: Variable number tandem repeat

Numbers

4v: Four variables

5-MTHF: 5-methyltetrahydrofolate

5,10-MTHF: 5,10-methylenetetrahydrofolate

5,10-CH-THF: 5,10-methenyltetrahydrofolate

7-OH-MTX: 7-hydroxymethotrexate

10-CHO-THF: 10-formyltetrahydrofolate

Symbols

Alfa: α

Beta: β

Centimeter: cm

Day: d

Decreased: ↓

Euro: €

Gram: g

Higher: >

Hour: h

Increased: ↑

Liter: L

Lower: <

Meter: m

Microgram: µg

Milligram: mg

Milliliter: mL

Millimeter: mm

Mole: mol

Percentage: %

Picogram: pg

Week: w

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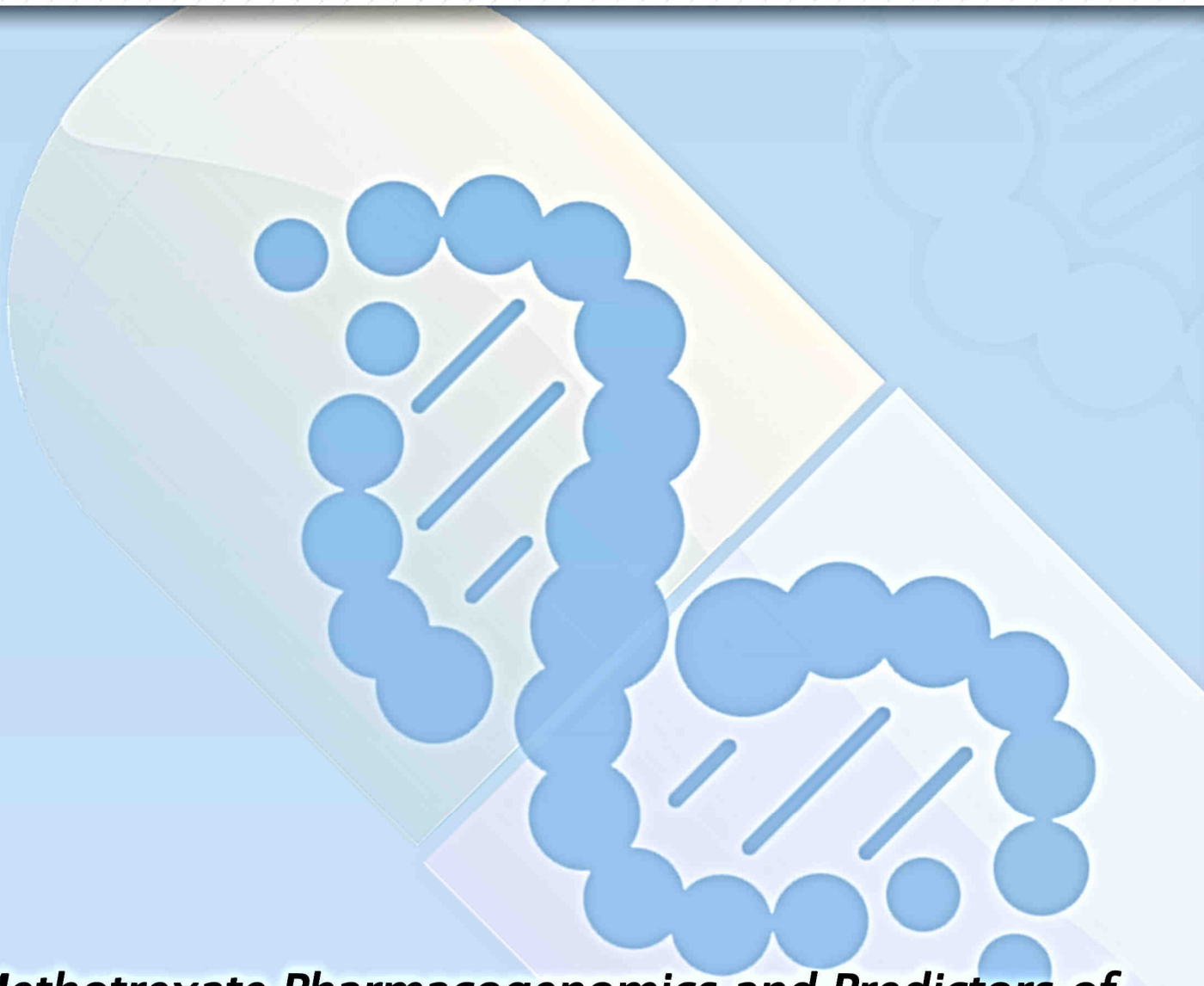

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

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CHAPTER I

INTRODUCTION

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER I - INTRODUCTION

This chapter provides a description of Rheumatoid Arthritis and Methotrexate, and exploits the applicability of Pharmacogenomics in the field of Personalized Medicine in Rheumatoid Arthritis patients.

1. RHEUMATOID ARTHRITIS

This section includes information about Rheumatoid Arthritis historical aspects, epidemiological data, natural history, pathogenesis, classification criteria, disease monitoring, disease burden and prognostic, and, therapeutic strategies.

1.1. General Concepts

Rheumatoid Arthritis (RA) is a complex, systemic autoimmune (AI) disease, characterized by a chronic inflammation of multiple peripheral joints, often symmetric, which leads to cartilage and bone destruction, progressive deformity and disability (1, 2).

1.2. Historical Aspects

The earliest evidence of RA comes from pre-Columbian North America installation, with symmetrical, polyarticular erosive arthritis reported in Native American remains from as far back as 4500 Before Christ (BC) in what is now Tennessee (3). Paleopathological evidence of RA exists only in skeletal remains from New World populations and RA was not documented in the Old World until the late 16th century, whereas other rheumatic diseases have been well described in biblical and ancient texts (4, 5). The lapse suggests that RA either migrated to Europe after New World colonization or arose as a response to a new environmental exposure (6). In 1591, the French physician Guillaume de Baillou applied the age-old catch-all term "rheumatism" to a condition characterized by soreness, inflammation,

stiffness and pain (6). Sporadic medical and literary depictions of RA-like illnesses followed, but the first medical literary evidence was penned in 1800 by Augustin Jacob Landré-Beauvais, a French medical student at Salpêtrière Hospital (7). He described, in nine female patients, a painful, inflammatory arthritis that left the hand joints swollen and deformed that he called "asthenic gout" ("La goutte asthénique primitive"), distinguishing it from classic gout based on inflammation pattern, female predominance, polyarticular involvement and its chronic course (7). In 1859, Alfred Garrod coined the term "Rheumatoid Arthritis" (8). Rubens seems to be the first painter to depict what seems to be RA of the hands in the mid-17th century (9). Paintings such as "The three graces" and "The miracle of St. Ignatius", show ulnar deviation, boutonniere deformities and metacarpophalangeal (MCP) swelling (9, 10) (Figure 1).

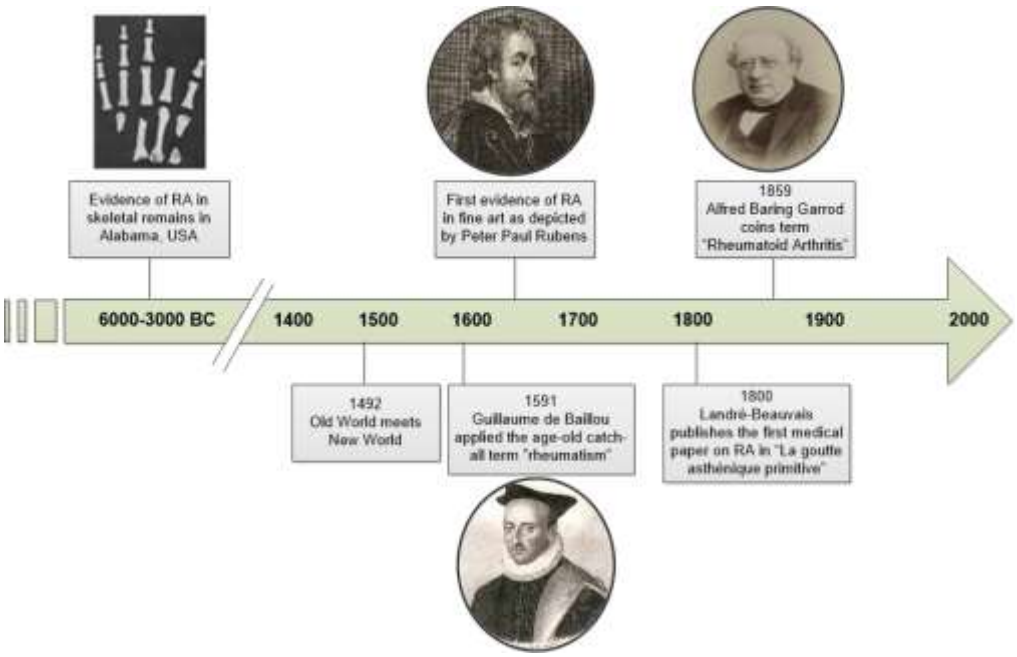


Figure 1. Historical, literary, artistic and paleopathological evidence of rheumatoid arthritis as a New World disease that has "spread" to the rest of the world.

(Adapted from Scher JU, Abramson SB. The microbiome and rheumatoid arthritis. Nature reviews Rheumatology. 2011;7(10):569-78)

BC: before Christ; RA: rheumatoid arthritis; USA: United States of America.

1.3. Epidemiology

Several studies have been reported during the last few decades about the incidence and prevalence of RA. As previously described, epidemiological evidence supports the notion that RA is a disease of the New World and, keeping with this theory, the last populations to be in contact with European conquistadors have a strikingly low prevalence of RA (6).

1.3.1. Prevalence

The worldwide prevalence of RA is relatively constant ranging from 0.5% to 1.1%, as reported in North European and North American populations (11-15). Southern European countries have slightly lower median prevalence rates (of 0.3% to 0.7%) compared to these countries (12, 16-18). However, there are some exceptions, such as Southern American, African and Asian populations, which have even more lower prevalence than Southern European countries (13, 19-21). In contrast, certain native American-Indian populations have an extremely increased disease prevalence (of 5.3% to 6.8%) (Figure 2) (13). In Portugal, the prevalence in 2011 was of 0.36%, in accordance to other Southern European countries (22).

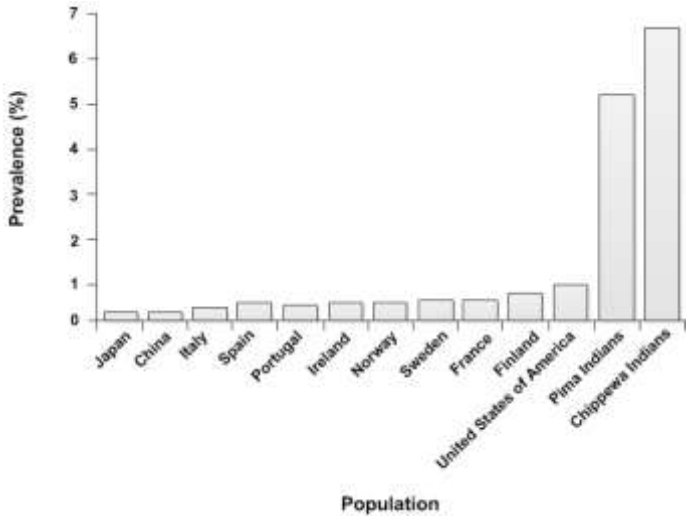


Figure 2. Prevalence of rheumatoid arthritis in some world countries.
(Adapted from Silman A, Pearson J. Epidemiology and genetics of rheumatoid arthritis. Arthritis research. 2002;4(Suppl 3):S265 - S72 and Branco JC, Canhao H. Epidemiological Study of Rheumatic Diseases in Portugal - EpiReumaPt. Acta Reumatol Port. 2011;36(3):203-4)

1.3.2. Incidence

Among all the countries, the incidence of RA is higher in females than in males, with a gender ratio from about 2:1 to 3:1 (23) and a peak of disease onset age occurring in the fifth decade of life (24). The annual incidence rates of RA vary between 20 to 60 cases *per* 100 000 inhabitants in North European and North American populations (11, 15, 25, 26), in Southern European countries is lower (9 to 24 cases *per* 100 000 inhabitants) (27, 28) and it was not reported or studies are outdated for other countries. In Portugal, the incidence in 2001 was reported as varying between 20 to 40 cases *per* 100 000 inhabitants (29).

1.3.3. Time trends in epidemiology

Limited data are available on time trends of RA incidence and prevalence. Several longitudinal studies for the same region suggested declines after the 1960s, mainly in women (25, 30). This trend is supposed to be influenced by some factors such as differences in studies methodologies and difficulties in differentiate RA from unspecified polyarthritis (misclassification), and thus should be interpreted with precaution (31). However, the changes over time may be true and the declining trend mainly observed in women (25), could be explained by the greater exposure to oral contraceptives (30), as further discussed in accordance to hormonal factors (see *Gender and Susceptibility genes* - 1.5.1. Prearthritis phase – 1.5. Pathogenesis).

1.4. Natural History

Natural history of RA varies considerably regarding disease onset, pattern of presentation, pattern of progression and clinical course.

1.4.1. Disease onset

Rheumatoid arthritis onset may be sudden and acute, gradual and insidious, or sub-acute between these extremes (32). A gradual onset is most common (at least 50% of cases) when compared to a sudden onset (10% to

25%) (32). Rheumatoid arthritis begins predominantly as an articular disease, and one or many joints may be affected (32). It may also start with an extra-articular or non-articular presentation, such as a local bursitis, tenosynovitis, carpal tunnel syndrome, or as a systemic presentation, with diffuse polyarthralgia or polymyalgia (32). Although the onset is predominantly articular, it is frequently associated with a variety of extra-articular features, including generalized weakness, anorexia, weight loss or fever (32). In some cases, fatigue alone or diffuse non-specific aching with other extra-articular features, such as pulmonary disease, may herald - by weeks or months - the onset of polyarthritis (32).

1.4.2. Pattern of presentation

There are six possible patterns of presentation, as follow: 1) gradual onset; 2) slow monoarticular presentation; 3) sudden acute polyarthritis; 4) acute monoarthritis; 5) local extra-articular features; and, 6) systemic extra-articular features (33). The most common early presentation is the gradual onset (33). It affects small peripheral joints (e.g. wrists, ankles or MCP, interphalangeal – IP - and metatarsophalangeal – MTP - joints) and it is usually symmetric, with considerable morning stiffness (33). The slow monoarticular presentation, which affects larger joints (e.g. shoulders or knees) and confines the symptoms to one or two joints, as also the sudden acute polyarthritis of shoulders, elbows, wrists, fingers, hips, knees, ankles, and feet, with intense joint pain, diffuse swelling and limitation, are less common (33). It is rare the acute monoarthritis of the knee, shoulder or hip, in which is presented a picture suggesting a septic, pseudo gout or gouty process with a characteristic severe joint pain (33). In addition, local extra-articular features (e.g. bursitis and tenosynovitis) and systemic presentation, particularly in elderly patients, may be observed (33).

1.4.3. Pattern of progression

Independently of the onset or presentation, patients' subsequent progress may follow different patterns. It may be a course that is brief and self-limited, episodic (palindromic) or prolonged and progressive, or

something intermediate (34). The severity may vary from mild to intense. Crises can be prolonged and smoldering or prolonged and progressive (34). There are at least three possible disease patterns (Figure 3): 1) Progressive: with increasing joint involvement; 2) Monocyclic: a single cycle with remission for at least 1 year and without reoccurrence; and, 3) Polycyclic: with either intermittent or continuing subtypes. The latter group shows smoldering activity with incomplete remission or progression (34).

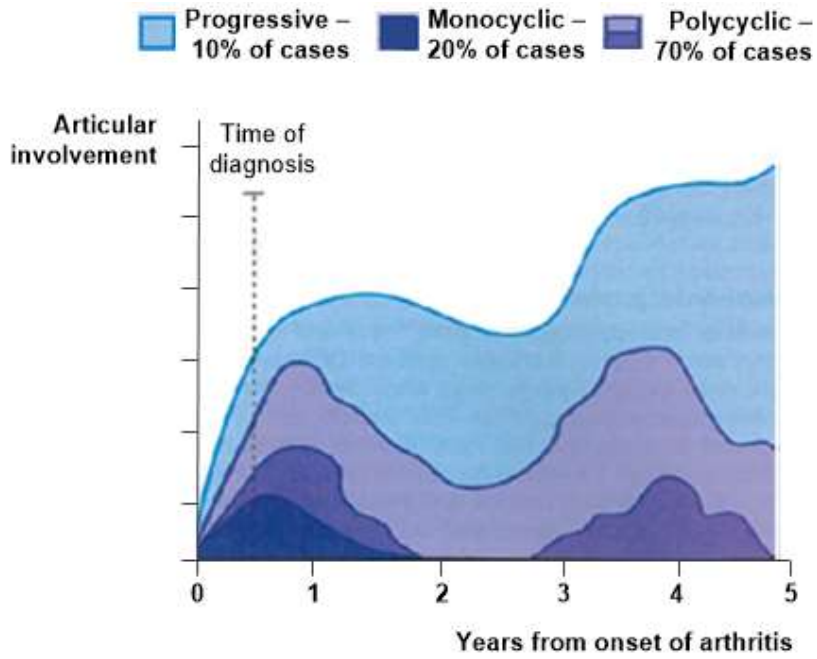


Figure 3. Patterns of the clinical (articular) course of rheumatoid arthritis.
(Adapted from Masi AT. Articular patterns in the early course of rheumatoid arthritis. The American journal of medicine. 1983;75(6A):16-26)

1.4.4. Clinical course

The clinical course of RA, which can be predicted by disease onset and patterns of presentation and progression, could provide insights into outcome variables and disease prognosis (32). In acute-onset pattern, patients with sudden disease onset are associated with a better functional outcome when compared with patients presenting a slow onset of disease

(32). Moreover, patients with gradual-onset disease are associated with worse prognosis when large joints and the first and second MTP joints are involved (32).

1.5. Pathogenesis

The mechanisms involved in the pathophysiology of RA are currently not fully established. Nevertheless, the innate immune system, responsible for the initial non-specific response to foreign antigens, seems to be involved early in RA (35, 36). The antigens which trigger these response have not yet been identified, but probably resemble or mimic synovial tissue antigens, resulting in a multistep progression until the development of RA: 1) Prearthritis phase; 2) Transitional phase; and 3) Clinical phase (37).

1.5.1. Prearthritis phase

The cause of RA is unknown, however, it seems that involves a complex factorial interplay among genotype, environmental triggers, and chance (24, 38) (Figure 4).

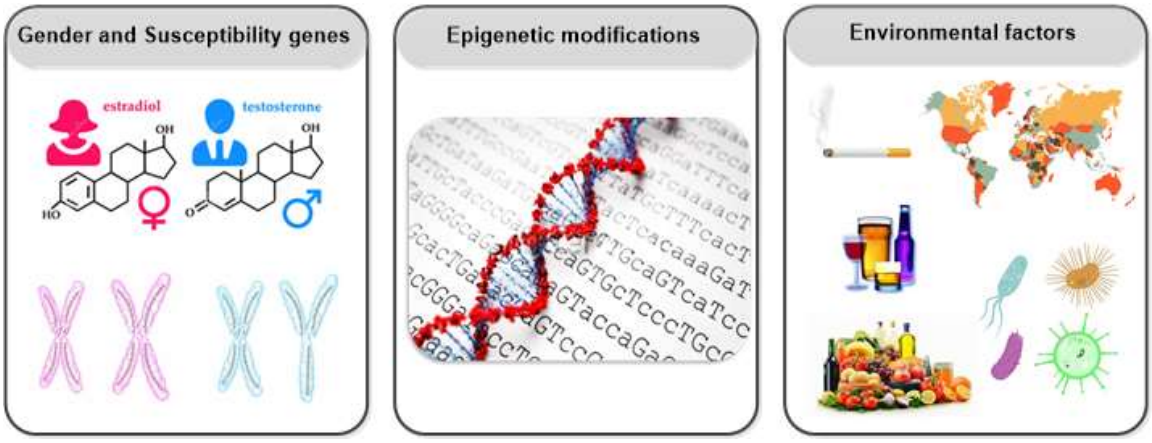


Figure 4. Prearthritis phase.

1.5.1.1. Gender and Susceptibility genes

Females are two to three more times affected than men (23) but the mechanism by which gender influences RA susceptibility is unknown. Nevertheless, it is suggested a possible role of hormonal fluctuations to which woman are exposed throughout life (39, 40). This is in agreement with the peak disease onset age - the fifth decade of life - a time of great hormonal changes, such as those occurring at the beginning of menopause phase, which could be possible disease risk factors (24). Sex hormones (estrogen, androgen, prolactin and testosterone) and common intermediate metabolites (dehydroepiandrosterone – DHEA, and estradiol - the most potent naturally occurring estrogen) have all been proposed as having a role in susceptibility to AI diseases, since they modulate the immune response via androgen and estrogen receptors (41). In addition, low levels of gonadal androgens (testosterone/dihydrotestosterone) and adrenal androgens (DHEA and its sulfate), as well as lower androgen/estrogen ratios, have been detected in body fluids of both male and female RA patients, supporting the possibility of a pathogenic role for the decreased levels of the immune-suppressive androgens (41). Since estrogen and prolactin are both proinflammatory hormones (41), longer lifetime period of menstruation leads to greater lifetime exposure to proinflammatory sex hormones; and, very irregular menstrual cycles, which are presumed to be a consequence of excess hormone production, were associated with an increased risk of RA (42). In addition, there is evidence of reduced levels of androgens (natural anti-inflammatory hormones) and progesterone in RA patients (43). Men with RA have low levels of testosterone, DHEA and estrone, while estradiol is increased and correlates with the inflammatory indices (43). Furthermore, females have enhanced immunoreactivity, with higher immunoglobulin levels and enhanced antibody (Ab) production to antigen (Ag) stimulation, and a predominant T-helper type 2 immune response when compared to men (39, 40).

In accordance to RA susceptibility genes, literature describes that siblings of individuals affected with RA have a higher risk of developing

disease when compared with unrelated individuals (44). This increased risk could be the result of either their shared genetic background or factors in their shared family environment (see *Environmental factors* - 1.5.1. Prearthritis phase – 1.5. Pathogenesis). These influences can be distinguished by comparing disease recurrence risks (disease concordance) in co-twins of affected monozygotic and dizygotic twins. Because both types of twins are assumed to share their common environment to a similar extent, a greater concordance in monozygotic (concordance rates of 15 to 30%) than in dizygotic twins (concordance rates of 5%) suggests a genetic influence (45). The elevated risk among family members was also demonstrated in a study based on a multiple-generation register (46).

The nature of much of the genetic risk in RA is becoming increasingly clear. The long-established association with the human leukocyte antigen (HLA), class II, DR beta 1 (*HLA-DRB1*) locus has been confirmed and alleles that contain a common amino acid motif in the *HLA-DRB1* region, termed shared epitope (SE), confer particular susceptibility mainly in positive patients for rheumatoid factor (RF), an autoantibody (auto-Ab) directed against the fragment, crystallizable (Fc) region, of immunoglobulin G (IgG) and anti-cyclic citrullinated peptide (anti-CCP), an auto-Ab directed against proteins containing citrulline (47). These findings suggest that some predisposing T-cell repertoire selection, Ag presentation, or alteration in peptide affinity has a role in promoting autoreactive adaptive immune responses (47). Other possible explanations for the link between RA and the SE include molecular mimicry of the SE by microbial proteins, increased T-cell senescence induced by SE–containing HLA molecules, and a potential proinflammatory signaling function that is unrelated to the role of the SE in Ag recognition (48, 49). Nevertheless, *HLA-DRB1* may explain only 30% of the population's genetic susceptibility to disease (47). Among other genes, the main RA susceptibility factor is the gene that codify for protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (*PTPN22*) (50). A missense cytosine (C)>thymine (T) substitution at nucleotide position 1856 of *PTPN22* leads to substitution of tryptophan (Trp) for arginine (Arg) at residue 620 of

the protein product (50). The resulting gain of function, with enhanced regulation of T-cell receptor (TCR) signaling during thymic selection, permits autoantigen (auto-Ag) specific T-cells to escape clonal deletion, thereby predisposing to autoimmunity (50). Other non-HLA genetic risk factors such as signal transducer and activator of transcription 4 (*STAT4*) (51) and cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) (52) have also been described.

1.5.1.2. Environmental factors

Environmental factors may affect RA susceptibility in individuals who share the same genetic background – epigenetic factors. Besides this, environmental factors include: socio-demographic conditions; cigarette smoking; alcohol consumption; diet; and, infectious agents.

Socio-demographic conditions

There is an increased prevalence of RA in the Pima and Chippewa Indians, in contrast with certain populations, including Southern American, African and Asian populations (13, 19-21) (see 1.3.1. Prevalence – 1.3. Epidemiology). The high frequency of RA in these populations strongly suggests a genetic basis of disease, since RA is a "New World disease", raising the possibility that RA is transmissible (53). Even so, possible environmental triggers such region particularities in diet (54), infections agents, cigarette smoking and sex hormones are thought to have an effect on RA, although not well known for now (53).

Furthermore, urbanized regions have been associated with an increased prevalence of RA. For example, in Taiwan, the prevalence of RA is higher among individuals living in an urban rather than a rural environment (55). Regions with a greater exposure to traffic pollution were related with an increased risk for RA (56). Moreover, the risk of developing RA may be also influenced by diet particularities of these regions (54) (57). High birth weight, mostly in developed countries, was positively associated with RA,

whereas increased breast feeding time, mostly in development countries, was protective (58).

Cigarette smoking

Cigarette smoking represents the most prominent environmental risk factor in RA (59-61) and an increased risk for RA has been observed among heavy smokers (60). In fact, the risk of RA increases after ten pack-years of smoking and remains elevated up to twenty years after smoking discontinuation (61). Multiple reports were developed in order to elucidate epigenetic modifications induced by cigarette smoking and, the most important genetic risk factor associated with RA, the *HLA-DRB1* SE–containing allele, appears to have robust interactions with smoking in disease susceptibility (59, 60, 62). The interaction between smoking and *HLA-DRB1* SE is most evident in seropositive RA (59, 60), particularly in disease that is characterized by positive serology for anti-CCP (59), and in heavy smokers (59, 60, 62).

Alcohol consumption

Alcohol consumption has been associated with decreased risk for developing RA, particularly for anti-CCP-positive patients (63). A dose-dependent effect was also observed whereby individuals with the highest consumption (≥5 drinks or 80g ethanol/w) had a decreased risk of RA on the order of 40-50% compared with those with low to no consumption (<0.5g ethanol/w) (64).

Diet

A diet rich in fish, olive oil, cooked vegetables and foods with high content of omega-3 fatty acids has been shown to protect against RA (54). Several studies found that populations of Southern Europe had milder forms of RA, with fewer extra-articular and radiological manifestations, when compared to other populations (54). This difference may be ascribable in part to the Mediterranean diet (54). Protein and red meat intake were found to increase the risk of inflammatory arthropathy (57), but a subsequent

study showed no association between protein, red meat and fish consumption with the risk of developing RA (65). Vitamins were also associated with RA. Vitamin D, an important modulator of the inflammatory response and of bone and mineral homeostasis (66), has been associated with a decreased risk for RA (67, 68) and vitamin K, which is found primarily in legumes and other vegetables, may inhibit the proliferation of fibroblast-like synoviocytes, thereby diminishing the severity of inflammation (69).

Infectious agents

Infectious agents such as Parvovirus B19 (B19V), Epstein–Barr virus (EBV), *Porphyromonas gingivalis* and gastrointestinal (GI) microbiome, have long been linked with RA, and although unifying mechanisms remain elusive, some form of molecular mimicry is postulated (70). Clearly, no single microorganism is responsible for triggering the development of RA possibly in individuals who carry genetic susceptibility factors to the disease (70). Evidence supporting a role for B19V includes the presence of viral deoxyribonucleic acid (DNA) in the synovial fluid, synovial cells, and/or synovial tissue of RA patients (71). Serums from RA patients contain high titles of EBV Ags and of Abs to latent and replicative EBV antigens (70, 72). In addition, EBV ribonucleic acid (RNA) has been identified in B-cells in synovial tissue from RA patients (70). Furthermore, RA appears to be associated with periodontal disease: *Porphyromonas gingivalis* expresses peptidyl arginine deiminase, type IV (PADI4), which is capable of promoting citrullination of mammalian proteins by the promotion of post-translational modifications (73). Finally, the GI microbiome is now recognized to influence the development of autoimmunity in articular models and, specific (and potentially tractable) clinical bacterial signatures that are associated with auto-Ab-positive RA are emerging (74). Other infectious agents (e.g. Cytomegalovirus - CMV, *Proteus* species, and *Escherichia coli* – *E. coli*) and their products (e.g., heat-shock proteins) have been also associated with the development of RA (70).

1.5.2. Transitional phase

From prearthritis phase results environment–gene interactions that promote loss of tolerance to self-proteins that contain a citrulline residue, which is generated by citrullination (48). Citrullination (conversion of peptidyl-arginine to peptidyl-citrulline) is a post-translational modification of proteins, also known as self-protein citrullination, that is thought to be important in the initiation of the AI response that leads to the development of RA (75). This anticitrulline response can be detected in T-cell and B-cell compartments and is probably initiated in secondary lymphoid tissues or bone marrow. Autoantibodies, such as RF and anti-CCP, are often (but not always) detected in patients before the development of arthritis; in some series, their levels are increased and epitope spreading occur as the onset of disease approaches (76).

Why the systemic loss of tolerance is linked to a localized onset of inflammation in the joint is still unclear (transition to arthritis). Localization of the inflammatory response occurs in the joint by virtue of poorly understood mechanisms that probably involve microvascular, neurologic, biomechanical, microtrauma-related mechanisms, or other tissue-specific pathways (48). It is possible that biologic features of the targeted auto-Ags (e.g. regulation of cellular metabolism in the case of α-enolase and glucose-6-phosphatase) may contribute (Figure 5).

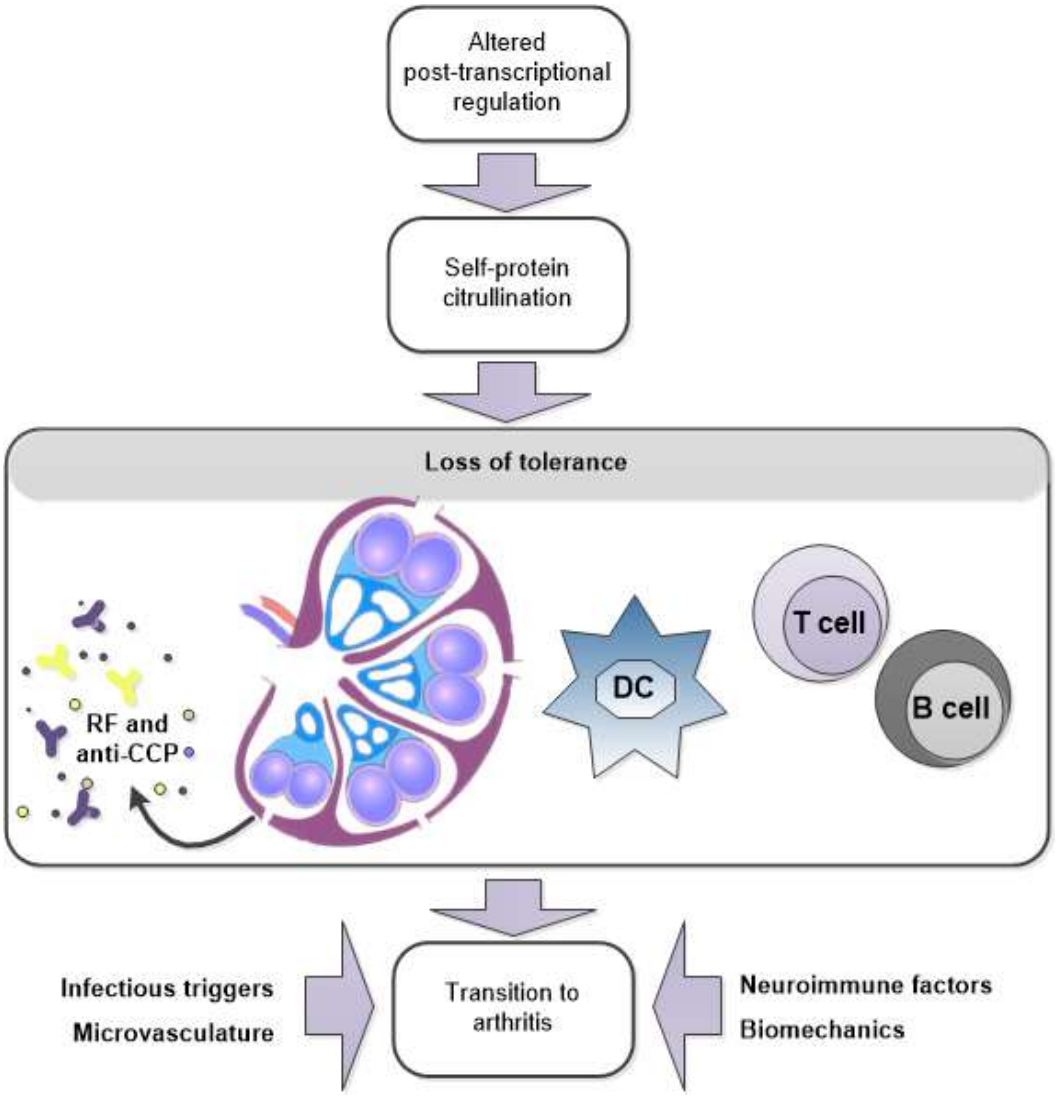


Figure 5. Transitional phase.

(Adapted from McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19)

Anti-CCP: anti-cyclic citrullinated peptide; DC: dendritic cell; RF: rheumatoid factor.

1.5.3. Clinical phase

Synovitis is initiated and perpetuated by positive feedback loops and in turn promotes systemic disorders that make up the "syndrome of rheumatoid arthritis" (48) (Figure 6).

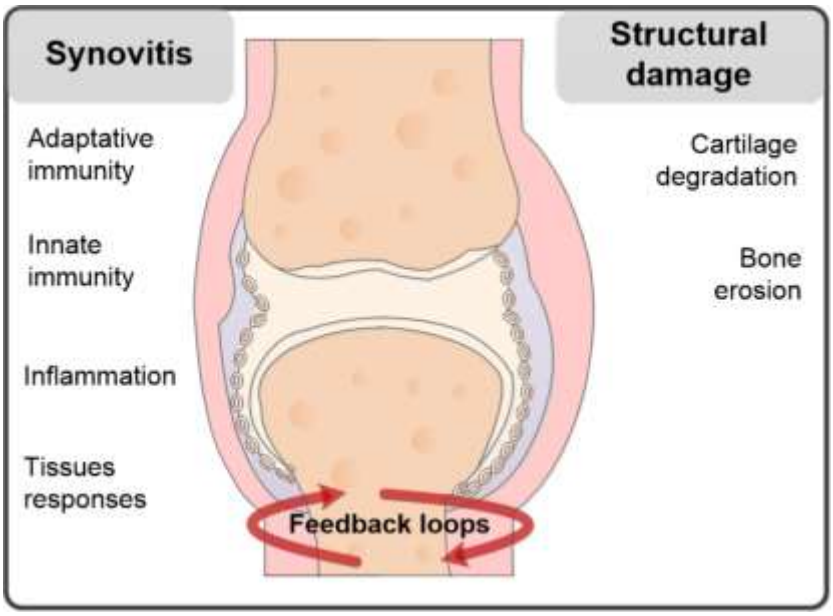


Figure 6. Clinical phase – Feedback loops between synovitis and structural damage.
(Adapted from McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19)

Antigen stimulation leads to a cascade of events in a genetically predisposed individual with a particular gender and in the presence of certain environmental factors. This cascade include the activation of antigen-presenting cells (APCs), such as dendritic cells (DCs), that start several costimulation-dependent interactions among DCs, T- and B-cells at lymph node (77). Thereafter, the adaptive immune system (Ag-specific immune responses) launches a sustained and specific attack on synovial tissue, which is now perceived as "foreign" (37). The immune response leads to the characteristic histological features of RA. This is characterized by synovial proliferation, neoangiogenesis (induced by local hypoxic conditions and cytokines), insufficient lymphangiogenesis, cell migration of T-cells (type-1 T helper – Th1 - and type-17 T helper – Th17), B-cells, macrophages and fibroblasts and increased expression of adhesion molecules and chemokine's (78, 79). This process is eventually followed by *pannus* formation, local joint destruction and auto-Abs production (e.g. RF and anti-CCP) by plasma cells (80). The inflammatory infiltrate secretes numerous proinflammatory

cytokines, glycoproteins that act via cell surface receptors to regulate cellular function by promotion of inflammation. Tumor necrosis factor -alpha (TNF-α), interleukin (IL) -1, IL-6 and IL-17 are the pivotal cytokines in the pathogenesis of RA since they are potent stimulators of fibroblast-like synoviocytes (FLS), chondrocytes and osteoclasts (37).

Fibroblast-like synoviocytes synthesize metalloproteinases (MMPs), a family of enzymes responsible for degradation and reorganization of matrix, that promote the degradation of collagen-rich structures associated to joint tissues (bone, cartilage, ligaments and tendons), and other tissues (81). Since endogenous enzyme inhibitors (e.g. tissue inhibitors of MMPs) fail to reverse this destructive cascade and articular cartilage itself has limited regenerative potential, this process will lead to joint damage and systemic manifestations (81). These manifestations include not only extra-articular disease but accelerated atherosclerosis and consequent increased cardiovascular (CV) morbidity and mortality (80). In addition, FLS proliferation contributes directly to local cartilage destruction and the chronicity of synovial inflammation (37). This synovial inflammation chronicity is also promoted by the synthesis of prostaglandins, proteases and reactive oxygen species (ROS) intermediates by neutrophils, which reside mainly in synovial fluid (82).

Chondrocytes physiologically regulate matrix formation and cleavage: under the influence of synovial cytokines (particularly IL-1 and IL-17) and reactive nitrogen species (RNS), cartilage is progressively deprived of chondrocytes, which undergo apoptosis (37). These processes ultimately lead to the destruction of the surface cartilage and the radiographic appearance of joint-space narrowing (37).

Synovial cytokines, particularly macrophage colony-stimulating factor and receptor activator of nuclear factor kappa B ligand (RANKL), a molecule responsible for inducing osteoclastogenesis, promote osteoclast differentiation and invasion of the periosteal surface adjacent to articular cartilage (83). Tumor necrosis factor-α and IL-1, IL-6 and, potentially, IL-17 amplify osteoclast differentiation and activation, leading to bone erosion (84) (Figure 7).

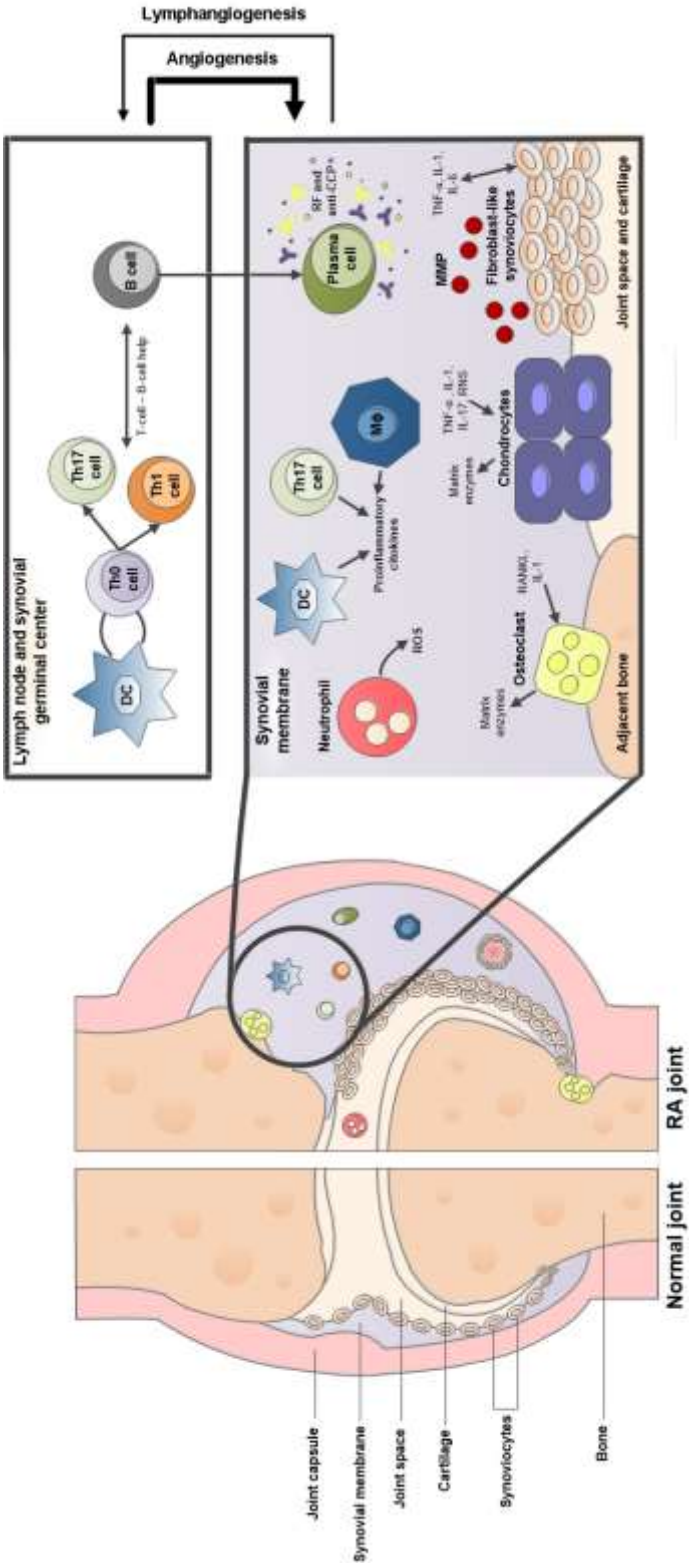


Figure 7. Putative mechanisms involved in rheumatoid arthritis pathophysiology.
Antigen stimulation leads to a cascade of events that include the activation of antigen-presenting cells, such dendritic cells (DCs), antigen presentation to type-0 T helper (Th0) cells and differentiation into type-1 T helper (Th1) cells and type-17 T helper (Th17) cells at lymph node and synovial germinal center. Local antigen-specific, T-cell–mediated B-cell help will occur initiating the adaptive and innate autoimmune responses to citrulline-containing self-proteins that will promote joint tissues damage, chronic synovitis and systemic effects. These responses include interactions shown among leukocytes, fibroblast-like synoviocytes, chondrocytes and osteoclasts, together with the molecular products of damage.

1.6. Classification Criteria

The 1987 American College of Rheumatology (ACR), formerly the American Rheumatism Association, criteria for the classification of RA were initially developed in 1957 by a committee of expert rheumatologists who compiled components of the physical examination and diagnostic testing that they considered important for RA diagnosis (85). Subjects were classified into four groups as follow: 1) "possible RA", 2) "probable RA", 3) "definite RA", and 4) "classic RA" (added in 1958) (85). In 1987 the criteria were revised, simplifying the classification to identify individuals with "definite RA" (85) (Table 1).

Table 1. 1987 ACR criteria for classification as "definite RA"*

1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.
2. Arthritis in 3 or more joint areas[§]	Soft tissue swelling or fluid (not body overgrowth) observed by a physician presenting simultaneously for at least 6 weeks.
3. Arthritis of hand joints	Swelling of wrist and MCP or proximal IP joints for at least 6 weeks.
4. Symmetric arthritis	Simultaneous involvement of the same joint area (defined in 2, above) on both sides of the body (bilateral involvement of proximal IP, MCP or MTP joints is acceptable without absolute symmetry) for at least 6 weeks.
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences, extensor surfaces, or in justarticular regions, observed by a physician.
6. Rheumatoid factor	Detected by a method that is positive in fewer than 5% of normal controls.
7. Radiographic changes	Typical of RA on posteroanterior hand and wrist radiographs; they must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (OA changes alone do not qualify).

*At least 4 criteria (in a total of 7) must be fulfilled for classification of RA; patients with 2 clinical diagnoses are not excluded.

[§]Possible areas: right or left proximal IP, MCP, wrist, elbow, knee, ankle and MTP joints.

ACR: American College of Rheumatology; IP: interphalangeal; MCP: metacarpophalangeal; MTP: metatarsophalangeal OA: osteoarthritis; RA: rheumatoid arthritis.

(Adapted from Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis and rheumatism. 1988;31(3):315-24)

Because of the cohort nature for which criteria were developed, the criteria performs best at distinguishing subjects with long duration and active RA from those with other arthritis (85). Yet, there is a strong interest in identifying the disease earlier in the clinical setting and for research studies since an early aggressive treatment for RA can break or slow the progression of synovitis and bone erosions, decreasing disease-related disability and increasing the rate of disease remission (86). However, criteria such as joint erosions and rheumatoid nodules (masses of inflammatory tissue with a central focus of necrosis, presumably the consequence of vascular inflammation, surrounded by chronic inflammatory cells) are often absent early in the disease, thus decreasing the sensitivity of the classification criteria (85).

In fact, the performance of 1987 ACR criteria on patients with early RA found a wide range of sensitivities from 25% to 90% and a specificity ranging from 60% to 90% (87, 88). Because of the lack of sensitivity in early disease, the 1987 ACR classification criteria for RA have been criticized (89). Therefore, since 1987, there have been several advances in the approach for diagnosing RA, most notably, the use of RF and of anti-citrullinated protein antibody (ACPA), tested as anti-CCP. Anti-cyclic citrullinated peptide and RF are involved in RA pathogenesis (1, 76) and have been shown to be specific markers for RA, particularly for subjects with early disease (anti-CCP: 94-100%; RF: 23-96%) (87, 90, 91). In relation to sensitivity of RF and anti-CCP, it is equivalent in both early and established disease (91).

Current criteria were developed in 2010 by the ACR and European League Against Rheumatism (EULAR) (89). In the new criteria, classification as "definite RA" is based on: synovitis in at least one joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of six or greater (of a possible ten) from the individual scores in four domains, as follow: 1) location and number of swollen and tender joints; 2) serologic levels of RF and anti-CCP; 3) elevated acute-phase reactants such C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) (related to

inflammation and disease activity); and 4) symptoms duration (89) (Table 2). These classification criteria enable the stratification of individuals groups into those with and without RA, in order to standardize recruitment into clinical trials and related studies, and provide the basis for a common approach to disease definition that can be used to compare across studies and centers (89). These criteria also provide the possibility of determine the group at highest risk for developing persistent or erosive RA and of aid diagnosis (89).

Table 2. 2010 ACR/EULAR criteria for classification as "definite RA"*

Domains	*Score*
Location and number of swollen and tender joints	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least one small joint)	5
Serology	
RF (-) and anti-CCP (-)	0
RF (+) or anti-CCP (+) (≤ 3 times than normal)	2
RF (+) or anti-CCP (+) (> 3 times than normal)	3
Acute-phase reactants	
Normal CRP and ESR levels	0
Elevated CRP and ESR levels	1
Symptoms duration	
<6 weeks	0
≥6 weeks	1

*Based in at least a total score of 6 or greater, the presence of synovitis in at least 1 joint and absence of an alternative diagnosis that better explains the synovitis.
ACR: American College of Rheumatology; anti-CCP: anti-cyclic citrullinated peptide; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; RA: rheumatoid arthritis; RF: rheumatoid factor.
(Adapted from Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Annals of the rheumatic diseases. 2010;69(9):1580-8)

1.7. Diagnosis

Rheumatoid arthritis is the paradigm of a systemic AI disease characterized by inflammatory polyarthritis. As a practical matter, a patient older than the age of eighteen who has symmetric joint pain and swelling in the hands and feet and morning stiffness is likely to have RA, especially if RF and/or anti-CCP findings are positive (89). Its early presentation can be quite similar to several other conditions that must be excluded and, thus, caution must be taken to avoid overdiagnosis of RA. On the other hand, early diagnosis is critical so that appropriate treatment can be administered and irreversible damage prevented. The hallmark of RA is symmetric synovial proliferation and tenderness of multiple joints, particularly the small joints of the hands and feet such the MCP, proximal IP and MTP joints (1, 2). Most patients experience joint stiffness or gelling for more than an hour in the morning (24). The blood of approximately 80% of RA patients contains RF (92). Rheumatoid nodules are quite specific for RA and occur in about 20% of patients, generally those with more severe disease and high-title RF (92). Therein lies the advantage for the 2010 ACR/EULAR classification criteria for the contemporary clinician: they provide diagnostic support for recognition of early RA, allowing the physician to make a definitive diagnosis without delay and thus to make a decision to administer disease-modifying antirheumatic drug (DMARD) therapy timely and before joint destruction occurs (89). Nevertheless, these criteria only provide a useful starting point for one to become familiar with the key clinical features of RA. In fact, the complex interplay between various risk factors for RA development makes this multifactorial disease very complex and its diagnosis extremely difficult. Therefore, no single physical finding or laboratory tests definitely confirm the existence of RA (24). In this way, the diagnosis is essentially clinical, which combines anamnesis, physical exam and diagnostic complementary exams (particularly, laboratory and imaging exams) (24).

Laboratory tests include blood count, quantification of RF and anti-CCP, measurement of ESR, quantification of CRP and evaluation of renal and liver functions (24).

The blood count allows, among other parameters, to assess the levels of hemoglobin, hematocrit, white blood cells, platelets and complement (24). The platelet count, white blood cells and complement may be elevated in patients with RA and, typically, these patients present a normochromic normocytic anemia or sometimes microcytic (24). The RF test is readily available and positive in about 80% of patients with RA (93). However, RF may be negative in early RA and positive in many other conditions, especially hepatitis C infection (92). Therefore, the advent of detection of anti-CCP has been a major advance in the diagnosis of early RA (88). Anti-cyclic citrullinated peptide is highly specific for RA and can be detected very early in disease (94). The combination of a positive anti-CCP test with a positive RF test further increases the specificity for RA (combination of these two tests: 99% specificity compared to 70% sensitivity for each of these tests) (76, 95). Thus, seropositivity for either one of these tests, in a patient with polyarthritis in the hands and feet, makes the diagnosis of RA quite likely. The ESR and CRP are the biomarkers most used to, in an indirect way, determine the presence and severity of inflammation, predict the functional status of the joints and assess disease activity (96). Typically, CRP and ESR are elevated (frequently, CRP >3mg/L and ESR >30mm/h) in patients with RA (24). As previously described, the 2010 ACR/EULAR classification criteria recognize the importance of the number of involved joints as well as of RF, anti-CCP, ESR and CRP (89).

The evaluation of renal and liver functions, performed by the determination of serum creatinine (SCr), estimated glomerular filtration rate (eGFR), urea, transaminases, albumin and coagulation factors, may be useful in the prediction of therapeutic impact on these organs (24).

In addition, other laboratory tests can be performed such the assessment of synovial fluid of affected joints, looking for inflammatory patterns; quantification of antinuclear antibodies (ANAs), which are positive in about 25% of patients, and of antineutrophil cytoplasmic antibodies (ANCAs); and, the analysis of urine, which can confirm the presence of

microscopic hematuria or proteinuria, usually present in patients with RA (24).

Concerning to imaging exams, radiographs of the hands and feet are sometimes diagnostic at presentation, and musculoskeletal ultrasound and magnetic resonance imaging can detect early evidence of synovitis and erosions not seen on radiographs, although the aim today is to prevent joint damage from occurring (97).

1.8. Disease Monitoring

Anamnesis and physical exam are also aimed to quantify the disease severity, such as intensity and duration of morning stiffness, assess patient's general condition (including the analysis of the presence of extra-articular manifestations), quantitative assessment of pain, functional testing of the joints, and others (98). Disease monitoring is performed taking into account the quantification of disease activity, joint damage, and health-related quality of life impact.

1.8.1. Quantification of joint damage

Several methods have been introduced for the annual or biannual scoring of plain radiographs (normally of the anatomic regions with the joints most frequently affected, such as hands, wrists and foots) in patients with RA for the quantification of joint damage.

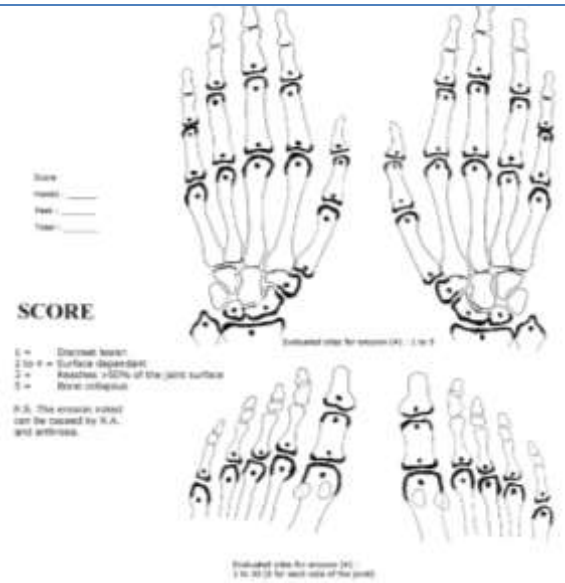
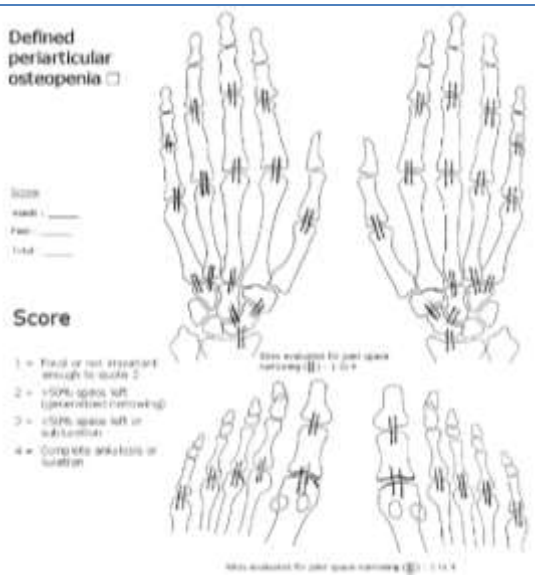
Nowadays, the two most widely used measures are based on the work of Sharp and Larsen, which provide a continuous quantitative scale rather than a limited qualitative measure of radiographic damage (99-102).

1.8.1.1. Sharp-van der Heijde score

The Sharp method modified by van der Heijde - Sharp-van der Heijde score (SvH) - involves separate scores for erosions and for joint space narrowing (JSN). Erosion score is evaluated in sixteen areas from each hand and wrist, and each side of the ten MTP and two IP joints of the big joints of the feet (103, 104) and erosions are classified as: 1) normal; 2) discrete

erosions; 3) larger erosions according to surface area involved; 4) erosions extending over middle of the bone; and, 5) complete collapse (103, 104). Joint space narrowing score includes fifteen areas from the hands and wrists and six areas from the feet (103) and is classified according to the original Sharp method as: 1) normal; 2) focal narrowing; 3) reduction of less than 50% of joint space; 4) reduction of greater than 50% of joint space; and, 5) ankylosis (Table 3) (102, 103, 105).

Table 3. Sharp-van der Heijde score

Erosion score		Joint space narrowing score	
			
Classification	**Score**	**Classification**	**Score**
Normal	0	Normal	0
Discrete erosions	1	Focal narrowing	1
Larger erosions according to surface area involved	2 or 3	Reduction of less than 50% of joint space	2
Erosions extending over middle of the bone	4	Reduction of greater than 50% of joint space	3
Complete collapse	5	Ankylosis	4
56 joints scored		42 joints scored	
Maximum erosion score: 160 - hands and wrists Maximum erosion score: 120 - feet		Maximum JSN score: 120 - hands and wrists Maximum JSN score: 48 - feet	
	Total: 280		Total: 168
Total maximum score: 448			

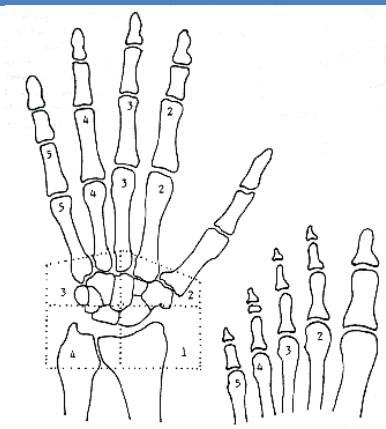
JSN: joint space narrowing.
(Adapted from http://rheumatology.usherbrooke.ca/radiographie_formulaire.pdf)

1.8.1.2. Larsen method

The Larsen method is based in a global score of each joint, including both erosions and JSN of all synovial joints, on a scale of zero to five according to reference radiographs (100). It is applied to standard radiographs in sixteen areas in both hands, eight areas in both wrists, and eight areas in both feet (106).

The classification is the follow: 1) intact bony outlines and normal joint space; 2) erosion less than 1mm in diameter or JSN; 3) one or several small erosions, diameter more than 1mm; 4) marked erosions; 5) severe erosions, where there is usually no joint space left, and the original bony outlines are partly preserved; and, 6) mutilating changes, where the original bony outlines have been destroyed (Table 4) (106).

Table 4. Larsen method



Classification	Score
Intact bony outlines and normal joint space	0
Erosion less than 1mm in diameter or JSN	1
One or several small erosions, diameter more than 1mm	2
Marked erosions	3
Severe erosions, where there is usually no joint space left, and the original bony outlines are partly preserved	4
Mutilating changes, where the original bony outlines have been destroyed	5
32 joints scored	
Total maximum score: 160	

JSN: joint space narrowing.

(Adapted from Larsen A. How to apply Larsen score in evaluating radiographs of rheumatoid arthritis in long-term studies. The Journal of rheumatology. 1995;22(10):1974-5)

Sharp and Larsen scores are correlated significantly and the minimal clinically important difference is roughly 1% of the maximum for both of the methods (107). Overall, the Larsen method is more easily scored and less time-consuming when compared to the Sharp method (107).

1.8.2. Disease activity measure

Although radiographic evidence of disease progression is a useful and specific way to evaluate disease progression and the effectiveness of treatment, it is less useful for routine monitoring in the office (108). The development of routine standardized measures of disease activity allows a "treat-to-target" strategy using pharmacologic therapy (109). These targets permit physicians and patients to set goals for treatment (109). Furthermore, they provide clinically meaningful and reliable estimates of disease activity with interpretation of multiple data points simultaneously, are less susceptible to selection bias related to the reporting of a single measurement and are preferable for statistical analysis in studies for quantifying disease activity, even in the lower ends of the scales (110-112). Therefore, a number of disease activity indexes have been developed, based on expert panel recommendations, for use in clinical trials and the office setting to standardize definitions and guide treatment (109).

Some of these commonly disease activity indexes used in daily practice are: 1) Disease Activity Score (DAS); 2) Disease Activity Score in twenty-eight joints (DAS28); 3) Simplified disease activity index (SDAI); 4) Clinical disease activity index (CDAI); 5) Patient activity score (PAS) and patient activity score-II (PASII); and, 6) Routine assessment of patient index data (RAPID) (108, 113). From all of these methods, one of the most used in clinical practice is DAS28 (108, 114).

The DAS28 is calculated attending to four variables (4v) as described by Prevoo *et al.*: 1) 28 tender joints count (TJC); 2) 28 swollen joints count (SJC); 3) ESR in the first hour (ranges from 0–100mm/h) or the CRP levels (range from 0.20–1440mg/L) (108, 113); and 4) patient global assessment of disease activity or global health on a visual analog scale (VAS) (114, 115) (Figure 8).

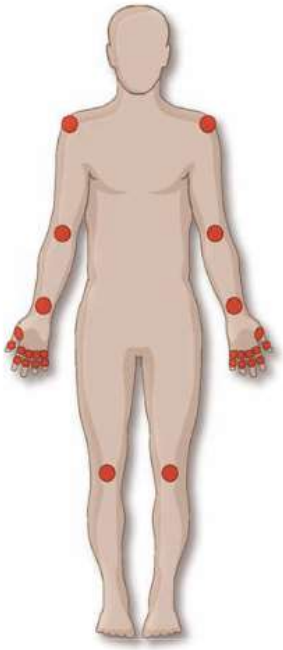


Figure 8. Twenty-eight regions used to the count of tender and swollen joints for Disease Activity Score 28. The joints used include: 2 in shoulder, 2 in elbow, 2 in wrist, 10 metacarpophalangeal, 10 proximal interphalangeal and 2 in knee.
(Adapted from The Institute for Continuing Healthcare Education. Disease activity score in 28 joints (DAS28) [06 June 2014]. Available from: http://www.iche.edu/newsletter/DAS28.pdf)

The DAS28 evaluation is carried out quarterly to semiannual and permits the disease activity interpretation in activity degrees as remission (DAS28≤2.6), low activity (2.6<DAS28<3.2), moderate activity (3.2<DAS28≤5.1) or high activity or uncontrolled disease (DAS28>5.1) (114). However, recent data from two large observational studies suggested that DAS28(CRP) scores tended to be lower than DAS28(ESR) scores and, thus, Inoue *et al.* suggested potential new thresholds for disease activity categories for DAS28(CRP) (116, 117).

Despite DAS28 have been extensively validated and endorsed by the ACR and EULAR for RA disease activity measurement in clinical trials and often considered the "gold standard" (113), some disadvantages are observed in clinical practice such as the need for a blood sample, time needed for physicians to perform joint counts, complicated mathematical

calculation of the composite score, and potential confusion from choosing among the multiple formulas available and interpreting into activity degrees (113).

1.8.3. Health-related quality of life impact

The ability to effectively measure health-related quality-of-life longitudinally is central to describing the impact of disease, treatment, or other insults upon the patient (118).

The Health Assessment Questionnaire (HAQ) is one of the most widely used comprehensive, validated, patient-oriented outcome assessment instruments for establishing health outcome assessment as a quantifiable set of reliable, valid and responsive hard data points (118). It's designed to capture prospectively and by protocol the long term influence of multiple chronic illnesses and to allow supplementation by additional measures for particular studies (118). The HAQ, that collects data on five generic patient-centered health dimensions, which is a comprehensive outcome measure that assesses a hierarchy of patient outcomes in four domains: 1) disability; 2) discomfort and pain; 3) adverse drug reactions (ADRs); and, 4) costs. Disability is assessed by the eight categories of dressing, arising, eating, walking, hygiene, reach, grip, and common activities (119, 120). Discomfort is determined by the presence of pain and its severity. Specific ADRs are classified according to their severity and whether the drug was stopped (119, 120). Costs are divided into direct (e.g., hospitalization, surgery, nursing home care, physician and health worker visits and medications) and indirect costs (e.g., those associated with productive days lost for the employed, housewives, students and retired persons, and changes in lifestyle and activities for the patient and family) (119, 120). Death, while obviously not a self-report outcome, is a requisite part of the conceptual model of patient outcome (119).

The HAQ is usually self-administered, but can also be given face-to-face in a clinical setting or in a telephone interview format by trained outcome assessors, and has been validated in these settings. The questionnaire is

typically mailed to patients every six months, and they are asked to complete it without additional instructions. Follow-up phone calls are sometimes needed to obtain missing data or to clarify ambiguous responses in the high-quality research data applications (119).

1.8.4. Limitations

However, and unfortunately, the above described tools have their limitations, namely the existence of errors in self-assessment of disease activity, due to the presence of other diseases which leads to deviations from the "true" activity, and the variability among health professionals (91). These limitations can be minimized if professionals make use of various tools and promote patients education about the impact that other diseases may have in the perception of pain and impaired function of the joints (91).

1.9. Disease Burden and Prognostic

The complex pathophysiology of RA leads to a synovitis associated with hyperplasia of synovial cells, *pannus* formation, excess of synovial fluid, cartilage damage and bone erosion (1) (Figures 6 and 7).

This inflammatory joint disease usually affects up to 80% of patients within one year of diagnosis and the eroded bone does not appear to be repaired, suggesting that the main goal should be to prevent this irreversible bone erosion (37, 89). The distinct pattern of bone and joint destruction in RA is translated in pain, stiffness (worse in morning) and swelling of involved joints, which difficult the daily living activities (1, 2). Additionally, there is also a persistent systemic inflammatory state that may promote a number of other extra-articular effects such lung fibrosis, osteoporosis, infections, and CV, hematological and GI diseases (31, 121, 122). These systemic complications related to the underlying disease process impact the average life expectancy of patients with RA (84). In accordance, life expectancy in females is reduced by ten years whereas in males is diminished by four years (123). Studies have demonstrated improvements in

survival rates over time (124, 125), however, others appointed that RA patients have not experienced improvements in survival rates over the past four decades, when compared to general population (126-128). No less important is the fact that about 20% of RA patients have approximately 2-fold to 4-fold higher risk of developing affective disorders, such as depression and anxiety, than general population (129). Furthermore, RA affects not only the patient but also the society in general, since the disease represents great economic losses, mostly caused by work withdrawal due to failure or pain associated with the disease, loss of work productivity and constant use of health care services (130). With continuous joint damage, mostly irreversible, there is a greater inevitability of worsening disability, not only due directly to RA but also to her complications (Figure 9).

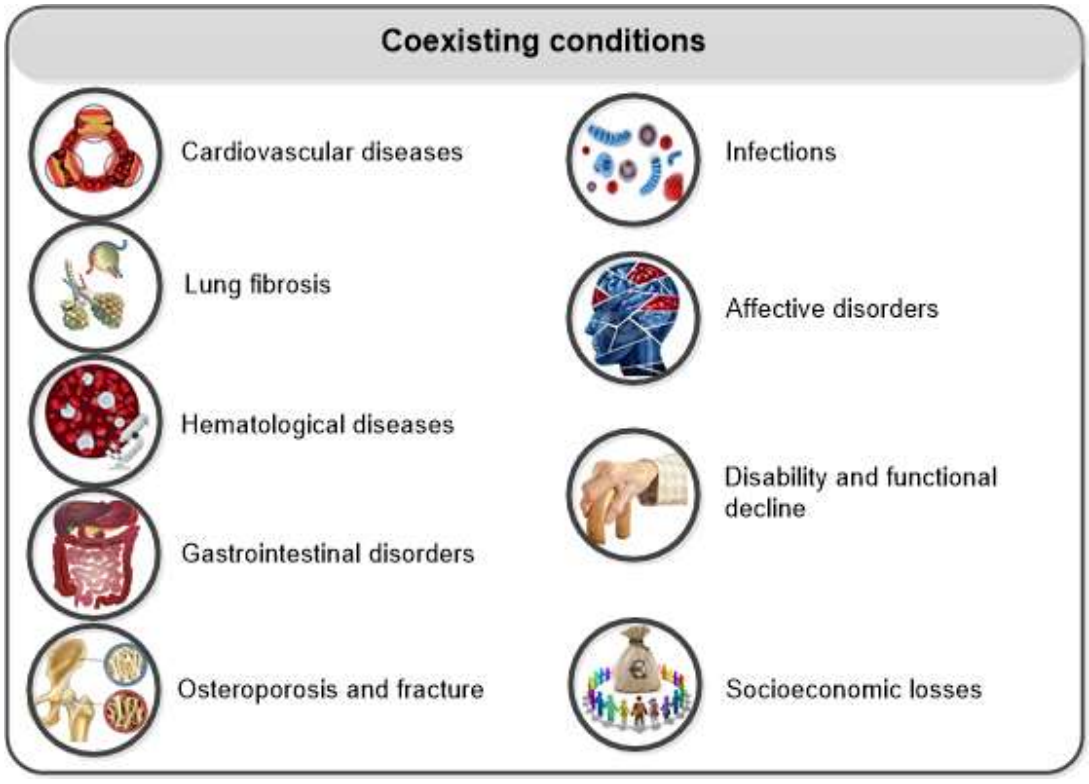


Figure 9. Rheumatoid arthritis coexisting conditions.

(Adapted from McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19)

1.10. Therapeutic Strategies

An earlier detection of RA and a rapid effective and more aggressive therapeutic institution are the key factors to achieve the disease remission and improve survival rates (81, 124, 125). Some data suggest that definitive treatment should be administered within three months of the onset of disease (131). From the foregoing, it is understood that the later treatment is started, the greater the extent of damage is later scored (131).

Normally, RA treatment is directed to control the inflammation and the pain, and ultimately, to retard or limit the joint destruction progression. Thus, the treatment is based both in non-pharmacologic and pharmacologic strategies (131) (Figure 10).

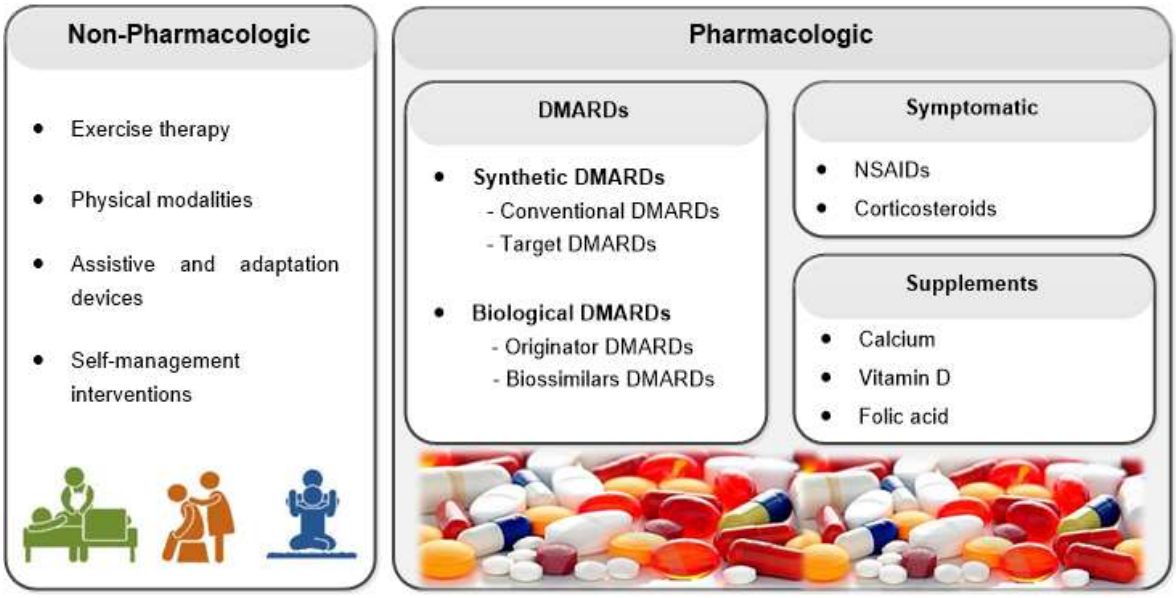


Figure 10. Non-pharmacologic and pharmacologic treatment strategies for rheumatoid arthritis.

DMARDs: disease-modifying antirheumatic drugs; NSAIDs: non-steroidal anti-inflammatory drugs.

1.10.1. Non-Pharmacologic treatment

Non-pharmacologic treatment includes a wide range of modalities, such exercise therapy, physical modalities, assistive and adaptation devices and

self-management interventions (132). Nowadays, the large majority of RA patients has been told to use and tried at least one type of non-pharmacologic treatment (132).

1.10.1.1. Exercise therapy

The objectives of exercise therapy in patients with RA are the restoration, preservation or improvement of joint range of motion, muscle strength, aerobic capacity and the performance of specific activities or skills (133).

1.10.1.2. Physical modalities

The physical modalities include electro-physical modalities (e.g. thermotherapy, electrotherapy, laser therapy and spa therapy) and manual therapy (e.g. massage), used to facilitate and restore movement and function (134).

1.10.1.3. Assistive and adaptation devices

Assistive and adaptation devices of the physical environment (e.g. orthoses and special shoes and inserts) are frequently prescribed to ease pain, overcome joint limitations, compensate for muscle weakness and enhance safety with the ultimate aim to prevent or reduce dependence (134).

1.10.1.4. Self-management interventions

Self-management interventions are seen as key components of rheumatologic care, in particular of RA (135), aimed to directly and/or indirectly improve health-related quality of life, healthcare utilization and perceived self-efficacy (136-139). These include: 1) educational and psychosocial interventions, such as, techniques to deal with problems and promotion of adequate decision making; 2) lifestyle interventions, such as appropriate exercise for maintaining and improving strength, flexibility and endurance, and making adequate nutrition; and, 3) treatment interventions for appropriate use of medications (136-139).

1.10.2. Pharmacologic treatment

The pharmacologic management of RA rests primarily on the use of symptomatic therapy, supplements and DMARDs.

1.10.2.1. Symptomatic therapy

Usually, as symptomatic therapy, are used the non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (24). Non-steroidal anti-inflammatory drugs are a heterogeneous group of drugs aimed for controlling the inflammation and pain of affected joints by inhibition of cyclooxygenase-2 (COX-2) (24). Usually they are administered orally in low-doses and for a short period of time in order to minimize the possible occurrence of undesirable ADRs (24). Corticosteroids are fast action anti-inflammatory drugs since are responsible for decreasing cytokines synthesis and inhibiting lymphocyte function (140). Nevertheless, and because its ADRs, they are generally administered orally in low-dose (≤ 5mg), which allows their continuously use over several years (140).

1.10.2.2. Supplements

Corticosteroids chronic use requires supplementation with calcium and vitamin D to circumvent osteopenia appearance (93, 141). Methotrexate (MTX) use requires folic acid supplementation to prevent the occurrence of ADRs-related to MTX, and folic acid is usually administered twenty-four hours after MTX administration (142, 143).

1.10.2.3. Disease-modifying antirheumatic drugs

Disease-modifying antirheumatic drugs are commonly characterized by their capacity to reduce or reverse signs and symptoms, disability, impairment of quality of life, inability to work, and progression of joint damage and thus to interfere with the entire disease process (144). They are a heterogeneous drugs group that form two major classes: synthetic chemical compounds - synthetic DMARDs (sDMARDs) - and biological agents - biological DMARDs (bDMARDs), which could be used in monotherapy or in

combination (1). Some conventional sDMARDs (csDMARDs) include chemical agents such as MTX, sulfasalazine (SSZ) hydroxychloroquine (HCQ), cyclosporine (CCP) and leflunomide (LEF), whereas targeted sDMARD (tsDMARD) include new chemical agents such as tofacitinib (1, 145). Regarding to bDMARDs, these can be divided into biological originator DMARDs (boDMARDs) that include the TNF-α inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab and infliximab), non-TNF-α inhibitors (tocilizumab, abatacept, rituximab and anakinra), and biosimilars DMARDs (bsDMARDs), such as the recently approved bs-infliximab (145, 146).

Despite all of these drugs, the most used and cost-effective DMARD for RA treatment is MTX (147).

2. METHOTREXATE

This section provides information about Methotrexate historical aspects, chemical characteristics, pharmacokinetics and pharmacodynamics.

2.1. Historical Aspects

The introduction of high-dose MTX in the treatment regimen of children with acute lymphoblastic leukemia by Sidney Farber in 1948 resulted from the observation that folic acid antagonists interfered with the normal growth of cells (148). Only in 1951, Gubner demonstrated that low-dose MTX (up to 25mg/w) could be effective in controlling disease activity both in patients with psoriatic arthritis and RA (149). In 1988 this drug was approved by US Food and Drug Administration (FDA) for RA treatment. In the late of 1980s, low-dose MTX started to be used in juvenile idiopathic arthritis (JIA) patients with polyarthritis (150) and in ankylosing spondylitis (AS) (151). Moreover, low-dose MTX have been used in other rheumatic diseases, such as Felty's syndrome (152), ANCA-associated vasculitis (153), polymyalgia rheumatic/giant cell arthritis (GCA) (154), inflammatory myopathy (155) and

systemic lupus erythematosus (SLE) (156), and also in dermatological diseases, such as psoriasis (157).

2.2. Chemical Characteristics

Methotrexate (2,4-diamino-N10-methyl propyl glutamic acid), a Biopharmaceutical Classification Systems class III drug, is an antifolate negatively charged drug with low permeability (ClogP = 0.53) and poor aqueous solubility (0.01mg/ml). Structurally, it's composed by three parts as follows: 1) pteridine ring, 2) *p*-aminobenzoic acid and 3) glutamic acid (158, 159) (Figure 11).

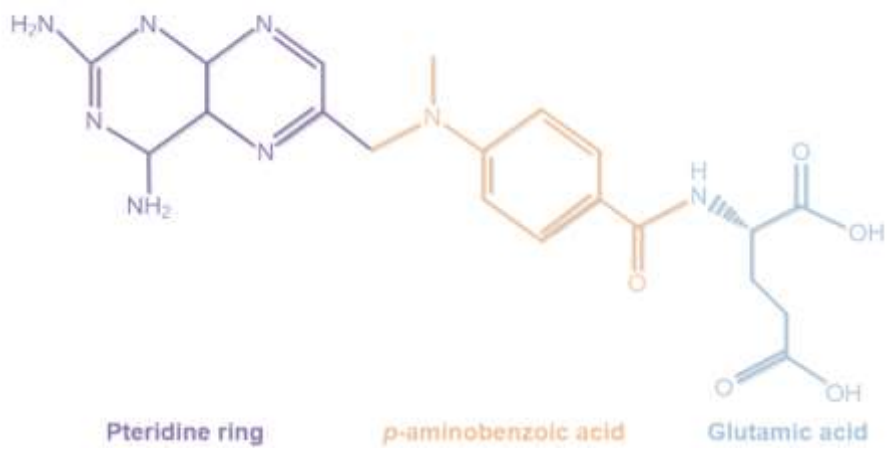


Figure 11. Methotrexate chemical structure. Methotrexate is composed by a pteridine ring, *p*-aminobenzoic acid plus glutamic acid.

Methotrexate is a weak bicarboxylic acid (pKa value = 4.7-5.5) with a molecular weight of 454.5g/mol ($C_{20}H_{22}N_8O_5$), structurally similar to folic acid, differing only by the substitution of a hydroxyl group for an amine in the pteridine ring (at C4 carbon) and the addition of a methyl group on tenth nitrogen of *p*-aminobenzoic acid (159-161) (Figure 12).

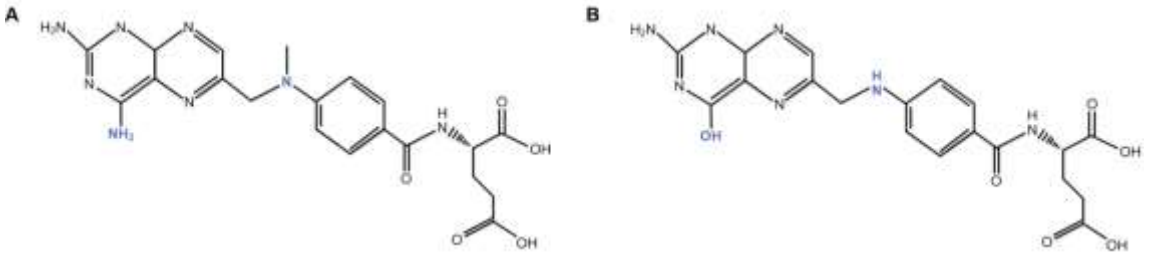


Figure 12. Chemical structures of Methotrexate (A) and Folic Acid (B). Methotrexate and Folic Acid chemical structures are similar, differing in the pteridine ring (by substitution of a hydroxyl group for an amine) and on the 10(th) nitrogen of *p*-aminobenzoic acid (by addition of a methyl group).

2.3. Pharmacokinetics

Pharmacokinetics (PK) describes what the body does to a drug. Pharmacokinetics parameters result from the absorption, distribution, metabolism and excretion of drugs and provide information about the rates of these processes and systemic exposure of drugs (162).

The bioavailability of MTX is relatively high but can vary depending of the dose and administration route (enteral or parental) (163, 164). MTX absorption in gastrointestinal tract (GIT) occurs via proton-dependent active transport, which is partially shared with folic acid (163). Methotrexate follows saturable (Michaelis-Menten) kinetics and, thus, its absorption becomes dose dependent in GIT (165). Consequently, at 7.5mg/w, enteral and parenteral absorption is the same, but at doses of 15mg/w or more, enteral absorption may decrease by as much as 30% compared with parenteral dosing (163, 166). Bioequivalent doses of MTX were compared and mean intramuscular bioavailability was nearly 76% compared with 33% via *per os* (PO) route (167). Therapeutic and toxic plasma concentrations of MTX are 0.005 and 0.01μg/ml in low-dose therapy; and, on high-dose therapy, those concentrations are 2.27 and 4.54μg/ml at 24h, 0.23 and 0.45μg/ml at 48h, and 0.02 and 0.04μg/ml at 72h (168). Methotrexate shows high inter- and intrapatient variability in both serum and cerebrospinal fluid (CSF) (169). Due to its erratic GI absorption, it has been suggested that doses ≥25mg should be administered parenterally (170). Factors that influence its absorption

include food intake, oral non-absorbable antibiotics, flow transit through GIT (such diarrhea and constipation) and intestinal pathology (163, 170, 171). After absorption, 10% of MTX is converted in the liver, by aldehyde oxidase, to 7-hydroxymethotrexate (7-OH-MTX), an active metabolite (161). Approximately 50% of MTX is albumin-bound, whereas 90% to 95% of 7-OH-MTX is albumin-bound (163). Nevertheless, other drugs such NSAIDs alter MTX and 7-OH-MTX binding to plasmatic proteins and impair MTX hepatic metabolism (161). The half-life of MTX in the serum ranges from 6 to 8 hours after administration and, MTX is undetectable in the serum by 24 hours (161). The majority of both MTX and 7-OH-MTX is excreted in the urine, although a small portion is also excreted in the bile (161, 163, 172). Renal clearance is likely due to a combination of filtration and secretion in the proximal tubule with subsequent reabsorption in the distal tubule (173). Therefore, renal insufficiency can lead to toxicity caused by impaired clearance of MTX. Excretion of MTX is reduced by interactions with weak organic acids such as NSAIDs (161). These interactions are generally only clinically relevant at the typical higher weekly maintenance MTX dosage range (up to 25mg/w) but not at the lower doses, commonly used to begin RA treatment (166).

During PK processes, a drug molecule passes through several biological membranes. The extent of drug movement through these membranes is generally affected by the physicochemical properties of a drug, namely size, lipophilicity and charge (or degree of ionization) (162). In addition, membrane transporters have a significant role in facilitating or preventing drug movement (174). Transporters may be classified as influx (uptake into cell) and efflux (out of cell) transporters, which are typically located either at the basolateral or apical membrane in polarized cells. Interplay of influx and efflux transporters together with phase I and II metabolism may be required for the sequential traverse of the basolateral and apical membranes (175). Therefore, drug transporters can be regarded as completing the phase I and II enzyme-based detoxification system; drug uptake delivers the drug to the

detoxification system to facilitate metabolism, whereas drug efflux decreases the load on detoxification enzymes (176).

2.3.1. MTX cellular transport

In enterocytes, at apical membrane, MTX is absorbed through active transport mediated predominately by solute carrier (SLC) family 19 member 1 (SLC19A1), followed by SLC family 46 member 1 (SLC46A1) (161, 163, 177). Methotrexate may also bind, with lower affinity than folic acid, to folate receptor alpha (FOLR1), which transports MTX via endocytosis (178). Methotrexate efflux from enterocytes can occur to intestinal tract lumen mediated by adenosine triphosphate (ATP)-binding cassette (ABCs) transporters, i.e., by ATP-binding cassette sub-family C member 2 (ABCC2), ATP-binding cassette sub-family B member 1 (ABCB1) and ATP-binding cassette sub-family G member 2 (ABCG2); or to bloodstream by ATP-binding cassette sub-family C member 1 (ABCC1) and ATP-binding cassette sub-family C member 3 (ABCC3) (179). In bloodstream, MTX can be distributed for hepatocytes or renal cells (148). Hepatic uptake of MTX involves SLC19A1, SLC organic anion transporter family member 1B1 (SLCO1B1) and SLCO family member 1B3 (SLCO1B3) (173). Most of this drug, inside the hepatocytes, re-enters into bloodstream by ABCC3 and ABC sub-family C member 4 (ABCC4) and, the remaining, is oxidized to 7-OH-MTX or is excreted into the bile duct by ABCC2, ABCB1 and ABCG2 (173, 174). Systemic clearance of MTX happens primarily through renal glomerular filtration and active secretion over the proximal tubular cells (173). Some renal transporters have affinity to MTX and 7-OH-MTX, which allow their entrance in renal cells by solute carrier family 22 member 6 (SLC22A6) and solute carrier family 22 member 8 (SLC22A8), in basolateral membrane, and by SLC family 22 member 11 (SLC22A11) and SLCO family member 1A2 (SLCO1A2), in apical membrane (179, 180). Moreover, SLC family 16 member 7 (SLC16A7) has been described as having a moderate to low expression in plasma membrane of kidney but its function on MTX transport remains unclear (181, 182). Furthermore, MTX excretion through urinary tract can be mediated by ABCB1, ABCC2, ABCC4 and ABCG2 (147, 161, 180) (Figure 13).

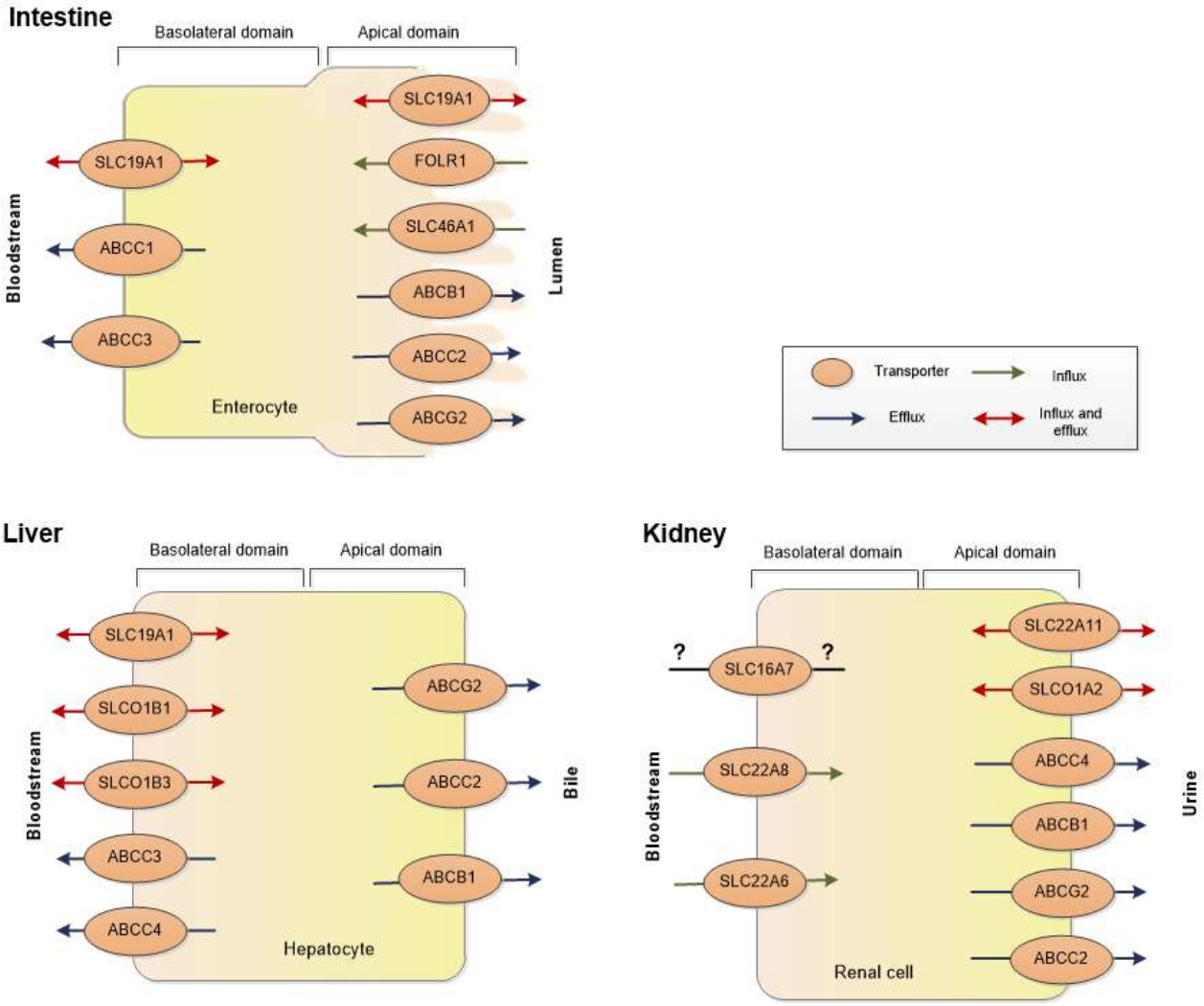


Figure 13. Methotrexate transporters cells location and representation of their influx and/or efflux function.

ABCB1: adenosine triphosphate (ATP)-binding cassette sub-family B member 1; ABCC1: adenosine triphosphate (ATP)-binding cassette sub-family C member 1; ABCC2: adenosine triphosphate (ATP)-binding cassette sub-family C member 2; ABCC3: adenosine triphosphate (ATP)-binding cassette sub-family C member 3; ABCC4: adenosine triphosphate (ATP)-binding cassette sub-family C member 4; ABCG2: adenosine triphosphate (ATP)-binding cassette sub-family G member 2; ATP: adenosine triphosphate; FOLR1: folate receptor alpha; SLC16A7: solute carrier family 16 member 7; SLC19A1: solute carrier family 19 member 1; SLC22A6: solute carrier family 22 member 6; SLC22A8: solute carrier family 22 member 8; SLC22A11: solute carrier family 22 member 11; SCL46A1: solute carrier family 46 member 1; SLCO1B1: solute carrier organic anion transporter family member 1B1; SLCO1B3: solute carrier organic anion transporter family member 1B3; SLCO1A2: solute carrier organic anion transporter family member 1A2.

2.4. Pharmacodynamics

Pharmacodynamics (PD) describes the extent and time course of the effects of drugs on physiological and pathophysiological processes (162). The relationship between drug concentration and drug effect is important in determining PD (162). Thus, once inside the cells MTX is metabolized to methotrexate polyglutamates (MTXPGs) by a sequential addition of glutamic acid residues via the enzyme folylpolyglutamate synthetase (FPGS) (163, 183). Nevertheless, gamma-glutamyl hydrolase (GGH) enzyme removes the glutamic acid residues of MTXPGs and, consequently, MTX can be transported out of the cells by efflux transporters (163). Polyglutamation of MTX enhances the intracellular retention of MTX promoting the inhibition of folate, methionine and adenosine pathways and also of purines and pyrimidines *de novo* synthesis, crucial for the MTX anti-inflammatory and antiproliferative effects (161, 163, 184) (Figure 14) (see 2.4.1. MTX action mechanism: the role of the principal pathways).

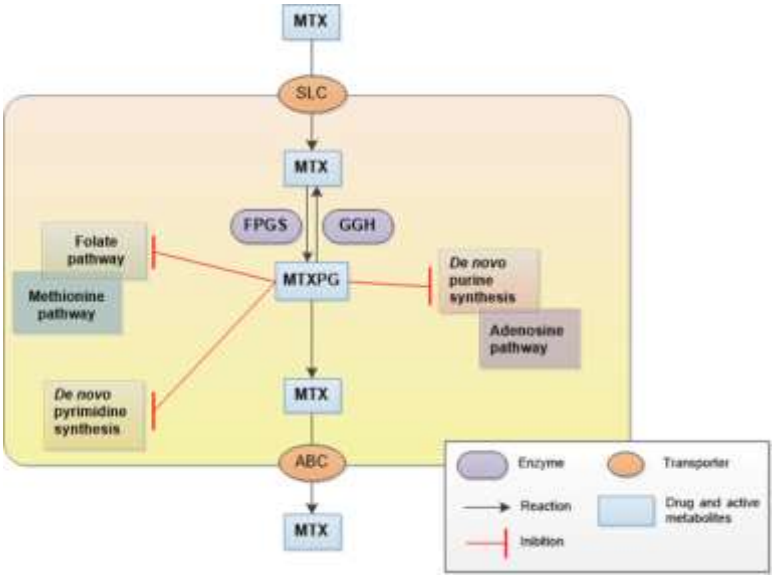


Figure 14. Methotrexate pharmacodynamics. Left panel represents the intervention of MTX in *de novo* pyrimidine synthesis, folate and methionine pathways. Right panel shows the effect of MTX in *de novo* purine synthesis adenosine pathway. These action mechanisms contribute to MTX antiproliferative and anti-inflammatory effects.

ABC: adenosine triphosphate (ATP)-binding cassette; ADORA: adenosine receptor; FPGS: folylpolyglutamate synthetase; GGH: gamma-glutamyl hydrolase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SLC: solute carrier.

Other proposed mechanisms include: 1) modifications in the expression of cellular adhesion molecules; 2) reduction of proinflammatory cytokines production; 3) apoptosis induction, limiting synovial tissue hyperplasia; and, 4) alterations in humoral responses and in bone formation (161, 185).

2.4.1. MTX action mechanism: the role of the principal pathways

Methotrexate pharmacological effects (antiproliferative and anti-inflammatory effects) are primarily attributed to its ability to inhibit multiple enzymes involved in folate pathway and *de novo* nucleotides synthesis (179). This inhibitory capacity is then reflected in other pathways, such methionine and adenosine pathways, which along will contribute to MTX action mechanism (179).

2.4.1.1. Folate and Methionine Pathways

Methotrexate effects are achieved by MTX and MTXPGs high affinity binding to dihydrofolate reductase (DHFR) (186-188) leading to its inhibition and, consequently, preventing the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF). Since THF is essential for the generation of folate cofactors, required for *de novo* purine and pyrimidine synthesis (161, 189) (see *De novo nucleotides synthesis pathways*), lower THF levels result in the depletion of metabolically active intracellular folates, accumulation of homocysteine and reduction in methionine (161, 189). Thus, is hypothesized that MTX, by decreasing THF levels, indirectly increases ROS, leading to apoptosis (185). Furthermore, THF can be transformed into 10-formyltethrahydrofolate (10-CHO-THF) by methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), which can convert 10-CHO-THF into 5,10-methenyltetrahydrofolate (5,10-CH-THF) and then into 5,10-methylenetetrahydrofolate (5,10-MTHF). The 5,10-MTHF can regenerate the THF via serine hydroxymethyltransferase 1 (SHMT1) or can be converted to 5-methyltetrahydrofolate (5-MTHF) via methylenetetrahydrofolate reductase (MTHFR). The conversion of 5-MTHF to THF is mediated by methionine synthase (MS), in the presence of methylcob(III)alamin, with simultaneously

remethylation of homocysteine to methionine (190, 191). Additionally, methionine synthase reductase (MTRR), maintains adequate levels of methylcob(III)alamin, the activated cofactor for MS. Additionally, methionine can be transformed into S-adenosyl methionine (SAM), catalysed by methionine adenosyltransferase (MAT), and then to S-adenosyl homocysteine (SAH), catalysed by methyltransferases (190). Furthermore, S-adenosylhomocysteinase (AHCY) catalyses the reversible hydrolysis of SAH to adenosine and homocysteine without added cofactors, leading to accumulation of homocysteine and adenosine, and, reduction in methionine (192) (Figure 15).

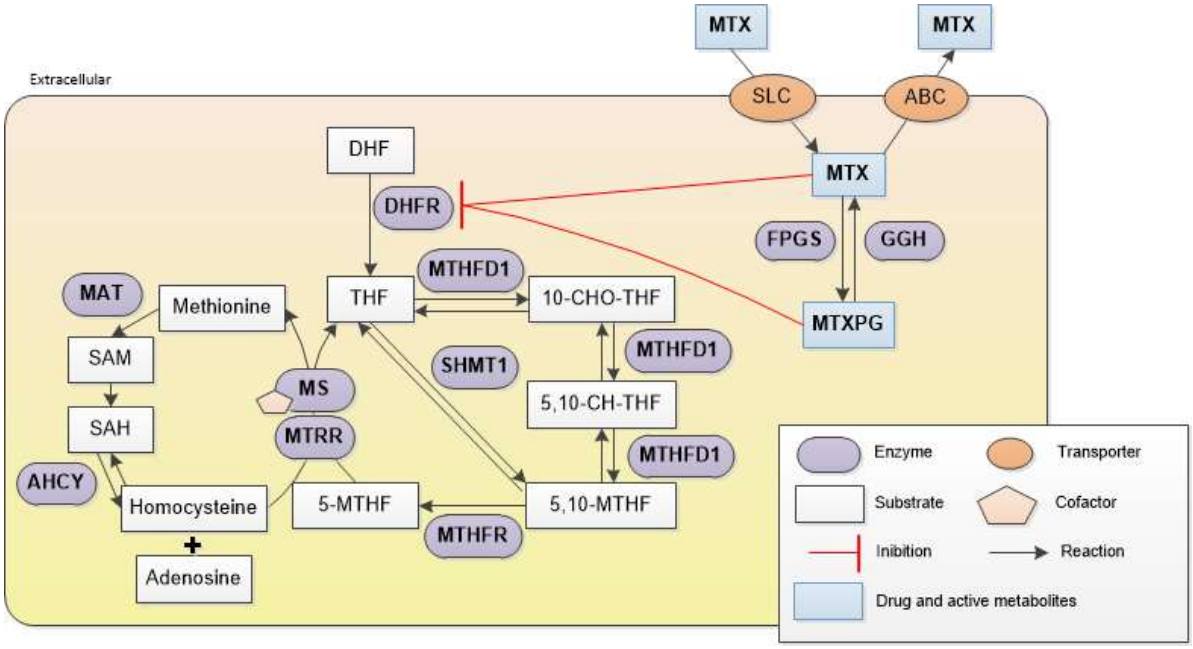


Figure 15. Methotrexate action mechanism - Folate and Methionine Pathways.

5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; 5,10-CH-THF: 5,10-methenyltetrahydrofolate; 10-CHO-THF: 10-formyltetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; AHCY: S-adenosylhomocysteinase; DHF: dihydrofolate; DHFR: dihydrofolate reductase; FPGS: folylpolyglutamate synthetase; GGH: gamma-glutamyl hydrolase; MAT: methionine adenosyltransferase; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTRR: methionine synthase reductase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SAH: S-adenosyl homocysteine; SAM: S-adenosyl methionine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; THF: tetrahydrofolate.

2.4.1.2. De novo nucleotides synthesis pathways

Nucleotides (pyrimidines and purines) are crucial for DNA and RNA synthesis as well as for additional cellular processes where they, for example, serve as energy carriers or signaling molecules cofactors, necessary for normal cellular proliferation (193-195). Intracellular nucleotide pools are controlled by *de novo* synthesis pathways, where nucleotides are synthesized from small metabolites, that work in coordination with salvage pathways, in which nucleotides are synthesized from intermediates in the degradative pathway for nucleotides (193). Salvage pathways are used to recover bases and nucleosides that are formed during DNA and RNA degradation. This is important in some organs because some tissues cannot undergo *de novo* synthesis (193).

Pyrimidine synthesis pathway

Methotrexate antiproliferative effect is partly achieved by intracellular inhibition of thymidylate synthase (TS) by MTXPGs (161, 163, 196). Thymidylate synthase is a key protein for the *de novo* pyrimidine synthesis and is responsible for the simultaneous conversion of deoxyuridine monophosphate (dUMP) and 5,10-MTHF to deoxythymidine monophosphate (dTMP) and DHF. Subsequently, the dTMP is phosphorylated to deoxythymidine triphosphate (dTTP) and used for the DNA synthesis and repair (161, 197) (Figure 16).

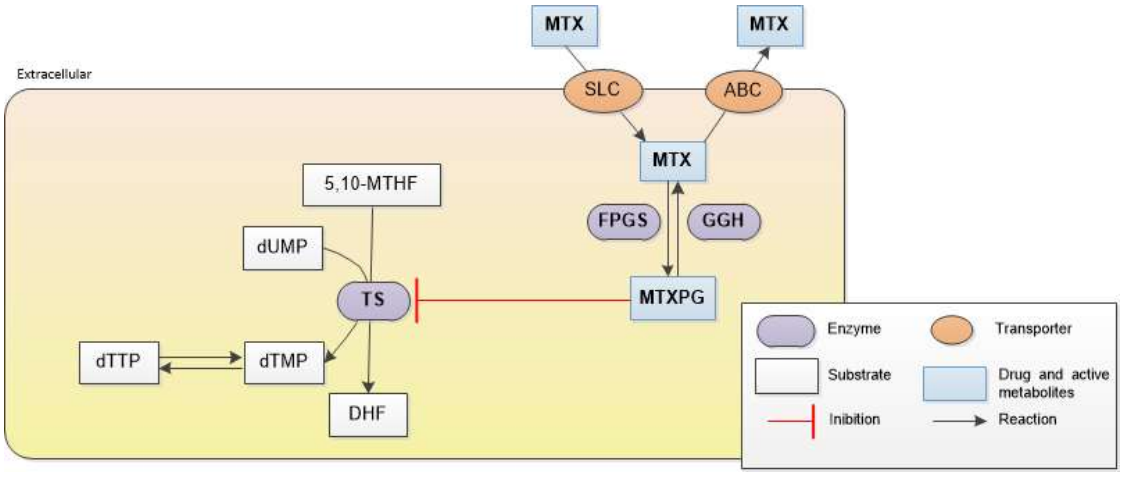


Figure 16. Methotrexate action mechanism - Pyrimidine Pathway.

5,10-MTHF: 5,10-methylenetetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dTTP: deoxythymidine triphosphate; dUMP: deoxyuridine monophosphate; FPGS: folylpolyglutamate synthetase; GGH: gamma-glutamyl hydrolase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SLC: solute carrier; TS: thymidylate synthase.

Purine synthesis pathway

The *de novo* purine synthesis pathway inhibition by MTXPGs contributes to MTX antiproliferative and anti-inflammation effects (194, 195). Methotrexate polyglutamates inhibits three enzymes of this pathway as follow: 1) phosphoribosyl pyrophosphate amidotransferase (PPAT); 2) glycineamide ribonucleotide formyl transferase (GART); and, 3) 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) (194, 195) (Figure 17). Phosphoribosyl pyrophosphate amidotransferase is responsible for catalyzing the first step of the *de novo* purine synthesis pathway by the simultaneous conversion of 5-phosphoribosyl-1-pyrophosphate (PRPP) and glutamine into 5-phosphoribosyl amine (PRA) and glutamate, respectively (198). The 5-phosphoribosyl amine is converted into glycineamide ribonucleotide (GAR) by incorporation of glycine mediated by GAR synthase (GARS) (199). Glycineamide ribonucleotide formyl transferase, also inhibited by MTXPGs, simultaneously converts GAR into formyl glycineamide ribonucleotide (FGAR) and 10-CHO-THF into THF. The FGAR is then used in several reactions still being converted into aminoimidazole

carboxamide adenosine ribonucleotide (AICAR). The AICAR is converted to formyl-AICAR (FAICAR) by ATIC and, simultaneously, 10-CHO-THF is converted into THF (200) (Figure 17). The ATIC inhibition by MTXPGs seems to be stronger then PPAT and GART inhibition, contributing to intracellular accumulation of AICAR, which consequently will lead to adenosine increased levels (184, 200).

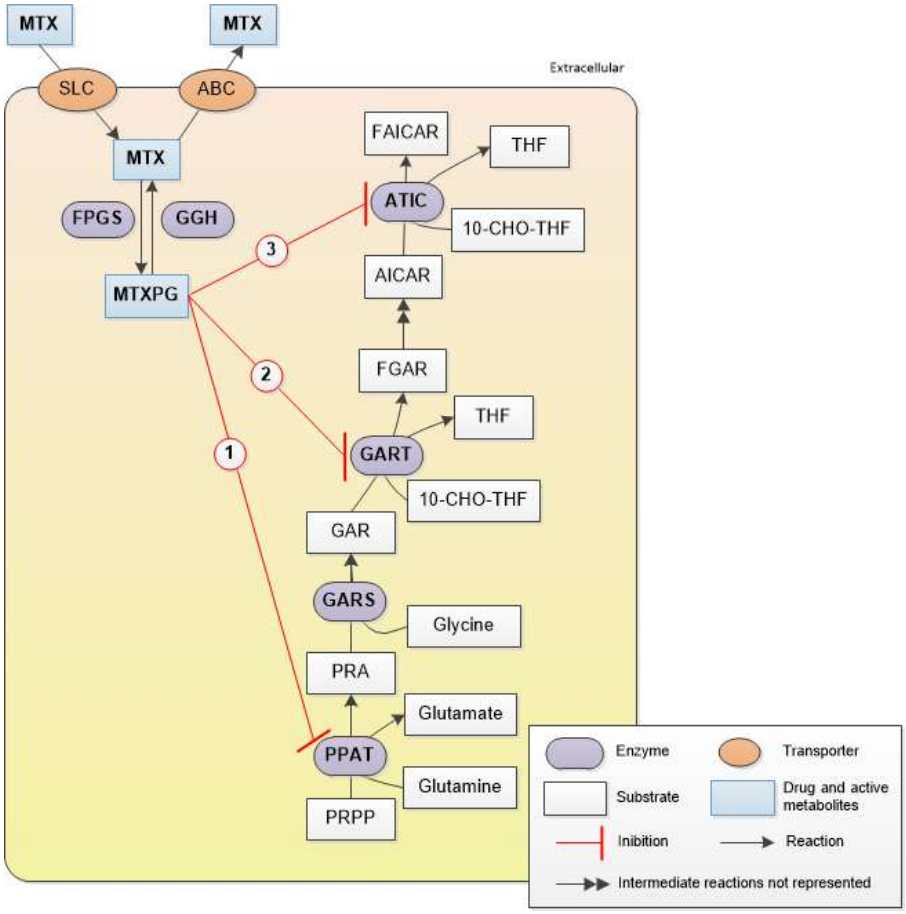


Figure 17. Methotrexate action mechanism - Purine Pathway.

10-CHO-THF: 10-formyltetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; AICAR: aminoimidazole carboxamide adenosine ribonucleotide; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; FAICAR: formyl-aminoimidazole carboxamide adenosine ribonucleotide; FGAR: formyl glycineamide ribonucleotide; FPGS: folylpolyglutamate synthetase; GAR: glycineamide ribonucleotide; GARS: glycineamide ribonucleotide synthase; GART: glycineamide ribonucleotide formyl transferase; GGH: gamma-glutamyl hydrolase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; PPAT: phosphoribosyl pyrophosphate amidotransferase; PRA: 5-phosphoribosyl amine; PRPP: 5-phosphoribosyl-1-pyrophosphate; SLC: solute carrier; THF: tetrahydrofolate.

2.4.1.3. Adenosine pathway

An alternative mechanism for MTX anti-inflammatory effect has gained favor, since has been supported by *in vitro*, *in vivo*, and clinical data studies that theorize that adenosine, a potent anti-inflammatory agent, is released in high concentrations from cells after treatment with MTX (166, 184, 201, 202). Literature hypothesizes that methionine pathway (192) (Figure 15) and AICAR accumulation, as a consequence of ATIC inhibition (200) (Figure 17), are the major responsible for adenosine increased levels. Accumulated AICAR inhibits adenosine monophosphate deaminase 1 (AMPD1) and adenosine deaminase (ADA) (184, 200). Since ADA catalyzes the hydrolysis of adenosine into inosine and AMPD1 catalyzes the deamination of adenosine monophosphate (AMP) to inosine monophosphate (IMP), the inhibition leads to an accumulation of AMP and adenosine (184, 200). Intracellular accumulation of AMP and adenosine results in an increase of these compounds in the extracellular space, where AMP is converted to adenosine, which binds to the specific adenosine receptor subtypes A1, A2a and A2b (ADORA1, ADORA2a and ADORA2b, respectively). Probably, there will be preponderance of ADORA2a receptor pathway compared to the other subtypes (200), yielding an increase of cyclic-AMP (cAMP) in the cell. The increased cAMP leads to immunosuppression by decreasing the secretion of proinflammatory mediators, such TNF-α and interferon-gamma (IFN-γ) (189, 203).

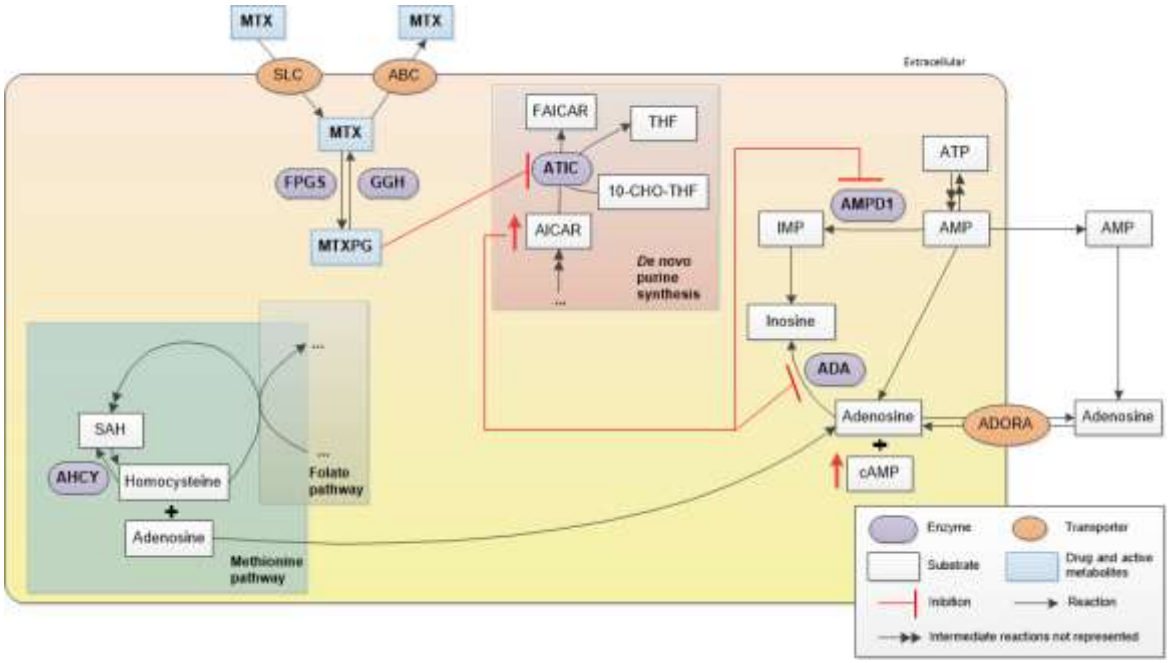


Figure 18. Methotrexate action mechanism - Adenosine Pathway.

10-CHO-THF: 10-formyltetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AHCY: S-adenosylhomocysteinase; AICAR: aminoimidazole carboxamide adenosine ribonucleotide; AMP: adenosine monophosphate; AMPD1: AMP deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; ATP: adenosine triphosphate; cAMP: cyclic-adenosine monophosphate; FAICAR: formyl-aminoimidazole carboxamide adenosine ribonucleotide; FPGS: folylpolyglutamate synthetase; GGH: gamma-glutamyl hydrolase; IMP: inosine monophosphate; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SAH: S-adenosyl homocysteine; SLC: solute carrier; THF: tetrahydrofolate.

With a better knowledge of the MTX action mechanism it's conceivable that it is most likely that some combination of all of these mechanisms is responsible for its potent anti-inflammatory and antiproliferative effects (201) (Figure 19).

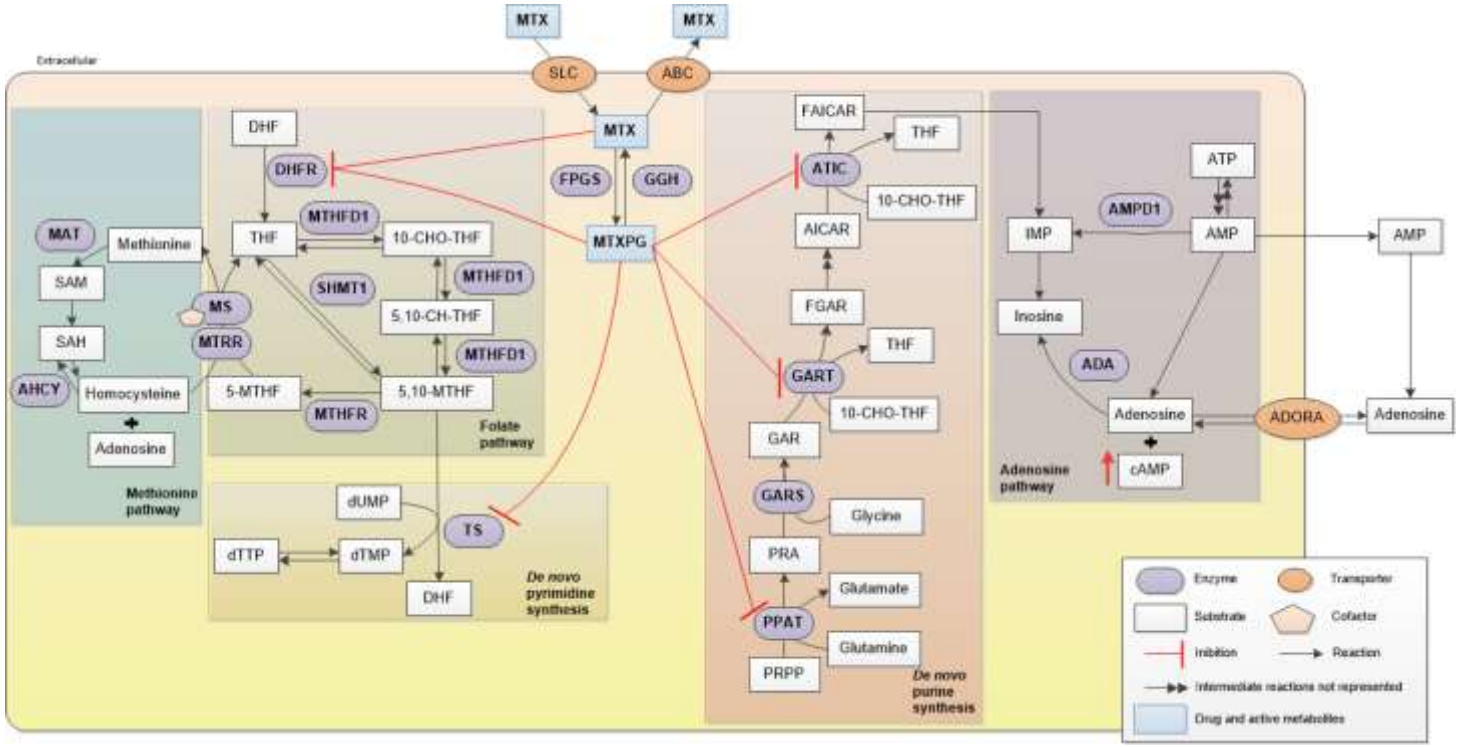


Figure 19. Methotrexate action mechanism.

5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; 5,10-CH-THF: 5,10-methenyltetrahydrofolate; 10-CHO-THF: 10-formyltetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AHCY: S-adenosylhomocysteinase; AICAR: aminoimidazole carboxamide adenosine ribonucleotide; AMP: adenosine monophosphate; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; ATP: adenosine triphosphate; cAMP: cyclic-adenosine monophosphate; DHF: dihydrofolate; DHFR: dihydrofolate reductase; dTMP: deoxythymidine monophosphate; dTTP: deoxythymidine triphosphate; dUMP: deoxyuridine monophosphate; FAICAR: formyl-aminoimidazole carboxamide adenosine ribonucleotide; FGAR: formyl glycineamide ribonucleotide; FPGS: folylpolyglutamate synthetase; GAR: glycineamide ribonucleotide; GARS: glycineamide ribonucleotide synthase; GART: glycineamide ribonucleotide formyl transferase; GGH: gamma-glutamyl hydrolase; IMP: inosine monophosphate; MAT: methionine adenosyltransferase; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTRR: methionine synthase reductase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; PPAT: phosphoribosyl pyrophosphate amidotransferase; PRA: 5-phosphoribosyl amine; PRPP: 5-phosphoribosyl-1-pyrophosphate; SAH: S-adenosyl homocysteine; SAM: S-adenosyl methionine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; THF: tetrahydrofolate; TS: thymidylate synthase.

2.5. Adverse Drug Reactions and Contraindications

Toxicity development related to MTX could be caused by several factors such as MTX dose and administration route (163, 164); erratic GI absorption (170); concomitant administered drugs or diseases that conditioned MTX hepatic metabolism and/or renal clearance (161); and, cellular folate status (204). Since the cell populations that divide more require more folates, and folates are important to prevent toxicity occurrence, those cells are most vulnerable to toxicity (142, 143, 204). Therefore, the first and most affected organic systems are the gastrointestinal and hematopoietic, as also skin and subcutaneous tissues and testicular tissue involved in spermatogenesis (205).

Considering low-dose MTX, the observed ADRs are minor and the most frequent ADRs being gastrointestinal disorders (particularly nausea and anorexia) (206), hepatobiliary disorders (particularly transaminases serum elevation), and blood and lymphatic system disorders (207, 208). Not frequently, respiratory, thoracic and mediastinal disorders, as also infections and infestations can occur (209).

Because of teratogenic effect, MTX in contraindicated in pregnancy and breastfeeding (208). Furthermore, MTX should be used with precaution in the event of liver disorder and/or renal insufficiency (208).

3. PHARMACOGENOMICS AND PREDICTORS OF THERAPEUTIC OUTCOME

This section describes the potential of Pharmacogenomics moving towards to Personalized Medicine in Rheumatoid Arthritis.

Personalized medicine is based on using an individual's genetic profile to make the best therapeutic choice by facilitating predictions about whether that person will benefit from a particular medicine or to be prone to suffer serious side effects (210). The aim of personalized medicine is based on five assumptions (Figure 20).

Figure 20. Personalized medicine assumptions.

Drugs are generally tested on a large population of people and the average response is reported. This sort of evidence-based medicine (that is, medical decision making based on empirical data) relies on the law of averages; personalized medicine, on the other hand, recognizes that there are not two patients that are alike and, thus different therapeutic outcomes are possible (210). These inter- and intrapatient variability will define if the drug is safe and effective, safe and not-effective, unsafe and effective, or unsafe and not effective (211).

Since not all patients benefit from specific therapies, there is a strong and unmet need for pretreatment predictions on therapy outcome (211). This concept of personalizing treatment has raised interest for the discovery of clinically applicable pretreatment biomarkers to allow predictions on outcome before the start of treatment (210).

The study of individual patient therapeutic outcomes to drugs, either in accordance to clinical response and ADRs occurrence using genomic information is carried by Pharmacogenomics (PGx) (211-213). Together, PGx and personalized medicine aim to get drugs simultaneously safe and effective (Figure 21).

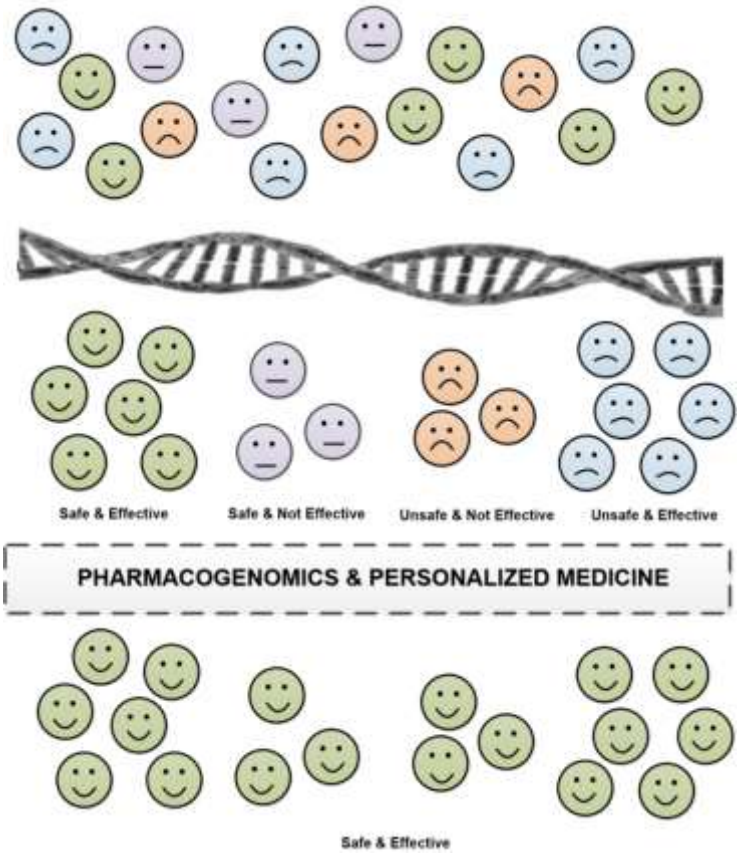


Figure 21. Drug therapeutic outcome profiles.

When a gene variant is associated with a particular therapeutic outcome in a patient, there is the potential for making clinical decisions based on genetics, for example by adjusting the dosage or changing the administration route. Scientists assess gene variants affecting an individual's therapeutic outcome the same way they assess gene variants associated with diseases: by identifying genetic *loci* associated with known drug therapeutic outcomes, and then testing individuals whose therapeutic outcome is unknown (210).

With the completion of the Human Genome Project, anticipation was high that genetic information would radically improve medicine, that ADRs would be more predictable, and that patients could be screened for likely drug clinical responses. But thus far, progress has been much slower than what the initial excitement suggested. A great deal of this delay relates to

the fact that an individual's response to drugs is multifactorial, resulting from multiple gene and environmental interactions. Modern approaches already include multigene analysis or whole-genome single nucleotide polymorphism (SNP) profiles, and these approaches are just coming into clinical use for drug discovery and development (213).

In rheumatology, genes and gene signatures may be associated with therapeutic outcome variability among patients (213). Indeed, RA patients do not form a homogenous population and several clinical subsets of RA, such as erosive *versus* non-erosive, anti-CCP seropositive *versus* seronegative, progressive *versus* mild-course, have been identified. Factors that possibly contribute to this variability include: 1) clinicopathological variables, which can be divided into patient-related (e.g. age, gender, ethnicity and comorbidities), disease-related (e.g. duration, activity, disability and biomarkers) and treatment-related (e.g. compliance, dose and concomitant drugs used); and 2) genetic factors, such genetic polymorphisms implicated in key MTX pathway genes (Figure 22).

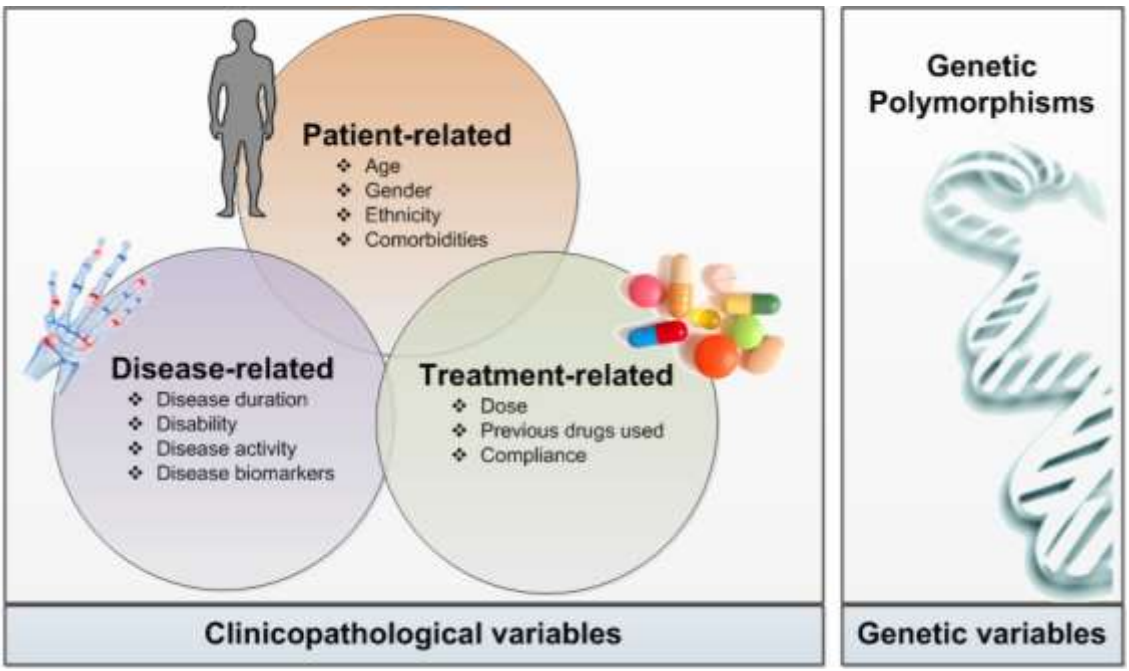


Figure 22. Factors that possibly influence Rheumatoid Arthritis disease course and Methotrexate therapeutic outcome.

Therefore, over the past decade, numerous PGx studies have been undertaken to decipher associations between polymorphisms in genes encoding proteins involved in MTX carrier-mediated transport systems and/or intracellular pathways with MTX therapeutic outcome in RA.

The literature reported PGx studies analyzing the association with MTX therapeutic outcome in RA and grouped by MTX pathways are described below.

3.1. Influx Carrier-Mediated Transport Pathway & Pharmacogenomics

SLC19A1

Reduced folate carrier protein (RFC1) or folate transporter (FOLT) is encoded by *SLC19A1*, located on chromosome 21q22.3 (214). It is a cell surface transmembrane protein and a bidirectional transporter of primarily reduced folates, including MTX, ubiquitously expressed in many tissues, showing particularly high expression in absorptive tissues (intestine, kidney and placenta) either on apical and/or basolateral membranes (215). The rs7499 leads to a change of a guanine (G) into an adenine (A) at position 16 (*SLC19A1* G*16A), and occurs in the 3´-untranslated region (UTR), thought to be important in messenger RNA (mRNA) stability, localization and translational efficiency (216). Despite there are no functional studies on this *SLC19A1* variant, there is one study in RA patients demonstrating an association between A allele and non-response to MTX, but no associations were related to ADRs occurrence (217). The rs1051266 is the most studied variation in *SLC19A1* and is characterized by a substitution of a G by an A at position 80, leading to the change of Arg into histidine (His) at codon 27 in the first transmembrane domain, a region implicated to carrier function (218). Literature described G homozygotes as associated with lower MTX influx activity in lymphocytes (219) and decreased MTXPGs levels in erythrocytes (220). Studies in RA patients, analyzing the impact of this SNP in

MTX therapeutic outcome, demonstrated an association of A homozygotes and A allele with better response to MTX (221-223) and for MTX-related overall toxicity (224, 225). Additionally, other studies demonstrated no associations with clinical response and/or toxicity to low-dose MTX (183, 224, 226-233). The rs1131596 leads a change of T into C in 5´UTR of *SLC19A1*, which is thought to modify gene transcription regulatory mechanisms, since it was found in the putative consensus sequence of the activating protein-1 transcription factor enhancer (228). Functional studies in RA patients demonstrated that C homozygotes were associated with a decreased SLC19A1 expression and influx capability (228) and, literature describes a trend for lower SLC19A1 expression in lymphoblastic cell lines related with C carriers (225). Regarding the influence in MTX therapeutic outcome, Bohanec Grabar *et al.* associated C carriers with a decreased risk for MTX therapy discontinuity, due to overall toxicity and infections in RA (225), but others showed no association with therapeutic outcome (228). The rs2838956 polymorphism, characterized by a substitution of an A for a G, is located in the fifth intron at position 707 (225) and its impact is currently unknown. Thus, functional studies are needed to evaluate the potential of this SNP on ribonucleic acid splicing, which may affect the protein structure and function (234). Nevertheless, there are studies relating this variant with MTX therapeutic outcome in RA: while Owen *et al.* associated G allele with non-response to MTX but not with toxicity (217); Bohanec Grabar *et al.* demonstrated that A allele has a trend to skin and subcutaneous tissue disorders, but no associations were observed regarding clinical response to MTX (225).

SLC46A1

Proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1), is encoded by *SLC46A1*, located on chromosome 17q11.2 and mostly expressed in apical membrane of small-intestine and kidney cells, acting as a key transporter for normal folate absorption and homeostasis (177). The

rs2239907 is characterized by a change of an A into a G at position 928 (A928G) in the 3´UTR region, which is reported as important for mRNA stability and translational efficiency (214, 216). The impact of this variant in SLC46A1 is currently unknown but Kato *et al.* investigated the role of this SNP in MTX therapeutic outcome, and found no significant associations with clinical response (231).

SLCO1B1

Liver-specific organic anion transporter 1 (LST1) or sodium-independent organic anion-transporting polypeptide 2 (OATP-2) is a bidirectional transporter with twelve transmembrane domains encoded by *SLCO1B1* located on chromosome 12p12 (235). It is mainly expressed in basolateral membrane of hepatocytes and plays a key role in the uptake of a wide variety of endogenous and exogenous anionic compounds, including MTX (236). Methotrexate hepatic uptake via SLCO1B1 was demonstrated as the major pathway for MTX clearance from systemic circulation (237). Moreover, SLCO1B1 mRNA has been detected also in other tissues, including small intestinal enterocytes (238). The rs2306283 is a common variant in *SLCO1B1* that results in a change of A into a G at position 88 and, substitution of asparagine into aspartate (Asp) at position 130 (235). *In vitro* studies associated G homozygotes with an increased transport activity for MTX (237) and Kato *et al.* showed no associations with MTX clinical response in RA (231). The rs4149056 is characterized by a change of a T into a C at position 521 leading to an amino acid substitution of valine (Val) into an alanine (Ala) at codon 174 (235). The C allele was associated with a loss-of-function phenotype, resulting in a decreased SLCO1B1 expression, reduced MTX influx and decreased MTX clearance from systemic circulation (235, 239). Nevertheless, Kato *et al.* reported no associations regarding this SNP and MTX clinical response (231).

3.2. Efflux Carrier-Mediated Transport Pathway & Pharmacogenomics

ABCB1

Multidrug resistance protein 1 (MDR1) or P-glycoprotein 1 (P-GP1) is encoded by *ABCB1* located on chromosome 7q21 (240). This energy-dependent unidirectional transporter, expressed in the apical membrane of many epithelial and endothelial cells, as well in lymphocytes, is a transmembrane protein with twelve regions that plays an important role in drug absorption and distribution within the body (230, 240, 241). Substrates include a variety of drugs, including MTX (164). The rs1045642 is one of the most studied SNPs in *ABCB1*, characterized by a substitution of a C to a T at position 3435 (C3435T) leading to a synonymous change of an isoleucine (Ile) at codon 1145 (214). The T homozygotes have been associated with decreased mRNA stability and enzyme expression, and thus to reduced efflux, and with increased intracellular levels of MTX (242). Pharmacogenetic studies have demonstrated that T homozygotes were associated with better response to MTX in RA (231, 243, 244). Nevertheless, Takatori *et al.* associated T homozygotes with non-response in RA patients (226). Regarding MTX-related toxicity in RA, an increased risk was associated to T homozygotes and to T carriers (224, 245), but Plaza-Plaza *et al.* associated T homozygotes with non-toxicity occurrence (233). Controversially, there are several studies that show no associations with either clinical response to MTX and/or MTX-related toxicity occurrence (224, 226, 230, 245, 246). The rs1128503 results in a T to C transition at nucleotide 1236 in exon 13 of *ABCB1* and results in a synonymous change of a glycine (Gly) at residue 412 in a cytoplasmic loop of ABCB1 (247). Literature bears no consensus with concern to the effect of this silent SNP in transporter function and thus, more studies are needed to elucidate this (247). In accordance to the influence of this SNP in MTX therapeutic outcome, Ranganathan *et al.* associated T homozygotes with increased risk for MTX-related toxicity in RA patients (246), while other studies did not present statistically significant

associations regarding the clinical response to MTX (231). The rs2032582 consists in a transversion of a G to a T or A at position 2677 in exon 21 (G2677A/T) corresponding to a replacement of Ala by threonine (Thr) or serine (Ser) in position 899 on the intracellular side of ABCB1, which results in a change from a lipophilic to a hydrophilic residue (248). The A/T alleles have been described as correlated with decreased protein expression, and thus, reduced efflux capability (248). Considering the studies analyzing the impact of this SNP in MTX therapeutic outcome, Kato *et al.* revealed a statistically significant association of A/T alleles with better response to MTX in RA patients (231) but Bohanec Grabar *et al.* did not show associations with MTX therapeutic outcome (224).

ABCC1

Multidrug resistance-associated protein 1 (MRP1) is encoded by *ABCC1* located on chromosome 16p13 (214). It is a polytopic membrane protein present in basolateral plasma membranes in all intestinal regions constituted by seventeen transmembrane regions (240, 249). It was reported as being expressed on cluster of differentiation 3 (CD3)-positive T cells in lymphocytic aggregates and in less extends in RA synovial tissue macrophages in the intimal lining layer and the synovial sublining and on endothelial cells (250). Moreover, it has been described as a transporter of several different drugs including MTX (251). The rs35592 is characterized by a substitution of a T to a C in an intron region of *ABCC1*. Its impact in transporter function is unknown and studies are needed to address this issue. Besides that, Stamp *et al.* presented no associations of this polymorphism with low-dose MTX therapeutic outcome (230). The rs2074087 polymorphism denotes a substitution of a C to a G in an intronic region (*ABCC1* IVS 18-30C>G) (214). Its functional impact is unknown and Ranganathan *et al.* demonstrated no significant associations of this SNP with MTX-related toxicity (246). The rs2230671 consists in a substitution at region 4002 of a G to an A that leads to a synonymous change of a Ser at codon

1334 (Ser1334Ser) (214). Literature describes G homozygotes as having increased mRNA levels in peripheral blood cells and, thus, possibly increased MTX efflux (252). However, no associations were observed in accordance to MTX-related toxicity occurrence (246).

ABCC2

Canalicular multispecific organic anion transporter 1 or multidrug resistance-associated protein 2 (MRP2) is encoded by *ABCC2* located on chromosome 10q24 (240). It is present in apical (brush border) membranes of hepatocytes and epithelial cells of the small intestine and kidney (240, 253). This protein is an anionic conjugate efflux pump, constituted by seventeen transmembrane regions, that plays a role in the excretion of both endogenous and exogenous compounds, and influences the PK of many drugs such as MTX (254). The ABCC2 seems to be the main determinant in the elimination of MTX and of 7-OH-MTX (254). The rs717620 is a SNP characterized by a substitution of a G to an A in the 5´UTR (214). Studies reporting the influence of this SNP in transporter function have associated A homozygotes with increased drugs clearance, including MTX (255). Nevertheless, studies evaluating the impact of this polymorphism in low-dose MTX therapeutic outcome have showed no influence in clinical response to MTX (231) or to MTX-related toxicity (246). The rs2273697 consists in the substitution of a G to an A at position 1249, which corresponds to a nonsynonymous change of a Val to an Ile at codon 417 (214). Literature describes the effect of A homozygotes as conditioning an *in vitro* increased efflux (256). Considering pharmacogenetic studies, Ranganathan *et al.* associated A homozygotes with increased risk for MTX-related gastrointestinal disorders (246). Nevertheless, other studies did not demonstrated associations of this SNP with low-dose MTX therapeutic outcome (230, 231). The rs4148396 is characterized by a change of a T to a C in an intronic region (*ABCC2* IVS 23+56T>C) (214). To the best of my knowledge there are no studies regarding the functional analysis of this

polymorphism in ABCC2. However, studies analyzing its impact in low-dose MTX therapeutic outcome associated C homozygotes with increased risk for MTX-related skin and subcutaneous tissue disorders (246), gastrointestinal disorders and overall toxicity (230). Regarding clinical response, no associations were reported by Stamp *et al.* in RA (230). The rs7080681 SNP is characterized by an alteration of a G to an A at position 1058 (G1058A) leading to a nonsynonymous amino acid substitution from Arg to His substitution at position 353 (214). The impact of this SNP in the function of ABCC2 is currently unknown. Regarding pharmacogenetic studies, Ranganathan *et al.* observed that A homozygotes were associated with an increased risk for MTX-related hepatobiliary disorders (246).

ABCG2

Multidrug resistance efflux transport ATP-binding cassette sub-family G member 2 or breast cancer resistance protein (BCRP) is expressed by *ABCG2* located on chromosome 4q22 (240). This transporter has six transmembrane regions and is present in apical membranes of several tissues, including hepatocytes, intestine and kidney (230, 240, 253) and also in synovial tissue macrophages, at the intimal lining layer and synovial sublining, and on endothelial cells in RA patients (250). Higher ABCG2 expression levels were associated with the persistence of infiltrated synovial tissue macrophages after treatment with MTX and a diminished response to DMARD therapy (250). It seems that, when ABCC2 is absent, ABCG2 plays a significant role in the elimination of MTX and 7-OH-MTX [116]. Moreover, ABCG2 seems to transport not only MTX but also MTXPGs, particularly those with two and three glutamic acid residues (257). The rs2231142 SNP is characterized by a change of a C to an A at position 421 (C421A), which corresponds to a substitution of a glutamine (Gln) to a lysine (Lys) at codon 141 (214). Functional studies have associated A homozygotes with reduced efflux capability (258) and pharmacogenetic studies reported A allele as associated with increased risk for MTX-related overall toxicity, but no associations were

observed regarding clinical response (230, 231). The rs17731538 is characterized by a change of a G to an A in an intronic region (214). There are no studies regarding the functional impact of this SNP in ABCG2 and Stamp *et al.* demonstrated no associations with either MTX clinical response or toxicity occurrence (230).

3.3. Polyglutamation Pathway & Pharmacogenomics

FPGS

Once inside the cells MTX is metabolized to MTXPGs by the sequential addition of glutamic acid residues via FPGS (163, 183). *Folylpolyglutamate synthetase* is located at chromosome 9q34 (214). Several SNPs have been studied, even though the functional significance is unknown, and significant associations with clinical response and/or toxicity to MTX have been observed in RA. For rs1054774, there is a substitution of a T for an A at 5´UTR region (214) and literature describes A allele as associated with an increased risk for MTX-related toxicity, but no associations were observed regarding clinical response to MTX (259). For rs1544105, which is characterized by a substitution of a C to a T at position 2572 in an intronic region (214), A carriers were associated with non-response to MTX (260). Another SNP, rs4451422, consists in an alteration of an A to a C at position 714 in 3´UTR (214), and the C allele for this SNP was related with increased risk for MTX-related toxicity (259). Additionally, no associations with MTX therapeutic outcome were described for rs10106, characterized by a T for a C substitution at 192 position in 3´UTR, rs7033913 C>T and rs10760503 A>G, both in an intronic region (261), and rs16887801, consisting in substitution of an A to a G at position 1994, in a 3´UTR (214, 262).

GGH

Gamma-glutamyl hydrolase enzyme removes the glutamic acid residues of MTXPGs and, consequently, MTX can be transported out of the cells by efflux transporters (163). Single nucleotide polymorphisms in *GGH* promoter region have been associated with changes in GGH expression as well in MTX polyglutamation turnover (rs719235, rs3758149) (263). In fact, for rs719235, which consists in a 5´UTR alteration of a G to a T at position 354 (214), T carriers were associated with non-toxicity to MTX but no associations were described for clinical response to MTX (264) or MTX-related toxicity in other studies (261). For rs3758149, that consists in an alteration of a C for a T at position 401 in 5´UTR (214), T carriers were associated with non-toxicity (183) and with non-response to MTX (222) but, one study described no association with clinical response to MTX (183). The rs11545078 is characterized by a substitution of a C to an T at position 452, leading to a substitution of Thr to an Ile at codon 151 (214). Literature describes T allele as associated with lower GGH activity, resulting in intracellular accumulation of long-chain MTXPGs (220, 262). Nevertheless, no associations were observed regarding the influence in MTX therapeutic outcome (230, 231, 259, 262, 264). Moreover, other SNPs with unknown functional significance have been studied. Davis *et al.* analyzed the impact of rs4617146 C>T, rs7010484 C>T and rs12548933 A>G, all in intronic regions, and, rs11988534 C>T at 3´UTR (214), and described no associations with MTX-related toxicity (261). Owen *et al.* studied the rs12681874, consisting in a substitution of a C to a T in an intronic region (214), and a better clinical response to MTX was associated to T allele, but no associations were reported regarding MTX-related toxicity (259).

3.4. Folate and Methionine Pathways & Pharmacogenomics

DHFR

The DHFR enzyme is a major MTX target and its gene (*DHFR*) is located at 5q11 chromosome (161, 189). The frequently studied polymorphisms in *DHFR* include rs1650697, characterized by a G to an A replacement in 5´-UTR at 473 position, and rs1232027, a substitution of an A to a G near the translation initiation site (214). Nevertheless, they were not associated with MTX clinical response or toxicity (227). Recently, three other SNPs were described as associated with MTX-related toxicity: the intronic' rs12517451 C>T and rs1643657 A>G, and rs10072026 T>C at 3´UTR (214, 259). The rs408626 (A-317G) consists in an A to a G substitution at 5´UTR in position 317 (214), and studies described an association between A homozygotes and non-response to MTX, but no associations were reported in relation to MTX-related toxicity (265). Additionally, rs7387 consists in a substitution of a T into A at 3´UTR (214) and A allele was associated with better response to MTX (260).

MTHFD1

The MTHFD1 is responsible for providing folate cofactors and its gene (*MTHFD1*) is located at chromosome 14q24 (161, 189). The G to A substitution at position 1958 leads an Arg to Gln substitution at codon 653 (rs2236225) and has unknown functional impact (230). Nevertheless, one study associated A allele with better response and with non-toxicity (266), while other associated A allele with an increased risk for MTX-related toxicity (230).

MTHFR

Methylenetetrahydrofolate reductase, an enzyme involved in folate pathway, is responsible for the conversion of 5,10-MTHF to 5-MTHF, a carbon

donor for the remethylation of homocysteine into methionine (191). Its gene (*MTHFR*), located at chromosome 1p36, plays an important role in MTX therapeutic outcome. The SNPs C677T (rs1801133) and A1298C (rs1801131) are described as the most important polymorphisms because of their influence in MTHFR activity and, consequently, in MTX action mechanism (161, 189). Nevertheless, results are still inconsistent. The rs1801133, responsible for a substitution of an Ala to a Val, leads to a thermolabile form of MTHFR with reduced activity (267). In fact, it has been suggested that T homozygotes and T carriers were associated with non-response to MTX (183, 227) and increased risk for MTX-related toxicity (233, 246, 268-271). But, other studies demonstrated no associations with MTX therapeutic outcome (183, 224, 227, 230, 231, 259, 261, 270, 272-276). The rs1801131 consists in a substitution of an A to a C at position 1298, leading to a change of a glutamine to an Ala at codon position 429 (214). It has been showed that C allele reduces MTHFR enzymatic activity (277) and several studies have investigated this SNP role in MTX therapeutic outcome; C carriers were associated with better response (231, 270) and with non-response (227) to MTX. In accordance to MTX-related toxicity, C homozygotes and C carriers were associated with an increased risk for MTX-related toxicity in some studies (227, 261, 275, 276), while other study associated with non-toxicity to MTX (224). Moreover, there are studies demonstrating no associations with MTX therapeutic outcome (224, 233, 259, 270, 271, 274). Another described SNP is the rs2066462. Consisting in a substitution of a C to a T, leading to a synonymous change of a Ser at position 352, the impact in protein expression is actually unknown and studies demonstrated no significant associations with MTX therapeutic outcome (270). Additionally, the rs2274976 consists in a substitution of an A to a G at 3´UTR region (214). Despite the unknown functional significance, G allele was associated with non-toxicity and no association were observed in relation to clinical response to MTX (270).

MS

In the presence of methyl(III)cobalamin, the conversion of 5-MTHF to THF is mediated by MS (also known as methyltransferase - MTR), which occurs simultaneously with the addition of a methyl group to homocysteine, forming methionine (190, 191). Its gene (*MS*) is located at 1q43 chromosome and the rs1805087, characterized by a substitution of an A to a G at 2756 position leading to a change of an Asp to a Gly at 919 codon, is considered to affect enzyme activity (278). Some studies associated G carriers with non-toxicity (183) and G homozygotes with increased risk for toxicity (279) to MTX, while others obtained no significant associations with clinical response and/or MTX-related toxicity occurrence (223, 231, 280).

MTRR

Methionine synthase reductase, which gene is located at 5p15 chromosome, maintains adequate levels of methylcob(III)alamin, the activated cofactor for MS (281). The rs1801394 consists of an alteration of an A to G at position 66 leading to a substitution of Ile into methionine at codon 22, and, G allele has been associated with lower MTRR activity *in vivo* (282). Moreover, some studies reported associations between G homozygotes and an increased risk for MTX-related toxicity (183), while others demonstrated no associations with MTX clinical response and/or MTX-related toxicity occurrence (183, 230, 231, 280).

SHMT1

Serine hydroxymethyltransferase 1 encodes a vitamin B6-dependent enzyme that plays a pivotal role in providing 1-carbon units for purine and thymidylate synthesis (283). Its gene (*SHMT1*) is located at 17p11 chromosome and the rs1979277, consisting in a C for a T substitution at 1420 position that leads to a change of leucine to phenylalanine at 474 codon (214). Literature reported T carriers as associated with increased

plasma and erythrocytes folate levels (284). In addition, T carriers were associated with MTX non-toxicity (269) and non-response (183). Other literature reports described no associations with MTX therapeutic outcome (183, 223, 230).

3.5. *De novo* Pyrimidine Synthesis Pathway & Pharmacogenomics

TYMS

Thymidylate synthase is a key protein for the *de novo* pyrimidine synthesis and is responsible for the simultaneous conversion of dUMP and 5,10-MTHF to dTMP and DHF (196, 285, 286). Subsequently, the dTMP is phosphorylated to dTTP and used for the DNA synthesis and repair (196, 285, 286). Thymidylate synthase is encoded by *TYMS*, located on chromosome 18p11 (214). Polymorphism rs34743033 is a 28 base pairs (bp) variable number tandem repeat (VNTR), located on 5´UTR (287). Is characterized by exhibiting a putative Enhancer box (E-box) sequence on the first 28bp repeat of 2R allele and on the two first repeats of 3R allele (288, 289). Therefore, a higher number of repeats should increase the amount of E-box binding sites for the upstream stimulating factors (USF), leading to an increased transcription of *TYMS* and, consequently, to higher TS levels (290). Studies demonstrated that 2R homozygotes were associated with better response (221, 291) as well as with non-response (183) to MTX. Moreover, Weisman *et al.* described 2R homozygotes with increased risk for MTX-related toxicity (269) while Bohanec Grabar *et al.* associated 2R carriers with non-toxicity to MTX (224). Other studies demonstrated no association with either clinical response to MTX and/or MTX-related toxicity occurrence (183, 223, 224, 259, 264, 291, 292). A SNP characterized by a C to G transition on the twelfth nucleotide of the second repeat of VNTR 3R allele (rs2853542) has been also described (289) and, in the presence of cytosine (3RC), the E-box seems to be disrupted, reducing the stimulation of transcription (in comparison to 3RG) and, thereby, decreasing TS levels (289). Since this SNP

occurs within the *TYMS* 28bp VNTR polymorphism, several studies have been performed combining the information from both *TYMS* enhancer region (TSER) polymorphisms (264, 286). Only one study in RA analysed the TSER polymorphisms, associating the 3RG homozygotes with non-response to MTX but, no association were observed in relation to MTX-related toxicity (264). Another important polymorphism in *TYMS* is a deletion-insertion polymorphism (DIP) of a 6bp sequence (TTAAAG) at 3´UTR (1494del6, rs34489327), which seems to affect a region of TS pre-mRNA that contains *cis* adenylate-uridylate-rich elements (AREs) (293, 294). These elements bind to a *trans* AU-rich factor 1 (AUF1), also named heterogeneous nuclear ribonucleoprotein D (hnRNPD), preferentially in the presence of deletion allele (6bp-), diminishing mRNA stability and, consequently, decreasing TS levels (293-295). Literature has reported deviating results. Some studies associated 6bp- allele with better response to MTX (291, 292), while others reported no associations with clinical response and/or MTX-related toxicity (223, 226, 246, 259, 291). Another *TYMS* polymorphism, the rs2853539, is a change of an A to a G in an intronic region (214). To the best of my knowledge there are no functional studies about this SNP but, Sharma *et al.* demonstrated that A homozygotes were associated with non-response to MTX (260).

3.6. *De novo* Purine Synthesis Pathway & Pharmacogenomics

ATIC

The 5-aminoimidazole-4-carboxamide ribonucleotide transformylase is an enzyme involved in the *de novo purine* synthesis pathway responsible for the conversion of AICAR into FAICAR (221). It is encoded by *ATIC,* which is located on chromosome 2q35 (214). The rs2372536, the most studied polymorphism in *ATIC*, is characterized by a substitution of a C to a G at position 347 leading, at protein level, to a change of a Thr into a Ser at codon 116 (214). Some studies have been associating the G homozygotes with

better response to MTX (183, 221), while other demonstrated G carriers as associated with non-response (280). Moreover, G homozygotes and G carriers were associated with MTX-related toxicity occurrence (183, 266, 269, 280), but, other studies revealed no associations with either clinical response to MTX and/or MTX-related toxicity (223, 226, 230, 231, 259, 260, 266, 296, 297). Some authors have studied the role of rs4673993, a SNP characterized by a T for a C alteration at position 675 in an intronic region, of which C allele has been associated with better response to MTX (273). In addition, C allele for rs12995526, in which occurs an intronic substitution of a T for a C at position 26293, and T allele for rs16853834, an intronic alteration of a C for a T, were described as associated with non-response to MTX, while T allele for rs3821353, characterized by a G to a T alteration in an intronic region, was associated with better response to MTX (259).

3.7. Adenosine Pathway & Pharmacogenomics

ADA

Adenosine is catabolized by ADA and blocking ADA induces the accumulation of adenosine (184, 200). Its gene is located at 20q13 chromosome and several genetic changes have been identified (214), some of them resulting in the substitution of one protein building block (amino acid) for another amino acid in the ADA enzyme; and others leads the enzyme to be unstable, or, prevent it from being produced at all (260). One of the most studied SNP is rs244076, a change of an A for a G at position 534, leading to a synonymous change of a valine at codon 178 (214). For this SNP, G carriers were described as associated with better response to MTX (260). The rs1799880, characterized by a C for a G substitution at intronic region, was also studied concerning the clinical response to MTX and no associations were observed (260).

ADORA2a

Adenosine receptor subtype A2a is member of the adenosine receptor group of G-protein-coupled receptors, responsible for the adenosine transport (184, 200). The *ADORA2a* is localized on chromosome 22q11.23 (214) and Hider *et al.* described several intronic SNPs with possible functional impact (rs2236624 C>T, rs2267076 T>C, rs2298383 C>T, rs3761422 C>T, and rs5760410 G>A) and, all were associated with increased risk for MTX-related toxicity (298). Sharma *et al.* studied rs5751876, that consists in a substitution of a T for a C at 1976 leading to synonymous alteration of a tyrosine at 361 codon, and associated C allele with better response to MTX (260).

AMPD1

Adenosine monophosphate deaminase 1 catalyzes the conversion of AMP to IMP (280). The *AMPD1* is located at 1p13 chromosome and rs17602729, characterized by a C for a T alteration at position 34, generates an AMPD1 enzyme with lower activity due to the non-synonymous change of a cysteine for an Arg at codon 12 (299). Thus, deficiency of AMPD1 could enhance adenosine release (280). Pharmacogenetic studies demonstrated that T carriers were more likely to have a better response to MTX (266, 280) but also presented an increased risk for MTX-related toxicity (230). Other studies demonstrated no associations with MTX clinical response and/or toxicity (230, 259, 260, 266, 280, 297).

Pharmacogenetic studies relating to MTX therapeutic outcome in RA patients are summarized in Table 5.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Influx carrier-mediated transport pathway										
SLC19A1	rs7499	G>16A	3´UTR	Owen *et al.* (2013)	R	309	UK	A allele: ↓ response	NS	(232)
FOLT	rs1051266	G80A	Exon (Arg27His)	Dervieux *et al.* (2004)	R	108	US	AA: ↑ response	NA	(221)
				Dervieux *et al.* (2006)	P	48	US	NS	NS	(183)
				Takatori *et al.* (2006)	R	124	Japan	NS	NS	(226)
				Wessels *et al.* (2006a)	R	205	Netherlands	NS	NS	(227)
				Chatzikyriakidou *et al.* (2007)	R	106	Greece	NS	NS	(228)
				Drozdzik *et al.* (2007)	P	174	Poland	NS	NS	(229)
				Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	A allele: ↓ toxicity	(224)
				James *et al.* (2008)	P	98	Australia	A allele: ↑ response	NA	(223)
				Hayashi *et al.* (2009)	P	87	Japan	AA: ↑ response	NA	(222)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
				Bohanec Grabar *et al.* (2012)	R	212	Slovenia	NA	A carrier: ↓ toxicity	(225)
				Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
				Owen *et al.* (2013)	R	309	UK	NS	NS	(232)
				Plaza-Plaza *et al.* (2012)	P	67	Spain	NA	NS	(233)
	rs1131596	T>43C	5´UTR	Chatzikyriakidou *et al.* (2007)	R	106	Greece	NS	NS	(228)
				Bohanec Grabar *et al.* (2012)	R	212	Slovenia	NA	C carrier: ↓ toxicity	(225)
	rs2838956	A>G	Intron	Bohanec Grabar *et al.* (2012)	R	212	Slovenia	NA	NS	(225)
				Owen *et al.* (2013)	R	309	UK	G allele: ↓ response	NS	(232)
SLC46A1	PCFT	rs2239907	A>928G	3´UTR	Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
SLCO1B1	LST1	rs2306283	A388C	Exon (Asn130Asp)	Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
		rs4149056	T>174C	Exon (Val174Ala)	Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein		rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Efflux carrier-mediated transport pathway											
ABCB1	MDR	rs1045642	C3435T	Exon (Ile1145Ile)	Pawlik *et al.* (2004)	R	92	Poland	TT: ↑ response	NA	(243)
	P-GP				Drozdzik *et al.* (2006)	P	174	Poland	TT: ↑ response	NA	(244)
					Takatori *et al.* (2006)	R	124	Japan	TT: ↓ response	NS	(226)
					Ranganathan *et al.* (2008)	R	222	US	NA	NS	(246)
					Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	TT: ↑ toxicity	(224)
					Kooloos *et al.* (2010)	R	205	Netherlands	NS	T allele: ↑ toxicity	(245)
					Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
					Kato *et al.* (2012)	R	55	Japan	TT: ↑ response	NA	(231)
					Plaza-Plaza *et al.* (2012)	P	67	Spain	NA	T allele: ↓ toxicity	(233)
		rs11285O3	C1236T	Exon (Gly412Gly)	Ranganathan *et al.* (2008)	R	222	US	NA	TT: ↑ toxicity	(246)
					Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
		rs2032582	G2677A/T	Exon (Ala899Ser/Thr)	Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	NS	(224)
					Kato *et al.* (2012)	R	55	Japan	AA/AT/TT: ↑ response	NA	(231)
ABCC1	MRP1	rs35592	T>C	Intron	Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
		rs20744087	G>C	Intron	Ranganathan *et al.* (2008)	R	222	US	NA	NS	(246)
		rs2230671	C4002G	Exon (Ser1334Ser)	Ranganathan *et al.* (2008)	R	222	US	NA	NS	(246)
ABCC2	MRP2	rs717620	G>A	5´UTR	Ranganathan *et al.* (2008)	R	222	US	NA	NS	(246)
					Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
		rs2273697	G1249A	Exon (Val417Ile)	Ranganathan *et al.* (2008)	R	222	US	NA	AA: ↑ toxicity	(246)
					Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
					Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid – transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein		rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Efflux carrier-mediated transport pathway											
ABCC2	MRP2	rs4148396	T>C	Intron	Ranganathan *et al.* (2008)	R	222	US	NA	CC: ↑ toxicity	(246)
					Stamp *et al.* (2010)	P	200	New Zealand	NS	CC: ↑ toxicity	(230)
		rs7080681	G1058A	Exon (Arg353His)	Ranganathan *et al.* (2008)	R	222	US	NA	AA: ↑ toxicity	(246)
ABCG2	BCRP	rs2231142	C421A	Exon (Gln141Lys)	Stamp *et al.* (2010)	P	191	New Zealand	NS	A allele: ↑ toxicity	(230)
					Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
		rs17731538	G>A	Intron	Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
Polyglutamation pathway											
FPGS	FPGS	rs10106	T>192C	3´UTR	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
		rs1054774	T>A	5´UTR	Owen *et al.* (2012)	R	309	UK	NS	A allele: ↑ toxicity	(259)
		rs1544105	C2572T	Intron	Sharma *et al.* (2009)	R	281	India	A carrier: ↓ response	NA	(260)
		rs4451422	A>714C	3´UTR	Owen *et al.* (2012)	R	309	UK	NS	C allele: ↑ toxicity	(259)
		rs7033913	C>T	Intron	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
		rs10760503	A>G	Intron	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
		rs16887801	A>1994G	3´UTR	van der Straaten *et al.* (2007)	R	352	Netherlands	NS	NS	(262)
GGH	GGH	rs719235	G-354T	5´UTR	Jekic *et al.* (2013)	R	184	Serbia	NS	T carrier: ↓ toxicity	(264)
					Davis *et al.* (2014)	R	319	US	NA	NS	(261)
		rs35758149	C-401T	5´UTR	Dervieux *et al.* (2006)	P	48	US	NS	T carrier: ↓ toxicity	(183)
					Hayashi *et al.* (2009)	R	87	Japan	T allele: ↓ response	NA	(222)
		rs4617146	C>T	Intron	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
		rs7010484	C>T	Intron	Davis *et al.* (2014)	R	319	US	NA	NS	(261)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; AIRC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST-1: liver specific transporter 1; MCT2: monocarboxylic acid transporter 2; MRP: multidrug resistance associated protein; MS: methionine synthase; MTR: methyltransferase; MTHFR: methylenetetrahydrofolate dehydrogenase 1; MTHFD1: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Polyglutamation pathway										
GGH GGH	rs11545078	C452T	Exon (Thr151Ile)	van der Straaten *et al.* (2007)	R	352	Netherlands	NS	NS	(262)
	rs11545078	C452T	Exon (Thr151Ile)	Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
				Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)
				Jekic *et al.* (2013)	R	184	Serbia	NS	NS	(264)
	rs11988534	C>T	3´UTR	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
	rs12548933	A>G	Intron	Davis *et al.* (2014)	R	319	US	NA	NS	(261)
	rs12681874	C>T	Intron	Owen *et al.* (2012)	R	309	UK	T allele: ↑ response	NS	(259)
Folate and methionine pathways										
DHFR DHFR	rs7387	T>A	3´UTR	Sharma *et al.* (2009)	R	281	India	A allele: ↑ response	NA	(260)
	rs408626	A-317G	5´UTR	Milic *et al.* (2012)	R	125	Serbia	G carrier: ↑ response	NS	(265)
	rs1232027	G35289A	Intron	Wessels *et al.* (2006a)	R	205	Netherlands	NS	NS	(227)
	rs1643657	A>G	Intron	Owen *et al.* (2012)	R	309	UK	NS	G allele: ↓ toxicity	(259)
	rs1650697	G-473A	5´UTR	Wessels *et al.* (2006a)	R	205	Netherlands	NS	NS	(227)
	rs10072026	T>C	3´UTR	Owen *et al.* (2012)	R	309	UK	NS	C allele: ↓ toxicity	(259)
	rs12517451	C>T	Intron	Owen *et al.* (2012)	R	309	UK	NS	T allele: ↑ toxicity	(259)
MTHFD1 MTHFD1	rs2236225	G1958A	Exon (Arg653Gln)	Bohanec Grabar *et al.* (2010)	R	211	Slovenia	A allele: ↑ response	A allele: ↓ toxicity	(266)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	A allele: ↑ toxicity	(230)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver specific transporter 1; MCT2: monocarboxylic acid transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Folate and methionine pathways										
MTHFR	rs1801131	A1298C	Exon (Glu429Ala)	Wessels *et al.* (2006a)	R	205	Netherlands	C carrier: ↓ response	C carrier: ↑ toxicity	(227)
				Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	CC: ↓ toxicity	(224)
				Taraborelli *et al.* (2009)	R	84	Italia	NS	NS	(274)
				Xiao *et al.* (2010)	R	110	China	C carrier: ↑ response	NS	(270)
				Mena *et al.* (2011)	R	70	Mexico	NA	C allele: ↑ toxicity	(275)
				Cáliz *et al.* (2012)	R	468	Spain	NA	NS	(271)
				Choe *et al.* (2012)	R	167	Korea	NA	CC: ↑ toxicity	(276)
				Kato *et al.* (2012)	R	55	Japan	C carrier: ↑ response	NA	(231)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)
				Plaza-Plaza *et al.* (2012)	P	67	Spain	NA	NS	(233)
				Davis *et al.* (2014)	R	319	US	NA	C carrier: ↑ toxicity	(261)
	rs1801133	C677T	Exon (Ala222Val)	Aggarwal *et al.* (2006)	R	150	India	NS	NS	(272)
				Dervieux *et al.* (2006)	P	48	US	TT: ↓ response	NS	(183)
				Kim *et al.* (2006)	R	385	Korea	NA	T carrier: ↑ toxicity	(268)
				Weisman *et al.* (2006)	R	214	US	NA	TT: ↑ toxicity	(269)
				Wessels *et al.* (2006a)	R	205	Netherlands	T carrier: ↓ response	NS	(227)
				Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	NS	(224)
				Ranganathan *et al.* (2008)	R	222	US	NA	T allele: ↑ toxicity	(246)
				Lee *et al.* (2009)	R	262	US	NS	NA	(273)
				Taraborelli *et al.* (2009)	R	84	Italia	NS	NS	(274)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
				Xiao *et al.* (2010)	R	110	China	NS	T carrier: ↑ toxicity	(270)
				Mena *et al.* (2011)	R	70	Mexico	NA	NS	(275)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid – transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Folate and methionine pathways										
MTHFR MTHFR	rs1801133	C677T	Exon (Ala222Val)	Cáliz *et al.* (2012)	R	468	Spain	NA	T allele: ↑ toxicity	(271)
				Choe *et al.* (2012)	R	167	Korea	NA	NS	(276)
				Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)
				Plaza-Plaza *et al.* (2012)	P	67	Spain	NA	T allele: ↑ toxicity	(233)
				Davis *et al.* (2014)	R	319	US	NA	NS	(261)
	rs2066462	C>T	Exon (Ser352Ser)	Xiao *et al.* (2010)	R	110	China	NS	NS	(270)
	rs2274976	A>G	3´UTR	Xiao *et al.* (2010)	R	110	China	NS	G allele: ↓ toxicity	(270)
MTR MS	rs1805087	A2756G	Exon (Asp919Gly)	Dervieux *et al.* (2006)	P	48	US	NS	G carrier: ↓ toxicity	(183)
				Wessels *et al.* (2006b)	R	205	Netherlands	NS	NS	(280)
				Yackov *et al.* (2007)	R	86	Israel	NA	GG: ↑ toxicity	(279)
				James *et al.* (2008)	P	98	Australia	NS	NA	(223)
				Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
MTRR MTRR	rs1801394	A66G	Exon (Ile22Met)	Dervieux *et al.* (2006)	P	48	US	NS	GG: ↑ toxicity	(183)
				Wessels *et al.* (2006b)	R	205	Netherlands	NS	NS	(280)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
				Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
SHMT1 SHMT1	rs1979277	C1420T	Exon (Leu474Phe)	Dervieux *et al.* (2006)	P	48	US	T carrier: ↓ response	NS	(183)
				Weisman *et al.* (2006)	R	214	US	NA	T carrier: ↓ toxicity	(269)
				James *et al.* (2008)	P	98	Australia	NS	NA	(223)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	NS	(230)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; AITC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid-transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
De novo pyrimidine synthesis pathway										
TYMS TS	rs34743033	28bp	(CCGCGCCACTTCG	Kumagai *et al.* (2003)	R	167	Japan	2R2R: ↑ response	NS	(291)
		VNTR	CCTGCCTCCGTCC	Dervieux *et al.* (2004)	R	108	US	2R2R: ↑ response	NA	(221)
			CG)/2/3/4/5/9	Dervieux *et al.* (2006)	P	48	US	2R2R: ↓ response	NS	(183)
				Weisman *et al.* (2006)	R	214	US	NA	2R2R: ↑ toxicity	(269)
				Bohanec Grabar *et al.* (2008)	R	213	Slovenia	NS	2R carrier: ↓ toxicity	(224)
				James *et al.* (2008)	P	98	Australia	NS	NA	(223)
				Inoue *et al.* (2009)	P	36	Japan	NS	NA	(292)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)
				Jekic *et al.* (2013)	R	184	Serbia	NS	NS	(264)
	rs34743033 + rs2853542	TSER	VNTR + SNP C>G	Jekic *et al.* (2013)	R	184	Serbia	3RG3RG: ↓ response	NS	(264)
	rs2853539	A>G	Intron	Sharma *et al.* (2009)	R	281	India	AA: ↓ response	NA	(260)
	rs33489327	1494del6	-/TTAAAG	Kumagai *et al.* (2003)	R	167	Japan	6bp-allele:↑response	NS	(291)
				Takatori *et al.* (2006)	R	124	Japan	NS	NS	(226)
				James *et al.* (2008)	P	98	Australia	NS	NA	(223)
				Ranganathan *et al.* (2008)	R	222	US	NA	NS	(246)
				Inoue *et al.* (2009)	P	36	Japan	6bp-allele:↑response	NA	(292)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; AIIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr.: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver specific transporter 1; MCT2: monocarboxylic acid transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein		rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
De novo purine synthesis pathway											
ATIC	ATIC	rs2372536	C347G	Exon (Thr116Ser)	Dervieux *et al.* (2004)	R	108	US	GG: ↑ response	NA	(221)
					Dervieux *et al.* (2006)	P	48	US	GG: ↑ response	GG: ↑ toxicity	(183)
					Takatori *et al.* (2006)	R	124	Japan	NS	NS	(226)
					Weisman *et al.* (2006)	R	214	US	NA	GG: ↑ toxicity	(269)
					Wessels *et al.* (2006b)	R	205	Netherlands	G carrier: ↓ response	G carrier: ↑ toxicity	(280)
					James *et al.* (2008)	P	98	Australia	NS	NA	(223)
					Dervieux *et al.* (2009)	R	255	US	NS	NS	(296)
					Sharma *et al.* (2009)	R	281	India	NS	NA	(260)
					Bohanec Grabar *et al.* (2010)	R	211	Slovenia	NS	C allele: ↑ toxicity	(266)
					Stamp *et al.* (2010)*	P	200	New Zealand	NS	NS	(230)
					Fransen *et al.* (2012)	P	75	Netherlands	NS	NA	(297)
					Kato *et al.* (2012)	R	55	Japan	NS	NA	(231)
					Owen *et al.* (2012)	R	309	UK	NS	NS	(259)
		rs3821353	G>T	Intron	Owen *et al.* (2012)	R	309	UK	T allele: ↑ response	NS	(259)
		rs4673993	T675C	Intron	Lee *et al.* (2009)	R	262	US	C allele: ↑ response	NA	(273)
		rs12995526	T26293C	Intron	Owen *et al.* (2012)	R	309	UK	C allele: ↓ response	NS	(259)
		rs16853834	C>T	Intron	Owen *et al.* (2012)	R	309	UK	T allele: ↓ response	NS	(259)
Adenosine pathway											
ADA	ADA	rs244076	A534G	Exon (Val178Val)	Sharma *et al.* (2009)	R	281	India	G carrier: ↑ response	NA	(260)
		rs1799880	C>G	Intron	Sharma *et al.* (2009)	R	281	India	NS	NA	(260)
ADORA2a	ADORA2A	rs2236624	C>T	Intron	Hider *et al.* (2008)	R	309	UK	NS	T carrier: ? toxicity	(298)
		rs2267076	T>C	Intron	Hider *et al.* (2008)	R	309	UK	NS	C allele: ↑ toxicity	(298)
		rs2298383	C>T	Intron	Hider *et al.* (2008)	R	309	UK	NS	T carrier: ? toxicity	(298)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LSTI: liver-specific transporter 1; MCT2: monocarboxylic acid – transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MS: methionine synthase; MTR: methyltransferase; MTRR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

Table 5. Pharmacogenetic studies for therapeutic outcome to methotrexate in rheumatoid arthritis (cont.)

Gene/Protein	rs ID	Alleles	Region (aa change)	Study (year)		Cases	Population	Clinical Response	Toxicity	Ref.
Adenosine pathway										
ADORA2A	rs3761422	C>T	Intron	Hider *et al.* (2008)	R	309	UK	NS	T carrier: ↑ toxicity	(298)
	rs5751876	T1976C	Exon (Tyr361Tyr)	Sharma *et al.* (2009)	R	281	India	C allele: ↑ response	NA	(260)
	rs5760410	G>A	Intron	Hider *et al.* (2008)	R	309	UK	NS	G carrier: ↑ toxicity	(298)
AMPD1	rs17602729	C34T	Exon (Cys12Arg)	Wessels *et al.* (2006b)	R	205	Netherlands	T allele: ↑ response	NS	(280)
				Sharma *et al.* (2009)	R	281	India	NS	NA	(260)
				Bohanec Grabar *et al.* (2010)	R	211	Slovenia	T allele: ↑ response	NS	(266)
				Stamp *et al.* (2010)	P	200	New Zealand	NS	T allele: ↑ toxicity	(230)
				Franssen *et al.* (2012)	P	75	Netherlands	NS	NA	(297)
				Owen *et al.* (2012)	R	309	UK	NS	NS	(259)

↑: increased; ↓: decreased; A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP binding cassette; ADA: adenosine deaminase; ADORA: adenosine receptor; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; Chr: chromosome; del: deletion; DHFR: dihydrofolate reductase; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance associated protein; MS: methionine synthase; MTR: methionine synthase reductase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; NA: not analyzed; NS: not statistically significant; OAT: organic anion transporter; P: prospective; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; R: retrospective; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SHMT1: serine hydroxymethyltransferase 1; SLC: solute carrier; T: thymine; Thr: threonine; TS: thymidylate synthase; TYMS: thymidylate synthase (gene); UTR: untranslated region; Val: valine; VNTR: variable number tandem repeat.

For RA, very inconsistent results were obtained in conducted studies, raising skepticism about the clinical utility of the reported genetic polymorphisms, possibly due to the complex interplay between pathogenesis and MTX pharmacology in RA.

As not all RA patients are the same, there is an increasing need to establish personalized medicine in rheumatology, as well as in other fields in medicine. This will only happen when clinical and molecular properties of each patient are well characterized. Therefore, and besides there have been major advances in personalized medicine field, we are still very far from finding the optimal genetic biomarkers.

CHAPTER II

OBJECTIVES

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER II – OBJECTIVES

This chapter presents the main and specific objectives of this work.

Considering the ultimate key challenges of Pharmacogenomics in the field of Personalized Medicine, the general objective of this work was to evaluate the influence of clinicopathological and genetic variables, as potential predictors of methotrexate therapeutic outcome, both regarding clinical response and adverse drug reactions occurrence, in rheumatoid arthritis Portuguese patients.

Among the specific objectives, this work aimed to:

1) **Select the study population with rheumatoid arthritis according to the defined inclusion and exclusion criteria;**
2) **Reclassify rheumatoid arthritis patients according the 2010 criteria of the American College of Rheumatology and the European League Against Rheumatism;**
3) **Construct a database with the collected clinicopathological data from the selected population;**
4) **Define the endpoints to be measured - methotrexate therapeutic outcome, expressed as clinical response to methotrexate and as methotrexate-related toxicity, according to well defined standards and guidelines;**
5) **Analyze the influence of the clinicopathological variables in methotrexate therapeutic outcome prediction;**
6) **Select potential genetic polymorphisms for methotrexate therapeutic outcome prediction in rheumatoid arthritis;**
7) **Investigate the role of *solute carriers* and *adenosine triphosphate-binding cassette transporters* polymorphisms on the occurrence of methotrexate-related toxicity;**

8) Study the association between *thymidylate synthase* polymorphisms and methotrexate therapeutic outcome, both regarding clinical response and toxicity occurrence;

9) Elucidate the influence of *methylenetetrahydrofolate reductase* and *5-aminoimidazole-4-carboxamide ribonucleotide transformylase* polymorphisms in the clinical response to methotrexate;

10) Analyze and define a profile of clinicopathological variables and genetic polymorphisms that could be used to predict MTX response and/or toxicity occurrence and its concluding remarks, and reflect on future perspectives.

CHAPTER III

MATERIALS AND METHODS

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER III – MATERIALS AND METHODS

This chapter includes the information about the search strategy, study design (selection of participants, data and sample collection), genotyping procedures and statistical analysis.

1. SEARCH STRATEGY & SELECTION CRITERIA

For reviewing purposes it was developed a sensitive search strategy in the PubMed database using various combinations of search terms including 'ABCs', 'acute lymphoblastic leukemia', 'adverse drug reactions', 'association', 'clinical response', 'effectiveness', 'efficacy', 'expression', 'function', 'genetic', 'juvenile idiopathic arthritis', 'low-dose', 'methotrexate', 'molecular epidemiology', 'pharmacogenetics', 'pharmacogenomics', 'personalized medicine', 'polymorphism', 'psoriasis', 'rheumatoid arthritis', 'single nucleotide polymorphisms', 'SLCs', 'SNPs', 'survival', 'susceptibility', 'therapeutic outcome', 'thymidylate synthase', 'toxicity', 'transporters', 'TS' and 'TYMS'. Moreover, the search strategy also included rs identification number, SNP or gene/protein denomination of proteins involved in MTX transport-mediated system or action mechanism. All English-language and Portuguese-language articles published were identified. Bibliographies of retrieved articles were screened for cross-references to identify additional publications not indexed in the search databases.

Were selected, for inclusion in MTX transport-mediated system review, articles that met the following criteria:

- **aimed for disease models where MTX is used in low-dose (rheumatoid arthritis, psoriasis and juvenile idiopathic arthritis);**
- **included genetic polymorphisms in genes encoding for proteins involved in MTX transport-mediated system;**
- **analyzed genetic polymorphisms effects on protein function or expression levels;**

- studied genetic polymorphisms association with MTX therapeutic outcome, regarding clinical response and/or toxicity occurrence; and,
- explored pharmacogenomics and personalized medicine.

Manuscripts were excluded if their full text was not available or explored disease models where MTX is used in high-dose (such for example, acute lymphoblastic leukemia).

Likewise, were selected, for inclusion in *TYMS* review, articles that follow the criteria below:

- aimed for disease models where MTX is used independently of dose;
- included genetic polymorphisms in *TYMS*;
- analyzed genetic polymorphisms effects on TS function or expression levels;
- studied genetic polymorphisms association with MTX therapeutic outcome, regarding clinical response, toxicity occurrence and/or survival;
- investigated genetic polymorphisms association with risk for developing diseases; and,
- explored molecular epidemiology, pharmacogenomics and personalized medicine.

Manuscripts were excluded if their full text was not available.

2. CHARACTERIZATION OF THE STUDIED POPULATION

A retrospective study was performed between January 2009 and December 2012 at the São João Hospital Center (Porto, Portugal) in a cohort of consecutive Caucasian patients (≥ 18 years) with RA treated with MTX. Patients were excluded from the study if had drug abuse history, recent pregnancy or desire to become pregnant during the study. The study was submitted to the local Ethical Committee (reference 33/2009), procedures were considered to be according to the standards of the Helsinki Declaration and all patients provided an informed written consent (300).

Patients were diagnosed with RA, classified according the 1987 criteria of the ACR and reclassified according the 2010 criteria of the ACR and EULAR (89). After RA diagnosis, all patients were treated with 10mg PO/w of MTX in monotherapy. This dose was increased 5mg at each three weeks if the patient did not meet the EULAR criteria for response; in other words, whenever the patient presented a DAS28>3.2. At three months, if patient was still without response, the administration route was changed from PO to subcutaneous (SC) maintaining the MTX dose. Moreover, if patients presented GI toxicity with PO route, independently of MTX dose, the administration route was also changed to SC keeping the same dose. If in three months using SC, MTX patients presented GI toxicity or if, at the maximum tolerable doses, the patient did not meet the response criteria, MTX therapy was discontinued or was associated with LEF 20mg/d. Exceptionally, in the case of RA patients without poor prognosis factors that did not responded to MTX in monotherapy, MTX was combined with other csDMARDS such SSZ, HCQ and/or CCP before making the switch to LEF. After more three months, if patients continued without response in two successive evaluations and did not present any contraindication, therapeutic was changed by associating a bDMARD. The occurrence of MTX-related toxicity was registered at each visit and, according to the severity, MTX dose was decreased or MTX was discontinued. Due to the well-known protective effect of folic acid supplementation for the prevention of toxicity occurrence, in particular for GI disorders (142, 143), this supplement was prescribed to all patients and their regular compliance was registered. Other concomitant drugs, such as corticosteroids and NSAIDs were allowed during the study.

3. DATA COLLECTION & VARIABLE DEFINITION

Patient demographics, clinicopathological and treatment characteristics were collected from clinical records by the patients' clinicians during their regular visits to the hospital (Table 6).

3.1. Clinicopathological Variables Characterization

Table 6. Clinicopathological variables of population enrolled in the study

	At event Non-response	At event Toxicity
Patient-related		
Male, n (%)	37 (15.9)	37 (15.9)
Female, n (%)	196 (84.1)	196 (84.1)
Postmenopausal, n (%)	96 (49.0)	101 (51.5)
Age, mean ± SD, years	51.9 ± 11.9	52.0 (26.0-87.0)
BMI, median (IQR), Kg/m^2	27.1 (18.3-44.2)	26.2 (18.4-43.1)
Current smokers, n (%)	32 (13.7)	32 (13.7)
NPY*, median (IQR)	19.5 (0.8-120.0)	20.1 (0.8-120.0)
Comorbidity**, n (%)	126 (54.1)	126 (54.1)
Disease-related		
Diagnosis age, mean ± SD, years	40.3 ± 13.2	40.3 ± 13.2
Disease duration, median (IQR), years	8.0 (0.5-53.0)	10.0 (0.3-51.0)
RF positive, n (%)	131 (56.2)	131 (56.2)
Anti-CCP positive, n (%)	175 (75.1)	175 (75.1)
ANAs positive, n (%)	66 (28.3)	66 (28.3)
DAS28, mean ± SD	4.2 ± 1.3	4.1 ± 1.4
Individual variables - DAS28		
TJC (out of 28), median (IQR)	4.0 (0.0-27.0)	3.9 (0.0-26.0)
SJC (out of 28), median (IQR)	3.0 (0.0-24.1)	3.0 (0.0-24.1)
ESR, median (IQR), minutes (1st hour)	18.0 (1.0-92.0)	17.8 (1.0-91.0)
Global Health on VAS, median (IQR)	48.0 (0.0-100.0)	47.5 (0.0-100.0)
HAQ score, median (IQR)	1.25 (0.0-2.9)	1.24 (0.0-2.7)
HAQ ≤0.5, n (%)	39 (16.7)	39 (16.7)

*NPY = (number of cigarettes smoked per day x number of years smoking)/20.

**Comorbidity was defined as the presence of diabetes *mellitus*, hypertension, dyslipidemia and/or cardiac disorders beyond rheumatoid arthritis.

§Drugs co-administered with methotrexate when clinical response/toxicity to methotrexate was recorded.

#Patients with compliance to folic acid supplementation.

ANAs: antinuclear antibodies; Anti-CCP: anti-cyclic citrullinated peptide; bDMARDs: biological disease-modifying antirheumatic drugs; BMI: body mass index; DAS28: disease activity score 28; DMARDs: disease-modifying antirheumatic drugs; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; IQR: interquartile range; MTX: methotrexate; NPY: number of pack years; NSAIDs: non-steroidal anti-inflammatory drugs; RF: rheumatoid factor; SD: standard deviation; sDMARDs: synthetic disease-modifying antirheumatic drugs; SJC: swollen joints count; TJC: tender joints count; VAS: visual analog scale.

Table 6. Clinicopathological variables of population enrolled in the study (cont.)

	At event Non-response	At event Toxicity
***Treatment-related*[§]**		
Symptomatic		
Corticosteroids, n (%)	188 (80.7)	188 (80.7)
Daily dose in prednisolone equivalents, median (IQR), mg	5.0 (0.0-20.0)	5.0 (0.0-20.0)
NSAIDs, n (%)	170 (73.0)	170 (73.0)
Supplements		
Folic acid[#], n (%)	118 (50.6)	118 (50.6)
DMARDs		
MTX Monotherapy, n (%)	146 (62.7)	136 (58.4)
Combined MTX Therapy – sDMARDs, n (%)	59 (25.3)	44 (18.9)
Combined MTX Therapy – bDMARDs, n (%)	28 (12.0)	53 (22.7)
MTX administration characteristics		
Dose, median (IQR), mg/week	15.0 (2.5-25.0)	15.0 (2.5-25.0)
Treatment duration, median (IQR), months	28.0 (6.0-230.0)	47.0 (1.0-240.0)
Per os administration route, n (%)	201 (86.3)	210 (90.1)
Subcutaneous administration route, n (%)	32 (13.7)	23 (9.9)

*NPY = (number of cigarettes smoked per day x number of years smoking)/20.

**Comorbidity was defined as the presence of diabetes *mellitus*, hypertension, dyslipidemia and/or cardiac disorders beyond rheumatoid arthritis.

[§]Drugs co-administered with methotrexate when clinical response/toxicity to methotrexate was recorded.

[#]Patients with compliance to folic acid supplementation.

ANAs: antinuclear antibodies; Anti-CCP: anti-cyclic citrullinated peptide; bDMARDs: biological disease-modifying antirheumatic drugs; BMI: body mass index; DAS28: disease activity score 28; DMARDs: disease-modifying antirheumatic drugs; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; IQR: interquartile range; MTX: methotrexate; NPY: number of pack years; NSAIDs: non-steroidal anti-inflammatory drugs; RF: rheumatoid factor; SD: standard deviation; sDMARDs: synthetic disease-modifying antirheumatic drugs; SJC: swollen joints count; TJC: tender joints count; VAS: visual analog scale.

3.2. Therapeutic Outcomes Definition

Response / Non-Response

Methotrexate clinical response was recorded at the time of each visit. Non-response was defined if patients presented a DAS28>3.2 calculated and defined as described by Prevoo *et al.* (114) (see 1.8.2. Disease activity

measure - 1.8. Disease Monitoring), in two consecutive evaluations. Therefore, non-response to MTX had a minimum period of MTX therapy, at least, of six months. Additionally, response to MTX was defined when patients presented a DAS28≤3.2.

Toxicity / Non-Toxicity

Methotrexate-related toxicity was defined when patients presented any ADR related to MTX. At the time of each visit, physician directly asked the patient whether MTX-related ADRs were currently occurring and ADRs were recorded. The type of ADR was classified in System Organ Class (SOC) disorders, in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) (301). Additionally, non-toxicity occurrence related with MTX was defined when patients did not present any ADR related with MTX.

4. SAMPLE COLLECTION AND PROCESSING

Whole blood samples from each patient were obtained with standard venipuncture technique using ethylenediaminetetraacetic acid (EDTA) containing tubes. Genomic DNA was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer instructions. Total genomic DNA was quantified and its purity and integrity were analyzed using the NanoDrop® 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA).

5. GENOTYPING PROCEDURES

Genotyping procedures for studied genetic polymorphisms are presented in Table 7 and a representative genotyping result for each technique is shown in Figures 23-26. Several molecular biology techniques

were used, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFPL), Real-time PCR, automated sequencing and Sequenom® MassARRAY iPLEX. For quality control, 10% of the samples were randomly selected for a second analysis, and in case of PCR-RFLP and Real-Time PCR protocols, 10% of cases were confirmed by automated sequencing. Results were 100% concordant.

Table 7. Genotyping procedures for the studied genetic polymorphisms

Gene/ Protein	SNP/ rs ID	Molecular biology technique: Name	Molecular biology technique: Description	Primers or Context sequences/Results
Influx carrier-mediated transport pathway				
SLC19A1 RFC	G80A rs1051266	PCR-RFLP (221)	PCR: 1x DreamTaq©Green master mix (Thermo Scientific, USA), 0.3µM of each primer and 50-100ng of genomic DNA (final volume 50µL). Initial denaturation at 95°C, 5 min; 40 cycles with denaturation at 95°C, 15 sec., annealing at 58°C, 1 min; final extension at 72°C, 7 min. RFLP: At 37°C, 1 hour using CfoI (HhaI) (Life Technologies, USA).	F: 5'-AGT GTC ACC TTC GTC CCC TC-3' R: 5'-CTC CCG CGT GAA GTT CTT-3' Results: Individuals with the GG genotype presented three fragments (125bp, 68bp and 37bp), whereas individuals with the AA genotype presented two fragments (162bp and 68bp).
	C>A rs7499	Sequenom® MassARRAY iPLEX (302)	Sequenom® Assay Design 3.1 software was used to design the primers. Genomic sequence was amplified by multiplex PCR with forward and reverse primers. Amplified products were treated with Shrimp alkaline phosphatase and used for allele specific primer extension (iPLEX) reaction (Sequenom®, USA), the extend primer. Reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the MALDI-TOF MS. Genotypes were assigned based on the presence of mass peaks by the MassARRAY Typer v4.0 software (Sequenom®, USA). Results were manually inspected and verified.	F: 5'-ACG TTG GAT GAG AAC AGC TTC ATG ACT GCC-3' R: 5'-ACG TTG GAT GTT CCT CCG ACC CGC CGG TC-3' E: 5'-CTT CCT CTC CGG CCA TC-3'
	A>G rs2838956			F: 5'-ACG TTG GAT GAG GAG GAC TGT CAG GGT TAG-3' R: 5'-ACG TTG GAT GCT TAC CCT GAG CTC TGG AGT-3' E: 5'-GCT CTG GAG TCA CTG A-3'
	C>A rs3788200			F: 5'-ACG TTG GAT GCA CCT CCA CTG TCC CAA TAG-3' R: 5'-ACG TTG GAT GTC CCA GAC CCT AGA ATT GTG-3' E: 5'-TGA AAC TGA TGT GCA GC-3'
SLC16A7 MCT2	A>T rs3763980			F: 5'-ACG TTG GAT GAG ATT TGC TCA AGG GTT CAG-3' R: 5'-ACG TTG GAT GGC TTG CAA AGG AAA GGA AGG-3' E: 5'-TAG TAA AAT GCA ACG CAG AAG-3'
	T>G rs10877333			F: 5'-ACG TTG GAT GCG TGT CAT AAG CCA GAA TGC-3' R: 5'-ACG TTG GAT GCA TCA AGA GTT TCA AGA GAC-3' E: 5'-GCA AGA GTT TCA AGA GAG TGT ATA-3'
SLC22A6 OAT1	C>T rs11568626			F: 5'-ACG TTG GAT GAC AAC ACC CTG CAG AAC TTC-3' R: 5'-ACG TTG GAT GTT CTT GCT GAG GTT GGC ATC-3' E: 5'-GGG CAT CGG CAG GCG GG-3'
SLC22A11 OAT4	T>A rs11231809			F: 5'-ACG TTG GAT GGT CTT TGA ATC AGA CGT ATC-3' R: 5'-ACG TTG GAT GCT CAC CTG TTC CGT GAT TTC-3' E: 5'-TTA CTT CAT TCA TTC AAC AAA CAT GTA-3'

A: adenine; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; G: guanine; E: extend primer; F: forward primer; ID: identification; LST1: liver-specific organic anion transporter 1; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; OAT: organic anion transporter; PCFT: proton-coupled folate transporter; PCR: polymerase chain reaction; R: reverse primer; RFC: reduced folate carrier protein; RFLP: restriction fragment length polymorphism; SNP: single nucleoside polymorphism; T: thymine.

Table 7. Genotyping procedures for the studied genetic polymorphisms (cont.)

Gene / Protein	SNP/ rs ID	Molecular biology technique		
		Name	Description	Primers or Context sequences/Results
Influx carrier-mediated transport pathway				
SLC46A1 PCFT	G>A rs2239907	Sequenom® MassARRAY iPLEX (302)	Sequenom® Assay Design 3.1 software was used to design the primers. Genomic sequence was amplified by multiplex PCR with forward and reverse primers. Amplified products were treated with Shrimp alkaline phosphatase and used for allele specific primer extension (iPLEX) reaction (Sequenom®, USA), the extend primer. Reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the MALDI-TOF MS. Genotypes were assigned based on the presence of mass peaks by the MassARRAY Typer v4.0 software (Sequenom®, USA). Results were manually inspected and verified.	F: 5'-ACG TTG GAT GCA CTC ATT CAT CCT TTC CCC-3' R: 5'-ACG TTG GAT GCT GAG ATA CTT CCC ACT TCC-3' E: 5'-GGA ACT TCC TCA TAC ATT CTA CA-3'
SLCO1B1 LST1	T>C rs4149056			F: 5'-ACG TTG GAT GAA TCT GGG TCA TAC ATG TGG-3' R: 5'-ACG TTG GAT GCC AAT GGT ACT ATG GGA GTC-3' E: 5'-GGG TAA GCA TAT TAC CCA TGA AC-3'
Efflux carrier-mediated transport pathway				
ABCB1 MDR	C>T rs1045642	Sequenom® MassARRAY iPLEX (302)	Sequenom® Assay Design 3.1 software was used to design the primers. Genomic sequence was amplified by multiplex PCR with forward and reverse primers. Amplified products were treated with Shrimp alkaline phosphatase and used for allele specific primer extension (iPLEX) reaction (Sequenom®, USA), the extend primer. Reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the MALDI-TOF MS. Genotypes were assigned based on the presence of mass peaks by the MassARRAY Typer v4.0 software (Sequenom®, USA). Results were manually inspected and verified.	F: 5'-ACG TTG GAT GTA TGT TGG CCT CCT TTG CTG-3' R: 5'-ACG TTG GAT GGC TGA GAA CAT TGC CTA TGG-3' E: 5'-TGG GGT GTC ACA GGA AGA GAT-3'
	C>T rs1128503			F: 5'-ACG TTG GAT GCA CAG CCA CTG TTT CCA ACC-3' R: 5'-ACG TTG GAT GTT TCT CAC TCG TCC TGG TAG-3 E: 5'-CTG GTA GAT CTT GAA GGG-3'
	G>A/T rs2032582			F: 5'-ACG TTG GAT GCA TAT TTA GTT TGA CTC ACC-3' R: 5'-ACG TTG GAT GTG TTG TCT GGA CAA GCA CTG-3' E: 5'-AAA GAT AAG AAA GAA CTA GAA GGT-3'
ABCC1 MRP1	T>C rs35592			F: 5'-ACG TTG GAT GGA CGC CTG TGT CAT CTC AAA-3' R: 5'-ACG TTG GAT GCC TTG CCA ACT GAT GAG TTC-3' E: 5'-CTG AAC TGA GCA CCG CGG ATA AGA A-3'
	A>G rs246240			F: 5'-ACG TTG GAT GTG GTA AGC AAC AGG GCA AAC-3' R: 5'-ACG TTG GAT GAC GGA GCC TAA ATG TCC AGC-3' E: 5'-CGA CTA AAT GTC CAG CAG TAA GAG AT-3'

A: adenine; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; E: extend primer; F: forward primer; ID: identification; LST1: liver-specific organic anion transporter 1; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; OAT: organic anion transporter; PCFT: proton-coupled folate transporter; PCR: polymerase chain reaction; R: reverse primer; RFC: reduced folate carrier protein; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism; T: thymine.

Table 7. Genotyping procedures for the studied genetic polymorphisms (cont.)

Gene / Protein	SNP/ rs ID	Molecular biology technique		
		Name	Description	Primers or Context sequences/Results
Efflux carrier-mediated transport pathway				
ABCC1 MRP1	G>C rs2074087	Sequenom® MassARRAY iPLEX (302)	Sequenom® Assay Design 3.1 software was used to design the primers. Genomic sequence was amplified by multiplex PCR with forward and reverse primers. Amplified products were treated with Shrimp alkaline phosphatase and used for allele specific primer extension (iPLEX) reaction (Sequenom®, USA), the extend primer. Reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the MALDI-TOF MS. Genotypes were assigned based on the presence of mass peaks by the MassARRAY Typer v4.0 software (Sequenom®, USA). Results were manually inspected and verified.	F: 5'-ACG TTG GAT GTC ATG CTG TGC GTG ACC AAG-3' R: 5'-ACG TTG GAT GCA AGC TAG GCA GTC TCA CAC-3' E: 5'-CTC ACA CAT GTG CAC T-3'
	C>G rs2230671			F: 5'-ACG TTG GAT GAG CAT TCC CAC CAC ACC TG-3' R: 5'-ACG TTG GAT GAT CCG AAA TAA GCC CAG GGT-3' E: 5'-GAA GCC CAG GGT CAG GGA-3'
	G>A rs3784864			F: 5'-ACG TTG GAT GCT GCA TGT TGT TGA CTT GGG-3' R: 5'-ACG TTG GAT GGC AAT GGG AAG TAC TGA GAG-3' E: 5'-AGC TCA GCA AGC TAT CAA AGA ACT-3'
ABCC2 MRP2	G>A rs717620			F: 5'-ACG TTG GAT GCC TGT TCC ACT TTC TTT GAT-3' R: 5'-ACG TTG GAT GAG CAT GAT TCC TCG ACT GCG-3 E: 5'-CCC CTC TGG ACT GCG TCT GGA AC-3'
	C>T rs4148396			F: 5'-ACG TTG GAT GCT GAA CAG TGT TGT CTA GGG-3' R: 5'-ACG TTG GAT GAA GAA CTG TCA GGT GCT GTG-3' E: 5'-GAT ATA CTG AGG ATC TTT CTG A-3'
ABCG2 BCRP	C>A rs2231142			F: 5'-ACG TTG GAT GCG TCA TAG TTG TTG CAA GCC-3' R: 5'-ACG TTG GAT GTG ATG TTG TGA TGG GCA CTC-3' E: 5'-GGA TTA CGG TGA GAG AAA ACT TA-3'
	T>C rs13120400			F: 5'-ACG TTG GAT GCA GCA CAT ACT CCC TTT CAG-3' R: 5'-ACG TTG GAT GGT TGG AAT CAG GTG ATC AGG-3' E: 5'-AAA GGG AAA CCT TCA CCT-3'
	G>A rs17731538			F: 5'-ACG TTG GAT GGG AGT CTT GGG TTT AAG CAC-3' R: 5'-ACG TTG GAT GAT TAG CAC AGA GGC CAA TCC-3' E: 5'-TCC AAT CCT ATG ATC CTA TAC AAT TAT-3'

A: adenine; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; G: guanine; E: extend primer; F: forward primer; ID: identification; LST1: liver specific organic anion transporter 1; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance-associated protein; OAT: organic anion transporter; PCFT: proton coupled folate transporter; PCR: polymerase chain reaction; R: reverse primer; RFC: reduced folate carrier protein; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism; T: thymine.

Table 7. Genotyping procedures for the studied genetic polymorphisms (cont.)

Gene / Protein	SNP/ rs ID	Molecular biology technique		
		Name	Description	Primers or Context sequences/Results
Folate pathway				
MTHFR MTHFR	C677T rs1801133	PCR-RFLP (303)	PCR: 1x DreamTaq™Green master mix (Thermo Scientific, Lithuania), 0.3μM of each primer and 50-100ng of genomic DNA (final volume-50μL). Initial denaturation at 94°C, 5 min.; 30 cycles with denaturation for at 94°C, 1 min., annealing for at 57°C, 1 min., extension at 72°C, 15 sec.; final extension at 72°C, 10 min. RFLP: At 37°C, overnight, using HinfI (Thermo Scientific, Lithuania).	F: 5'- TGA ACG AGA AGG TGT CTG CGG GA-3' R: 5'-ACG ACG GTG CGG TCA CAG TG-3' Results: Individuals with the CC genotype presented one fragment with 198bp, whereas individuals with the TT genotype presented one fragment with 175bp.
***De novo* pyrimidine pathway**				
TYMS TS	28bp VNTR rs34743033	PCR (286)	PCR: 1x GoTaq® master mix (Promega, Wisconsin), 0.4μM of each primer, 5μL dimethyl sulfoxide (Sigma-Aldrich, Missouri) and 50-100ng of genomic DNA (final volume-50μL). Initial denaturation at 95°C, 5 min.; 40 cycles with denaturation at 95°C, 1 min., annealing at 60°C, 1 min., extension at 72°C, 2 min.; final extension at 72°C, 5 min.	F: 5'-GTG GCT CCT GCG TTT CCC CC-3' R: 5'-CCA AGC TTC GCT CCG AGC CGG CCA CAG GCA TGG CGC GG-3' Results: Individuals with the 3R3R genotype presented one fragment of 243bp and individuals with the 2R2R genotype presented one fragment of 215bp.
	C>G rs2853542	RFLP (286)	At 37°C using BsuRI (HaeIII) (Thermo Scientific, Lithuania) during 2 hours.	-
	1494del6 rs34489327	Automated sequencing (304)	PCR: 1x DreamTaq™ master mix (Thermo Scientific, Lithuania), 0.4μM of each primer and 50-100ng of genomic DNA (final volume-50μL). Initial denaturation at 95°C, 5 min.; 35 cycles with denaturation at 95°C, 30 sec., annealing at 58°C, 45 sec., extension at 72°C, 45 sec.; final extension at 72°C, 7 min. The PCR products were purified with USB® ExoSAP-IT (Affymetrix, USA) before cycle sequencing. Sequence reactions: carried out using the sequencing kit BigDye® Terminator v.3.1 (Life Technologies, USA) according to the manufacturer's specifications. The sequencing profile was 30 cycles at 96°C for 10 sec., 55°C for 10 sec. and 60° for 60 sec., and an extension cycle at 60°C for 10 min. The sequence products were purified with illustra Sephadex G-50 Fine DNA Grade (GE Healthcare, USA) columns, denatured with Hi-Di™ Formamide and run in an 3130xl Genetic Analyzer (Life Technologies, USA).	F: 5'-CAA ATC TGA GGG AGC TGA GT-3' R: 5'-CAG ATA AGT GGC AGT ACA GA-3' Results: Individuals with the 3RG3RG genotype presented two fragments with 66bp and 28bp, 3RC3RC presented one fragment with 94bp, 2RC3RG presented one fragment of 66bp and 2R3RC presented one fragment of 94bp.

A: adenine; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; G: guanine; E: extend primer; F: forward primer; ID: identification; LST1: liver specific organic anion transporter 1; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; OAT: organic anion transporter; PCFT: proton-coupled folate transporter; PCR: polymerase chain reaction; R: reverse primer; RFC: reduced folate carrier protein; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism; T: thymine.

Table 7. Genotyping procedures for the studied genetic polymorphisms (cont.)

Gene / Protein	SNP/ rs ID	Molecular biology technique		
		Name	Description	Primers or Context sequences/Results
De novo purine pathway				
ATIC ATIC	T675C rs4673993	Real-Time PCR (305)	1× TaqMan® Genotyping Master Mix (Applied Biosystems, USA), 900nM of each primer, 200nM of probes labeled with either FAM or VIC and 10ng of extracted DNA (final volume-5µL). Reactions performed on a 7300 Real-Time PCR System (Applied Biosystems, USA) by a TaqMan® SNP Genotyping Assay (C_362264_10) with fluorogenic binding probes. Thermal cycling conditions were: 95°C, 10 min.; 40 cycles at 95°C, 15 sec. and at 60°C, 1 min. Allelic discrimination was performed by measuring end-point fluorescence using ABI PRISM® Sequence Detection System (Version 1.2.3, Applied Biosystems, USA).	[VIC/FAM]: CCA ATT GAC TAC CCT CAG TTT TTT A[C/T] TCA GGA GCA GGA ATC AAC ATA AGT G

A: adenine; BCRP: breast cancer resistance protein; bp: base pairs; C: cytosine; G: guanine; E: extend primer; F: forward primer; ID: identification; LST1: liver-specific organic anion transporter 1; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance associated protein; OAT: organic anion transporter; PCFT: proton coupled folate transporter; PCR: polymerase chain reaction; R: reverse primer; RFC: reduced folate carrier protein; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism; T: thymine.

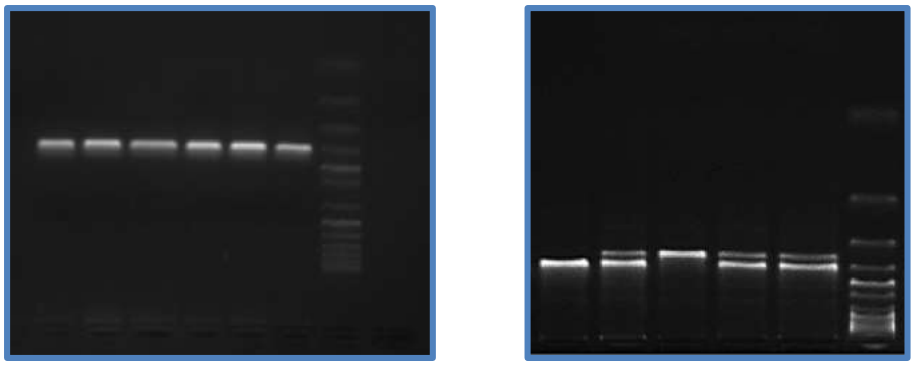


Figure 23. Electrophoresis gels of PCR and RFLP-PCR techniques of *MTHFR* C677T (rs1801133). Left: PCR product of 198bp + Gene ruler DNA ladder of 50bp. Right: PCR-RFLP products (1st band: CC genotype; 2nd, 4rd and 5th bands: CT genotype; 3th band: TT genotype) + Gene ruler DNA ladder of 50bp.

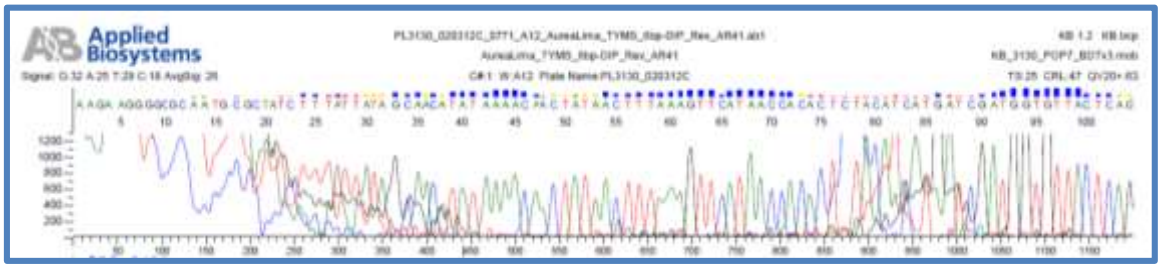


Figure 24. Automatic sequencing of *TYMS* 1494del6 (rs34489327). Example of 6bp+6bp+ genotype due to the presence of the sequence CTTTAA (or TTAAAC) from position 49 to 54.

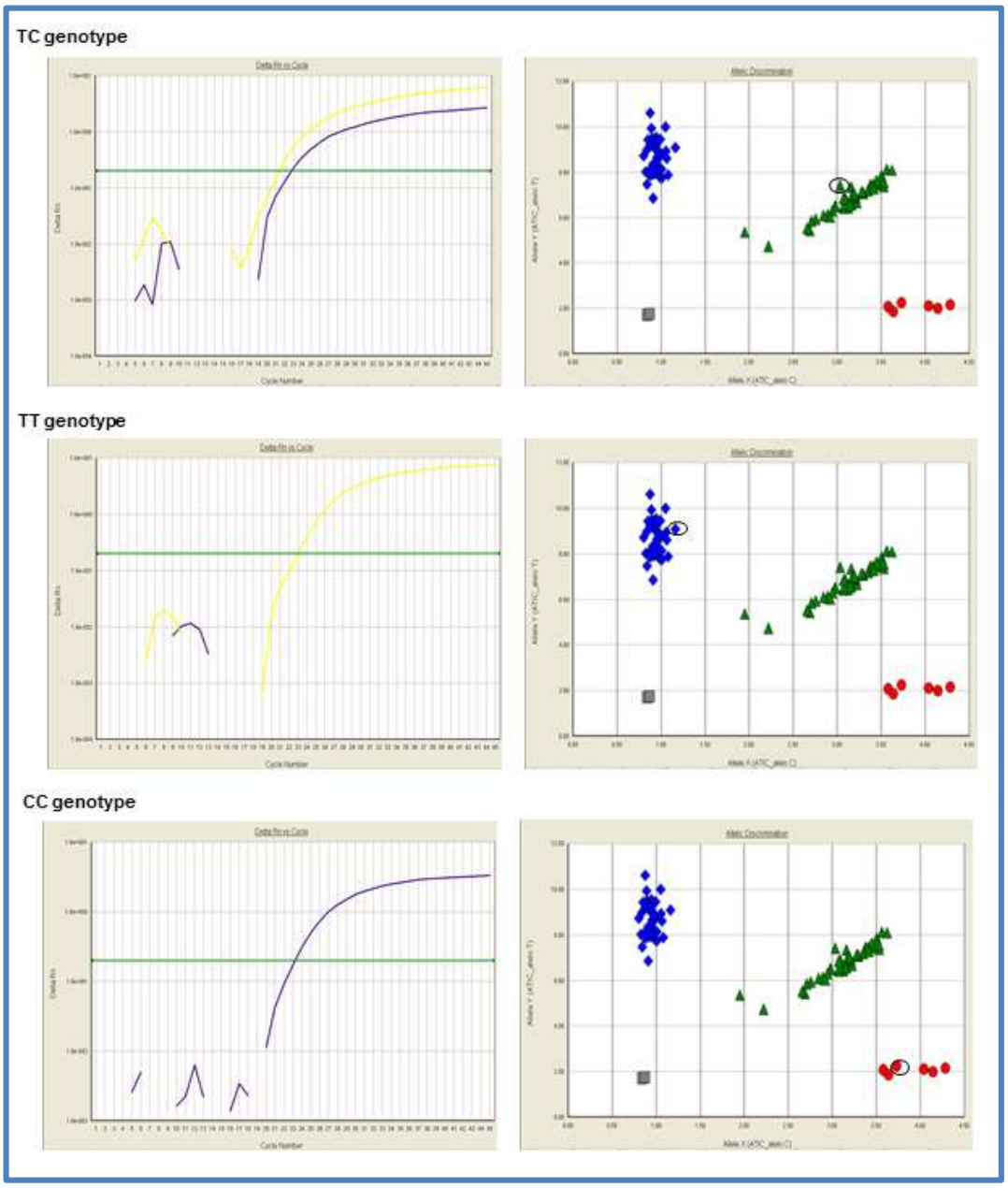


Figure 25. Real-time PCR technique of *ATIC* T675C (rs4673993). Upper: Results demonstrating a TC genotype due to the presence of two lines (yellow and purple) corresponding to T allele (left) and C allele (right). Middle: Results demonstrating a TT genotype due to the presence of one yellow line corresponding to T alleles. Lower: Results demonstrating a CC genotype due to the presence of one purple line corresponding to C alleles.

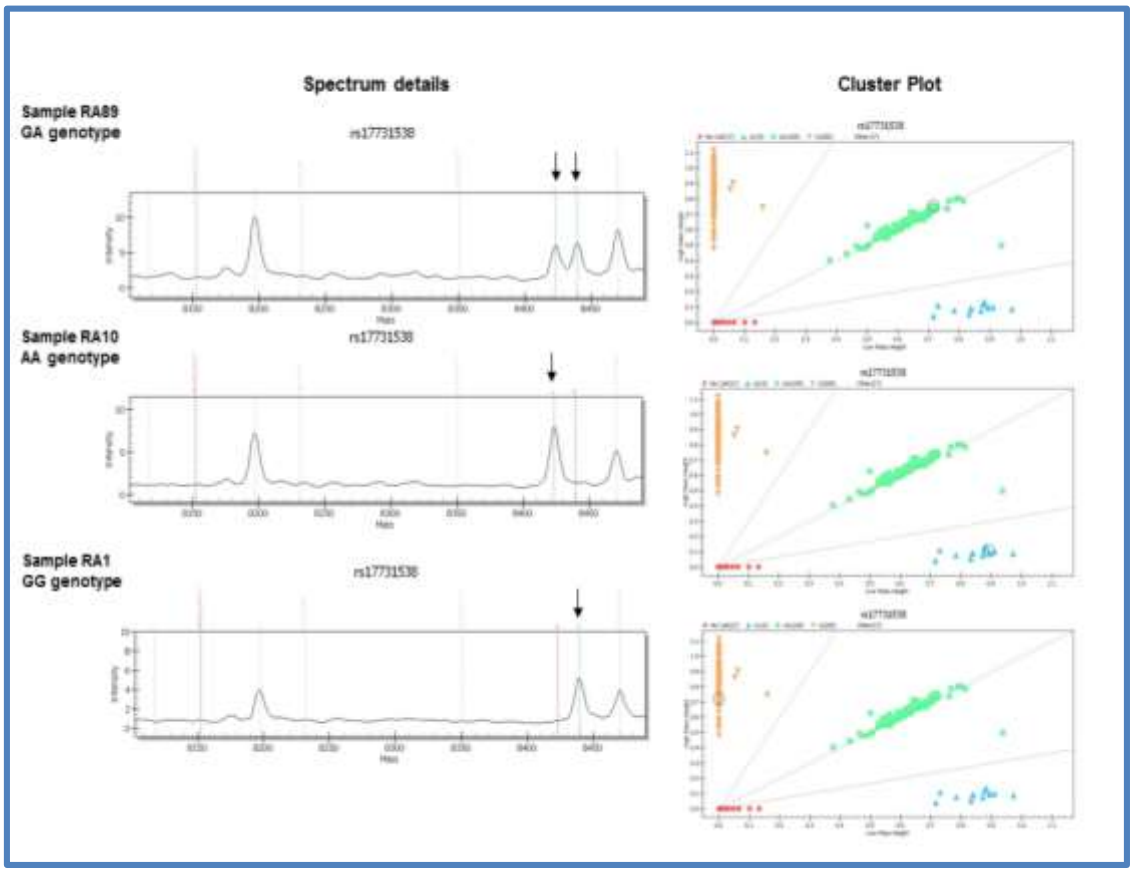


Figure 26. Sequenom® MassARRAY iPLEX technique of *ABCG2* G>A (rs17731538). Upper: Results demonstrating a GA genotype due to the presence of two peaks corresponding to A allele (left) and G allele (right). Middle: Results demonstrating a AA genotype due to the presence of one higher peak corresponding to A alleles. Lower: Results demonstrating a GG genotype due to the presence of one higher peak corresponding to G alleles.

6. STATISTICAL ANALYSIS

Statistical analysis was performed with either IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA), OpenEpi for Windows, Version 2.3.1 (306), SNPStats software (307), MedCalc® software for Windows, Version 13.1.2 (308) and Arlequin for Windows, Version 3.11 (309), when applicable.

6.1. Descriptive statistics

Genotype frequencies were assessed and tested for Hardy-Weinberg equilibrium (HWE) according to Rodriguez *et al.* (310). Quantitative variables description was expressed in mean ± standard derivation (SD) for normally distributed variables and median and interquartile range (IQR) for non-normally distributed variables, as evaluated by the Kolmogorov-Smirnov test. Categorical variables were described by number of cases (n) and percentages (%).

6.2. Univariate analysis

Pearson Chi-square test or Fisher's exact test were used to assess the association between the outcome groups (response *versus* non-response or toxicity *versus* non-toxicity) and the different categorical variables. Odds ratio (OR) and the correspondent 95% confidence intervals (CI) were calculated as a measure of the association between the categorical variables. For the comparison of quantitative variables between outcome groups, two sample t-tests, for normally distributed variables, and non-parametric Mann-Whitney *U* tests, for non-normally distributed variables, were applied. It was considered a probability (*P*) value of 5% or less as statistically significant. In the cases that univariate analysis was performed for clinicopathological variables, the variables that were statistically significant in this analysis were also adjusted in multivariate analysis.

6.3. Multivariate analysis

Multivariate analysis with binary logistic regression was used to identify which genotypes or clinicopathological variables could predict the occurrence of MTX-related toxicity and/or non-response to MTX. This analysis was performed in one or three-steps by adjusting to potential confounding variables, i.e. clinicopathological variables possibly influencing MTX-related toxicity, selected based on literature review and clinical significance (221, 311-316). When three steps were used, in the first step patient-related variables (age, gender and smoking) were considered; in a

second step, beyond patient-related variables, disease-related variables (diagnosis age and disease duration) were added; and in a third step, beyond patient and disease-related variables, treatment-related variables (folic acid supplementation, corticosteroids therapy, use of NSAIDs, other concomitant DMARDs used and MTX administration characteristics - dose, treatment duration and administration route) were also considered. When one step was used, the analysis corresponded only to the last step of the three-step analysis. In the case of carrier-mediated transport pathway, polymorphisms were adjusted for two extra patient-related variables concerning renal function (eGFR and SCr). It was considered a probability (*P*) value of 5% or less as statistically significant.

6.4. Haplotype analysis

Because haplotypes may have a particular significance in regard to functionality or as genetic markers for unknown functional variants, it appears more evident that, to better characterize the role of a candidate gene, the full haplotypic information should be exploited (317, 318). Therefore, haplotype analysis was performed, to assess of possible consequences on the phenotype in the copresence of several variants of the same gene. Haplotype analyses were performed by Arlequin for Windows, Version 3.11 (University of Berne, Bern, Switzerland) (309) with 100,000 number of steps in Markov chain or using a two-stage iterative method named expectation maximization algorithm (SNPStats software) (307). Linkage disequilibrium (LD) between SNPs in the same gene was estimated and expressed as *D´* coefficients. The measure was interpretable as the proportion of the maximum possible level of association between two *loci*, given the allele frequencies, ranging from 0 (linkage equilibrium) to 1 (complete LD) (26). Possible haplotypes were tested for association with risk for non-response to MTX and/or for MTX-related toxicity by taking the most frequent haplotype as reference.

6.5. Toxicogenetic risk index

In order to elucidate the cumulative impact of risk genotypes on MTX-related toxicity development, a toxicogenetic risk index (TRI) was created for each patient. The TRI resulted by the sum of risk genotypes from the SNPs in genes encoding for MTX membrane transport proteins that revealed to be statistically significant associated with MTX-related toxicity.

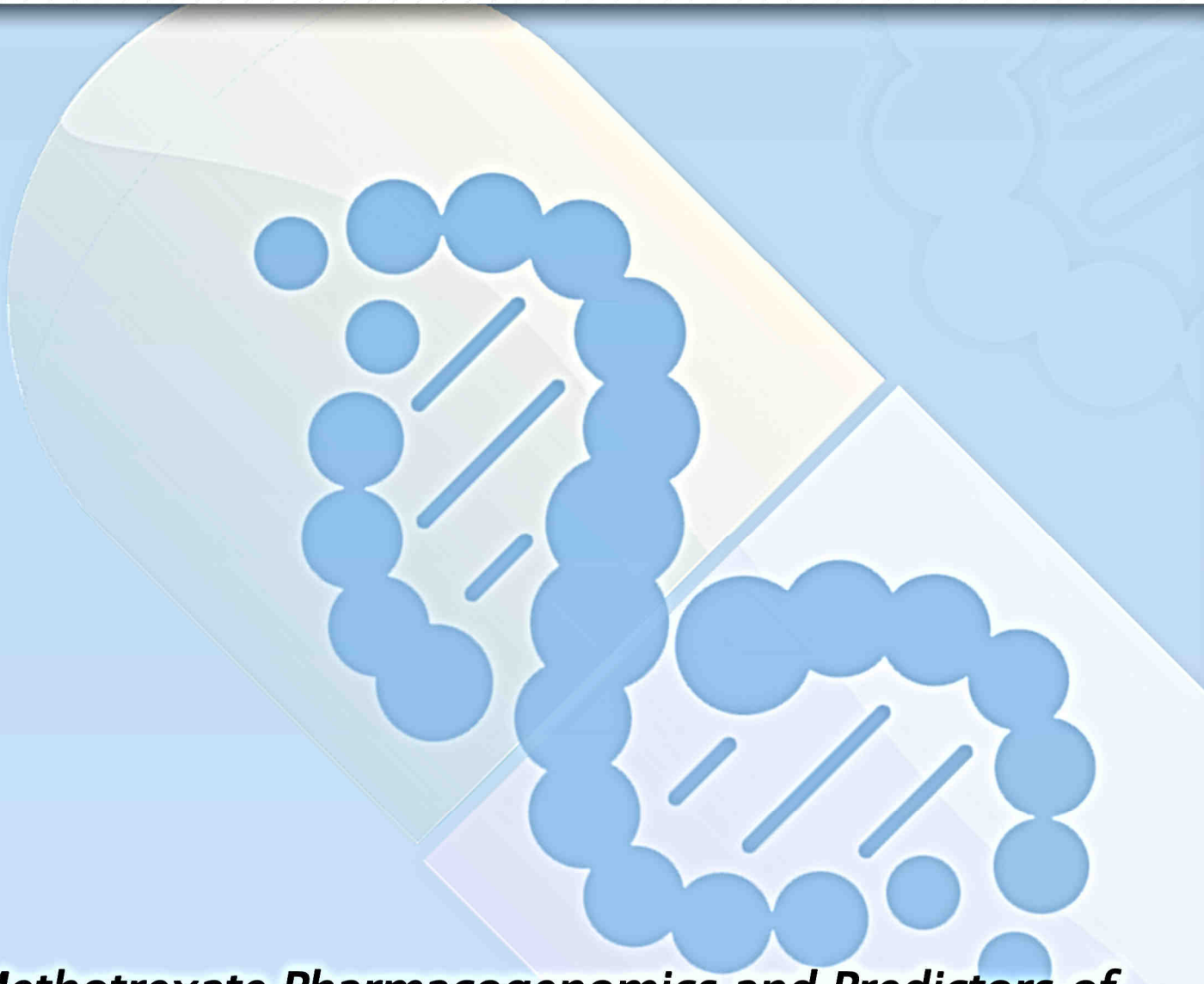
6.6. Other analyses

In order to understand the impact of the 5'UTR region in therapeutic outcome, polymorphisms in TSER were classified according to their theoretical TS functional status as previously described (286) and grouped by expression, as follow: low expression genotypes (2R2R, 2R3RC and 3RC3RC), median expression genotypes (2R3RG and 3RC3RG) and high expression genotype (3RG3RG).

When relevant, a Forest plot was performed using MedCalc® software for Windows, Version 13.1.2 (319). Additionally, and using IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA), a Cox proportional hazard regression model was used to adjust for potential confounders and, to correct for multiple comparisons, Bonferroni's method was applied in order to control the false positive rate, using a significance level of $\alpha=0.05/(n$ comparisons).

CHAPTER IV

GENETIC POLYMORPHISMS IN LOW-DOSE METHOTREXATE TRANSPORTERS: THE CURRENT RELEVANCE AS METHOTREXATE THERAPEUTIC OUTCOME BIOMARKERS



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER IV. GENETIC POLYMORPHISMS IN LOW-DOSE METHOTREXATE TRANSPORTERS: THE CURRENT RELEVANCE AS METHOTREXATE THERAPEUTIC OUTCOME BIOMARKERS

This chapter presents a comprehensive review about Methotrexate pharmacokinetics processes and the current data on genetic polymorphisms in genes encoding for low-dose Methotrexate membrane transporters, as also their influence in Methotrexate therapeutic outcome. The search strategy was performed given the clinical implications of widespread use of low-dose Methotrexate in rheumatic and dermatological diseases. In addition, the extent of Methotrexate movement through cellular membranes, determining the Methotrexate pharmacokinetics, is partly conditioned by membrane transporters, both influx and efflux. Therefore, functional activity and expression level of these proteins, possibly conditioned by genetic polymorphisms in genes encoding to those proteins, can determine the amount of intracellular and systemic Methotrexate and, consequently, its pharmacodynamics.

Genetic polymorphisms in low-dose methotrexate transporters: the current relevance as methotrexate therapeutic outcome biomarkers

ABSTRACT

Methotrexate (MTX) is used in low-doses to treat a variety of diseases. Although the mechanism responsible for its therapeutic action is unknown, MTX membrane transport proteins (influx and/or efflux) can be major determinants of pharmacokinetics, adverse drug reactions and clinical response profiles. With pharmacogenomics progress, the improvement of the prediction of patients' therapeutic outcome treated with low-doses of MTX will offer a powerful tool for the translational role of single nucleotide polymorphisms (SNPs) into clinical practice and will be essential to sustain a breakthrough in the field of personalized medicine. Therefore, this paper provides an update on the current data on SNPs in genes involved in low-dose MTX membrane transport proteins and their relevance as possible biomarkers of MTX therapeutic outcome.

Keywords: ATP-binding Cassette Transporters – ABCs; Low-dose; Methotrexate; Personalized Medicine; Pharmacogenomics; Therapeutic Outcome; Transporters; Single Nucleotide Polymorphisms - SNPs; Solute Carriers – SLCs

INTRODUCTION

The observation that folic acid antagonists interfered with normal cell growth lead back to 1951, when high-dose methotrexate (MTX) was introduced as a treatment option for children with acute lymphoblastic leukemia [1]. Then, in 1951, Gubner demonstrated that low-dose MTX (up to 25mg/week) could also be effective in controlling disease activity both in patients with psoriatic arthritis and rheumatoid arthritis (RA) [2] and, in the late 1980s, low-dose MTX debuted as treatment option in juvenile idiopathic arthritis (JIA) patients with polyarthritis [3] and in ankylosing spondylitis [4]. Low-dose MTX has been also used in other rheumatic diseases and dermatological diseases, such psoriasis [5].

Methotrexate (2,4-diamino-N10-methyl propyl glutamic acid) is an antifolate negatively charged drug with a molecular weight of 454.5g/mol ($C_{20}H_{22}N_8O_5$), low permeability and poor aqueous solubility [6]. Methotrexate is composed by a pteridine ring, *p*-aminobenzoic acid and glutamic acid, structurally differing from folic acid only by the substitution of a hydroxyl group for an amine in the pteridine ring (at C4 carbon) and the addition of a methyl group on the 10(th) nitrogen of *p*-aminobenzoic acid [6] (Figure 1).

Although therapeutic effects elicited by low-dose MTX treatment may involve mechanisms distinct from those observed with high-dose MTX treatment, there are overlapping mechanisms of action. These are primarily attributed to MTX ability to inhibit multiple enzymes involved in folate and *de novo* nucleotides synthesis pathways [7]. This ability is then reflected in other pathways such as methionine and adenosine pathways, which along, will contribute for MTX anti-inflammatory and antiproliferative effects [7]. The extent and time course of MTX effects on pathophysiological processes, described by pharmacodynamics (PD), are partly determined by MTX intracellular retention, which occurs as a consequence of MTX polyglutamates (MTXPGs) formation via folylpolyglutamate synthetase (FPGS) [8, 9]. Nevertheless, gamma-glutamyl hydrolase (GGH) can revert this process

and then MTX can be transported out of the cells by efflux transporters [9] (Figure 2).

For determining PD it is important to consider the relationship between drug effect and drug concentration [8]. The extent of drug movement through cellular membranes, determined by pharmacokinetics (PK), is partly affected by membrane transporters [9]. Therefore, functional activity and expression level of MTX membrane transport proteins, possibly altered by genetic polymorphisms, can determine the amount of MTX transported into or out of specific tissues [8]. These yet underlooked and subtle differences can be translated into interpatient therapeutic outcome variability, both regarding clinical response (effectiveness) and/or the occurrence of adverse drug reactions (toxicity) [6, 7]. Hence, and given the clinical implications of widespread use of low-dose MTX, this manuscript is devoted to provide an overview on MTX PK processes and an update on the current data on genetic polymorphisms in genes encoding for low-dose MTX membrane transporters as also their influence in MTX therapeutic outcome.

LOW-DOSE METHOTREXATE PHARMACOKINETICS

Pharmacokinetics generically describes what the body does to a drug and, PK parameters result from the release of the drug, its absorption, distribution, metabolism and excretion, and provides information about the rate of occurrence for these processes at a systemic level [8]. The bioavailability of MTX is relatively high but can vary depending of the dose and administration route [7]. At 7.5mg/week, enteral and parenteral absorption is similar, but at doses of 15mg/week or more, MTX enteral absorption may decrease by as much as 30% compared with parenteral dosing [7]. Enteral absorption is limited because MTX is absorbed from the gastrointestinal tract (GIT) primarily and predominantly from a saturable active transport system, the solute carrier (SLC) family 19 member 1 (SLC19A1) [7], later to be described in detail. Enteral absorption can also be affected by food intake, oral non-absorbable antibiotics, flow transit alteration through GIT (such diarrhea and constipation) and intestinal pathology [7]. After absorption, 10% of MTX is

converted in the liver, by aldehyde oxidase, to 7-hydroxymethotrexate (7-OH-MTX), an active metabolite [6]. Approximately 50% of MTX is albumin-bound, whereas 90-95% of 7-OH-MTX is albumin-bound [7]. Nevertheless, other drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) alter MTX and 7-OH-MTX binding to plasmatic proteins and impair MTX hepatic metabolism [6]. Methotrexate half-life in serum ranges from 6 to 8 hours after administration and MTX is undetectable by 24 hours [6]. The majority of both MTX and 7-OH-MTX is excreted in the urine, although a small portion is also excreted in the bile [6, 7]. Methotrexate renal clearance results from a combination of filtration and secretion processes, taken place in the proximal tubule, with subsequent reabsorption in the distal tubule [10]. Excretion of MTX can be reduced by renal insufficiency, leading to toxicity, and also by interactions with weak organic acids, such as NSAIDs [6]. However, these interactions have general clinical relevance only at the typical higher weekly maintenance MTX dosage range (up to 25mg/week) but not at the lower doses, commonly used to begin treatment [11]. Besides all of these factors influencing PK, other factors include physicochemical properties of a drug, namely size, lipophilicity and charge (or degree of ionization) [8]. In addition, membrane transporters *per se* have a significant role in facilitating or preventing drug movement. Interplay of influx and efflux transporters together with phase I and II metabolism may be required for the sequential traverse of the basolateral and apical membranes [12]. Drug transporters can, therefore, be regarded as completing the phase I and II enzyme-based detoxification system; drug uptake delivers the drug to the detoxification system to facilitate metabolism, whereas drug efflux decreases the load on detoxification enzymes [13].

Methotrexate cellular transport can occur via influx (uptake into cell) and efflux (out of cell) transporters, which are typically located either at the basolateral or apical membranes in polarized cells [9, 10]. In enterocytes, at apical membrane, MTX is primordially absorbed through active transport mediated by SLC19A1, also known as reduced folate carrier (RFC), a saturable bidirectional anion exchanger, followed by SLC family 46 member 1

(SLC46A1) and, in less extent, by endocytosis via folate receptor alpha (FOLR1) [6, 7, 14]. Methotrexate efflux from enterocytes to intestinal tract lumen can be mediated by ATP-binding cassette (ABCs) transporters, i.e., by ATP-binding cassette sub-family C member 2 (ABCC2), ATP-binding cassette sub-family B member 1 (ABCB1) and ATP-binding cassette sub-family G member 2 (ABCG2); or to bloodstream, by ATP-binding cassette sub-family C member 1 (ABCC1) and ATP-binding cassette sub-family C member 3 (ABCC3) [9]. Once in the bloodstream, MTX either enters in circulation, inside circulating cells [15], aims for affected cells [16] or is distributed towards hepatocytes or renal cells to be excreted [1]. The hepatic uptake of MTX involves SLC19A1, SLC organic anion transporter family member 1B1 (SLCO1B1) and SLCO family member 1B3 (SLCO1B3) [10]. Most of the hepatic distributed drug re-enters the sinusoidal bloodstream via ABCC3 and ABC sub-family C member 4 (ABCC4) and, the remaining, is oxidized to 7-OH-MTX or is excreted into the bile duct by ABCC2, ABCB1 and ABCG2 [10]. Systemic clearance of MTX happens primarily through renal glomerular filtration and active secretion over the proximal tubular cells [10]. Some renal transporters have affinity to MTX and 7-OH-MTX, allowing their entrance into renal cells by solute carrier family 22 member 6 (SLC22A6) and solute carrier family 22 member 8 (SLC22A8) present at basolateral membrane, and by SLC family 22 member 11 (SLC22A11) and SLCO family member 1A2 (SLCO1A2), expressed in apical membrane [9]. Moreover, SLC family 16 member 7 (SLC16A7) has been described as having a moderate to low expression in plasma membrane of kidney but its function on MTX transport remains unclear [17]. Furthermore, MTX excretion through urinary tract can also be mediated by ABCB1, ABCC2, ABCC4 and ABCG2 [6] (Figure 3).

METHOTREXATE PHARMACOGENOMICS: THE ROLE OF MTX-TRANSPORTERS

Multiple transporters and metabolic enzymes are involved in MTX PK and/or PD, prompting for the importance of genetic polymorphisms in genes encoding for related proteins. Assured about the presence of critical players in function and metabolism of MTX, it has been possible to study single

nucleotide polymorphisms (SNPs) in genes encoding for some of these proteins in order to determine their putative influence in MTX therapeutic outcome interpatient variability. Therefore, in the last few decades, numerous pharmacogenomics studies were conducted to decode associations of SNPs in genes encoding to MTX membrane transport proteins that could change MTX influx and/or efflux function and, consequently, could be responsible for interpatient MTX therapeutic outcome variability, both regarding clinical response and/or adverse drug reactions (ADRs) occurrence. The summary of the characteristics and putative effects of those SNPs in low-dose MTX transport function is presented in Table 1.

Membrane Influx Transporters

It has been previously accepted that MTX could enter cells by SLC19A1 and the presence of other influx transporters is not thoroughly explored. Nowadays, more transporters have been associated with MTX transport through cellular membranes and most of these transporters are related with MTX influx: SLC16A7, SLC19A1, SLC22A6, SLC22A8, SLC22A11, SLC46A1, SLCO1A2, SLCO1B1 and FOLR1. Table 2 summarizes the effect of SNPs in genes encoding for low-dose MTX influx transporters with interest to MTX therapeutic outcome.

SLC16A7

The SLC16A7, also known as monocarboxylate transporter 2 (MCT2), is a unidirectional proton-linked transport of monocarboxylates across the plasma membrane encoded by *SLC16A7*, located on chromosome 12q13 [17]. This transporter can mediate either influx or efflux depending of the prevailing substrate and pH gradients with net rates of monocarboxylate transport being determined by the difference between influx and efflux [17]. It is widely expressed in testis and has moderate to low expression in spleen, heart, kidney, pancreas, skeletal muscle, brain and leucocytes [17]. At the present, functional relationship of SLC16A7 expression and MTX mechanisms remains unclear.

rs3763980

This variant of *SLC16A7* results in a substitution of an adenine (A) with a thymine (T) and leads to an amino acid change from threonine (Thr) to a serine (Ser) at position 445 [18]. This polymorphism occurs in a predicted region encoding for a cytoplasmic domain, with implications in phosphorylation and signaling and, therefore, is potentially functional [18]. In fact, T carriers were associated with a reduced expression of SLC16A7 in transformed B cells from healthy donors and with better response to low-dose MTX in JIA patients [18]. In this study, non-response to MTX was defined as a failure to reach the 30% response improvement in American College of Rheumatology 70 pediatric criteria (ACRped70) for United Kingdom cohort and defined using the joints count alone for United States cohort [18]. In addition, literature demonstrated evidences about its linkage disequilibrium (LD) with *SLC16A7* T>G (rs10877333) [18].

rs10877333

This SNP is characterized by a substitution of a T to a Guanine (G) in an intron region of *SLC16A7* and, despite its impact in transporter function is unknown, there is one study that associates G allele with better response to MTX in JIA [18].

SLC19A1

Human SLC19A1, also known as folate transporter 1 or reduced folate carrier protein (RFC), is encoded by *SLC19A1*, located on chromosome 21q22.3 [19]. It is a cell surface transmembrane protein and a bidirectional transporter of primarily reduced folates, including MTX, ubiquitously expressed in many tissues, showing particularly high expression in absorptive tissues (intestine, kidney and placenta) either on apical and/or basolateral membranes [20].

rs7499

This SNP leads to a change of a G into an A at position 16 (*SLC19A1* G*16A), and occurs in the 3´-untranslated region (UTR), thought to be important in messenger ribonucleic acid (mRNA) stability, localization and translational efficiency [21]. Despite there are no functional studies on this *SLC19A1*

variant, there is one study in RA patients demonstrating an association between A allele and non-response to MTX, but no associations were related to toxicity occurrence [22]. In this study non-response to MTX was defined as physician statement of inefficacy plus failure to reduce erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP) by at least 20% with MTX therapy after three months at a minimum dose of 15mg/week and toxicity was defined as the occurrence of an ADR, which needs to be persistent or serious, leading to treatment cessation, the ADR had to resolve on treatment cessation and, in the case of GI disorders, recur after MTX re-challenge [22].

rs1051266

This SNP is the most studied variation in *SLC19A1* and is characterized by a substitution of a G by an A at position 80, leading to the substitution of arginine (Arg) into histidine (His) at codon 27 in the first transmembrane domain, a region implicated to carrier function [23]. Literature described G homozygotes as associated with lower MTX influx activity in lymphocytes [24] and decreased MTXPGs levels in erythrocytes [25]. Studies in RA patients analyzing the impact of this SNP in MTX therapeutic outcome demonstrated an association of A homozygotes with better response to MTX [26-28], and of A allele with better response to MTX when combined with sulfasalazine (SSZ) and hydroxychloroquine (HCQ) [29]. Nevertheless, in Dervieux *et al.* study, non-response was defined as a visual analog scale (VAS) score superior 2cm, obtained by physician's global assessment of disease activity [26]; Drozdzik *et al.* defined non-response when both tender joint count and swollen joint count improved <20% from baseline and, at least, three of the following criteria were not met: ≥20% improvement in VAS, in ESR, in physician's global assessment of disease activity, in patient's global assessment of disease activity, and in Health Assessment Questionnaire (HAQ) [27]; in Hayashi *et al.* work non-response was defined by the total inflammatory status during twelve months of MTX treatment calculated by the area under the curve (AUC) of serial CRP measurements [28]; and, James *et al.* defined non-response as an no improvement between baseline and twelve months based

on the Disease Activity Score 28 (DAS28) [29]. Moreover, de Rotte *et al.* associated A homozygotes with MTX non-response in JIA patients [30]. In this study MTX was used in monotherapy or combined with other disease-modifying antirheumatic drugs (DMARDs) and, non-response was defined by ACRped70 [31] and the use of anti-tumor necrosis factor-α (TNF-α) was a criteria for non-response [30]. Regarding MTX-related toxicity in RA patients, G allele was associated with an increased risk for GI disorders by Lima *et al.* [32] and for overall toxicity by Bohanec Grabar *et al.* [33, 34] while Warren *et al.* demonstrated that A homozygotes patients with psoriasis had an increased risk for MTX overall toxicity [35]. Nevertheless, Lima *et al.* defined toxicity as the occurrence of any ADR related to MTX treatment, independently of administration route and MTX dose, recorded at the time of each visit and, the type of ADR was classified in System Organ Class (SOC) disorders, in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) [32]; Bohanec Grabar *et al.* recorded the ADRs retrospectively from patients' files throughout the course of MTX therapy, ADRs were classified accordingly SOC disorders and considered as mild, moderate and serious [33, 34]; Warren *et al.* assessed the ADRs by clinical records, ADRs were classified accordingly SOC disorders enough to necessitate cessation of MTX or requiring treatment [35]. Additionally, other studies demonstrated no associations with clinical response and/or toxicity to low-dose MTX [22, 27, 33, 35-41].

rs1131596

The change of a T to a cytosine (C) in 5´UTR of *SLC19A1* is thought to alter gene transcription regulatory mechanisms, since it was found to be located in the putative consensus sequence of the activating protein-1 transcription factor enhancer [39]. Functional studies in RA patients demonstrated that C homozygotes were associated with a decreased SLC19A1 expression and influx capability [39] and in fact C carriers demonstrated a trend for lower SLC19A1 expression in lymphoblastic cell lines [34]. Regarding the influence in MTX therapeutic outcome, Bohanec Grabar *et al.* associated C carriers with a decreased risk for MTX therapy discontinuity, due to overall toxicity and

infections in RA [34], but others showed no association with therapeutic outcome [39]. This SNP was also described to be in high linkage disequilibrium with G80A (rs1051266) [34] and, therefore, haplotype studies may be important to be addressed when underlying differences in MTX therapeutic outcomes are compared.

rs2838956

The rs2838956 polymorphism, characterized by a substitution of an A for a G, is located in the fifth intron at position 707 (45525110A>G) [34] and its impact is currently unknown, thus functional studies are necessary to evaluate the potential of this SNP on ribonucleic acid (RNA) splicing, which may affect the protein structure and function [42]. Nevertheless, there are studies relating this variant with MTX therapeutic outcome in RA: while Owen *et al.* associated G allele with non-response to MTX but not with toxicity [22]; Bohanec Grabar *et al.* demonstrated that A allele has a trend to skin and subcutaneous tissue disorders, but no associations were observed regarding clinical response to MTX [34].

rs3788200

Despite there are no studies regarding the impact of this variant in SLC19A1 function and low-dose MTX therapeutic outcome, this intronic SNP characterized by substitution of a G for an A is potentially functional and has been demonstrated to be in LD with rs1051266, rs1131596 and rs4819130 [43].

SLC22A6

The SLC22A6, also known as organic anion transporter 1 (OAT1), is encoded by *SLC22A6*, located on chromosome 11q12.3 [44] and is mainly expressed in the basolateral membrane of renal cells in proximal tubules and plays a critical role in renal elimination of various endogenous anions and clinically important anionic drugs in humans, such as MTX [44, 45].

rs11568626

This SNP is characterized by substitution of C for a T, causing a substitution of an Arg to a His at codon 50 [19]. This change is not considered to be a

substantial chemical change but this residue does lie within the first extracellular loop of this transporter, a highly conserved structure within organic anion and cation transporters families [46]. This loop has previously been shown to be a potential glycosylation site, which is important for SLC22A6 function and cellular localization [46]. Literature describes T homozygotes as associated with a decreased expression of this transporter and, consequently, with reduced influx [46]. Nevertheless, there are no studies investigating the role of this SNP in low-dose MTX therapeutic outcome.

SLC22A8

The SLC22A8, also known as organic anion transporter 3 (OAT3), is encoded by the *SLC22A8*, located on chromosome 11q12.3 and is mainly expressed in the basolateral membrane of renal proximal tubules and can be found in luminal membrane of the choroid plexus in the brain [45].

rs45566039

This SNP is a change of a C to an A at position 445 leading to a substitution of Arg into Ser at a crucial functional location of the protein, with A homozygotes to be associated with loss of transport functionality [47]. To date, there are no studies investigating the role of this SNP in low-dose MTX therapeutic outcome.

SLC22A11

Human SLC22A11, also known as organic anion transporter 4 (OAT4), is encoded by *SLC22A11*, located on chromosome 11q13.1 and acts as a bidirectional transporter in the apical membrane of renal proximal tubular cells [48]. It has been appointed as having a major role in the tubular reabsorption of organic anions and secretion of MTX [48].

rs11231809

This SNP occurs in an intronic region and is characterized by a substitution of a T for an A [19]. It was previously been shown that A homozygotes were

associated with higher clearance of some drugs [49], but there are no studies regarding the functional influence of this SNP on MTX PK and/or PD.

SLC46A1

The SLC46A1 or proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1), is encoded by *SLC46A1*, located on chromosome 17q11.2 and mostly expressed in apical membrane of small-intestine and kidney cells, acting as a key transporter for normal folate absorption and homeostasis [14].

rs2239907

This SNP is characterized by a change of an A into a G at position 928 (A928G) in the 3´UTR region, which is reported as important for mRNA stability and translational efficiency [19, 21]. The impact of this variant in SLC46A1 is currently unknown but Kato *et al.* and de Route *et al.* investigated the role of this SNP in MTX therapeutic outcome, and found no significant associations with clinical response in RA and JIA, respectively [30, 40].

SLCO1A2

Human SLCO1A2, also known as solute carrier family 21 member 3 (SLC21A3) or sodium-independent organic anion-transporting polypeptide 1 (OATP-1), is encoded by *SLCO1A2*, located on chromosome 12p12.1 [50]. It has twelve transmembrane domains and transports a broad spectrum of substrates, including both endogenous and exogenous compounds such as MTX [50, 51]. This bidirectional transporter is present in several tissues including kidney (apical domains of the renal distal tubular cells), where it could be responsible for either reabsorption or secretion of drugs [50].

rs10841795

The T38C SNP occurs in exon 1 and causes a T to C change at position 38, which corresponds to an isoleucine (Ile) to Thr amino acid change at position 13 (Ile13Thr) [19]. Badagnani *et al.* speculated, from *in vitro* results, that *in vivo* C homozygotes should be associated with increased reabsorption of

MTX from distal tubule, leading to increased exposure to MTX and subsequent systemic toxicity [51]. However, there are no studies elucidating the role of this SNP in low-dose MTX PK and/or PD.

SLCO1B1

The SLCO1B1, liver-specific organic anion transporter 1 (LST1) or sodium-independent organic anion-transporting polypeptide 2 (OATP-2), is a bidirectional transporter with twelve transmembrane domains encoded by *SLCO1B1* located on chromosome 12p12 [52]. It is mainly expressed in basolateral membrane of hepatocytes and plays a key role in the uptake of a wide variety of endogenous and exogenous anionic compounds, including MTX [53]. Methotrexate hepatic uptake via SLCO1B1 was demonstrated as the *major* pathway for MTX clearance from systemic circulation [54]. Moreover, SLCO1B1 mRNA has been detected also in other tissues, including small intestinal enterocytes [55].

rs2306283

This common variant in *SLCO1B1* results in a change of A into a G at position 388 and, a change of an asparagine (Asn) into an aspartate (Asp) at position 130 [52]. *In vitro* studies associated G homozygotes with an increased transport activity for MTX [54] and Kato *et al.* showed no associations with MTX clinical response in RA, defined by DAS28 [40].

rs4149056

This SNP is characterized by a change of a T into a C at position 521 leading to an amino acid substitution of valine (Val) into an alanine (Ala) at codon 174 [52]. The C allele was associated with a loss-of-function phenotype, resulting in a decreased SLCO1B1 expression, reduced MTX influx and decreased MTX clearance from systemic circulation [52, 56]. Nevertheless, Kato *et al.* reported no associations regarding this SNP and MTX clinical response [40].

rs56387224

Characterized by a substitution of an A to a G at position 1295 (A1294G) this SNP leads to an amino acid change from Asn to an Asp at codon 432 [52]. In

fact, G homozygotes have been associated with a reduced influx capability mediated by SLCO1B1 [56]. To the best of our knowledge no studies have investigated the role of this SNP in low-dose MTX therapeutic outcome.

SLCO1B3

The SLCO1B3 or liver-specific organic anion transporter 3 (LST3), is encoded by *SLCO1B3* located on chromosome 12p12 [50] and is a member of the organic anion transporting polypeptide (OATP) superfamily responsible for transporting a wide range of xenobiotics including MTX [57]. This is a transmembrane bidirectional transporter localized in the basolateral domain of the plasma membrane in liver [10]. Expression and tissue distribution of SLCO1B3 and SLCO1B1 in liver is similar [53]. Since MTX is transported by both transporters [50] and there are no studies analyzing the contribution of SNPs in *SLCO1B3* for low-dose MTX therapeutic outcome, it seems important to conduct combined studies to address the importance of these SNPs.

FOLR1

The FOLR1, also identified as adult folate-binding protein (FBP), is encoded by *FOLR1*, which is located on chromosome 11q13.4 [58]. It is a variably expressed glycosylated membrane-associated protein present in the outer layer of plasma membrane facilitating unidirectional transport of folic acid, 5-methyltetrahydrofolate and MTX [58]. While folates enter primarily by FOLR1, MTX enters the cell primarily by SLC19A1 mediated transport [7]. Nevertheless, MTX also bind to FOLR1, although with reduced affinity when compared to folic acid [59]. FOLR1 and SLC19A1 are known to be expressed separately or simultaneously but FOLR1 may be up-regulated in a cell with increased metabolic activity [60]. For *FOLR1*, there are no published studies that have addressed this SNPs contribution for low-dose MTX therapeutic outcome. Nevertheless, the search for SNPs that could alter the functionality of this transporter and the analysis of associations between these SNPs and therapeutic outcome are important.

Membrane Efflux Transporters

Methotrexate is known as a substrate of ATP-binding cassette (ABC) efflux transporters which include: ABCB1, ABCC1, ABCC2, ABCC3, ABCC4 and ABCG2. The summary of literature analyzing the effect of SNPs in genes of low-dose MTX efflux transporters on therapeutic outcome is presented in Table 3.

ABCB1

The ABCB1, also identified as multidrug resistance protein 1 (MDR1) or P-glycoprotein 1 (P-gp), is encoded by *ABCB1* located on chromosome 7q21 [61]. This energy-dependent unidirectional transporter, expressed in the apical membrane of many epithelial and endothelial cells, is a transmembrane protein with twelve regions that plays an important role in drug absorption and distribution within the body [41, 61]. Substrates include a variety of drugs including MTX [62]. ABCB1 is also expressed in peripheral blood lymphocytes, which was markedly higher in RA patients in whom disease was refractory to treatment than in RA patients with a good clinical response to DMARD therapy [63].

rs1045642

This is one of the most studied SNPs in *ABCB1*, characterized by a substitution of a C to a T at position 3435 (C3435T) leading to a synonymous change of an Ile at codon 1145 [19]. The T homozygotes have been associated with decreased mRNA stability and enzyme expression, and thus, reduced efflux, and with increased intracellular levels of MTX [64]. Pharmacogenetic studies have demonstrated that T homozygotes were associated with better response to MTX [40, 65] and to MTX combined with SSZ [66] in RA and to MTX in monotherapy or combined with other DMARDs in JIA [30]. Nevertheless, Takatori *et al.* associated T homozygotes with non-response to MTX in monotherapy or combined with other DMARDs [36]. Moreover, Kato *et al.* used DAS28 to define the MTX clinical response [40]; Drozdzik *et al.* defined non-response when both tender joint count and swollen joint count improved <20% from baseline and, at least, three of the

following criteria were not met: VAS ≤20 mm, ≥20% improvement in ESR, in physician's global assessment of disease activity, in patient's global assessment of disease activity, and in HAQ [65]; in Pawlik *et al.* study non-response to MTX was defined as three swollen joints, morning stiffness >30 and ESR >25mm/h [66]; and, Takatori *et al.* defined non-response to MTX patients to whom MTX was administered continuously for at least three months and the last maintenance dosage of MTX was >6mg/week or those in whom MTX was substituted with other DMARDs attending to modified HAQ and biochemical findings (such as CRP, ESR and matrix metallopeptidase-3) [36]. Regarding MTX-related toxicity in RA, an increased risk was associated to T homozygotes by Bohanec Grabar *et al.* [33] and to T carriers by Kooloos *et al.* [67], but Plaza-Plaza *et al.* associated T homozygotes with non-toxicity [38]. Nevertheless, Bohanec Grabar *et al.* recorded the ADRs retrospectively from patients' files throughout the course of MTX therapy, ADRs were classified accordingly SOC disorders and considered as mild, moderate and serious [33]; Kooloos *et al.* evaluated the toxicity by counting each reported ADR and its consequences for the patient and treatment, ADRs were spontaneously reported by the patients or were reported as a result of nonspecific questioning on patients' wellbeing by the investigator, by physical examination or laboratory measurements during follow-up [67]; and, for Plaza-Plaza *et al.* MTX toxicity was considered when the patient experienced an increase in liver enzymes (AST, aspartate aminotransferase; and ALT, alanine aminotransferase) to values higher than or equal to three times the upper limit of normal (AST: 1–40 IU/L; ALT: 1–41 IU/L) in the absence of previous enzyme elevations, positive viral hepatitis markers, or alcohol consumption, other ADR experiences were also collected and each ADR was described by its duration, frequency, severity, cause, and specific therapy requirements [38]. Controversially, there are several studies that show no associations with either clinical response and/or toxicity occurrence [33, 36, 41, 67, 68].

rs1128503

This SNP results in a T to C transition at nucleotide 1236 in exon 13 of *ABCB1* and results in a synonymous change (GGT glycine, GGC glycine) at residue 412 in a cytoplasmic loop of ABCB1 [69]. Literature bears no consensus with concern to the effect of this silent SNP in transporter function and, thus, more studies are needed to elucidate this [69]. In accordance to the influence of this SNP in MTX therapeutic outcome, Ranganathan *et al.* associated T homozygotes with increased risk for toxicity in RA patients [68], while other studies did not present statistically significant associations regarding the clinical response to MTX [30, 40]. In Ranganathan *et al.* study, MTX toxicity information was collected retrospectively in specifically designed forms on nine individual disorders (alopecia, GI disorders, hepatotoxicity - elevation of AST or ALT above the upper limit of normal, leukopenia, pulmonary disorders, stomatitis, central nervous system (CNS) disorders, post-dosing reactions and rash), and attending to overall toxicity, which included patients who had experienced one or more of the individual toxicities [68].

rs2032582

This SNP consists in a transversion of a G to a T or A at position 2677 in exon 21 (G2677A/T) corresponding to a replacement of Ala by Thr or Ser in position 899 on the intracellular side of ABCB1, which results in a change from a lipophilic to a hydrophilic residue [70]. A/T alleles have been described as correlated with decreased protein expression, and thus, reduced efflux capability [70]. Considering the studies analyzing the impact of this SNP in MTX therapeutic outcome, Kato *et al.* revealed a statistically significant association of A/T alleles with better response to MTX in RA patients [40]. Nevertheless, other studies did not show associations with low-dose MTX therapeutic outcome [30, 33]. Since this SNP was described as being in linkage disequilibrium with C3435T (rs1045642) [70], haplotype analysis should be performed in order to evaluate the repercussions on ABCB1 expression level, transport function and MTX therapeutic outcome.

ABCC1

The ABCC1 or multidrug resistance-associated protein 1 (MRP1), is encoded by *ABCC1* located on chromosome 16p13 [19]. It is a polytopic membrane protein present in basolateral plasma membranes in all intestinal regions constituted by seventeen transmembrane regions [61, 71]. It was reported as being expressed on CD3-positive T cells in lymphocytic aggregates and in less extends in RA synovial tissue macrophages in the intimal lining layer and the synovial sublining and on endothelial cells [72]. Moreover, it has been described as a transporter of a number of different drugs including MTX [73].

rs35592

This SNP is characterized by a substitution of a T to a C in an intron region of *ABCC1*. Its impact in transporter function is unknown and studies are needed to address this issue. Besides that, Warren *et al.* demonstrated a significant association of C carriers with non-response to MTX in psoriasis, but no significant association was observed for MTX-related toxicity [35]. Other studies did not show any association of this polymorphism with low-dose MTX therapeutic outcome [30, 41].

rs246240

The rs246240 polymorphism consists in a substitution of an A to a G in an intronic region of *ABCC1* [19]. Similarly to the previous SNP, the impact of this SNP in ABCC1 function is unknown and further studies are necessary. Nevertheless, Warren *et al.* studied the influence of this SNP in MTX therapeutic outcome in psoriasis and associated G carriers with decreased MTX-related toxicity but no significant association was observed regarding clinical response [35].

rs2074087

This SNP denotes a substitution of a C to a G in an intronic region (*ABCC1* IVS 18-30C>G) [19]. Similarly to the previous SNP, the functional impact is unknown. Nevertheless, Ranganathan *et al.* demonstrated no significant associations of this SNP with MTX-related toxicity [68].

rs2230671

The rs2230671 consists in a substitution at region 4002 of a G to an A that leads to a synonymous change of a Ser at codon 1334 (Ser1334Ser) [19]. Literature describes G homozygotes as having increased mRNA levels in peripheral blood cells and, thus, possibly increased MTX efflux [74]. However, no associations were observed in accordance to MTX-related toxicity [68].

rs3784864

This SNP is characterized by a substitution of a G to an A in an intronic region [19]. To the best of our knowledge, its functional impact in ABCC1 is unknown. With concern to this SNP impact in therapeutic outcome, Warren *et al.* associated A carriers with decreased risk for MTX-related toxicity in psoriasis but no associations were observed in accordance to clinical response [35].

ABCC2

The ABCC2, encoded by *ABCC2* located on chromosome 10q24, is a well-known canalicular multispecific organic anion transporter 1 or multidrug resistance-associated protein 2 (MRP2) [61]. It is present in apical (brush border) membranes of hepatocytes and epithelial cells of the small intestine and kidneys [48, 61]. This protein is an anionic conjugate efflux pump, constituted by seventeen transmembrane regions, that plays a role in the excretion of both endogenous and exogenous compounds, and influences the PK of many drugs such as MTX [75]. For the plasma PK of 7-OH-MTX after MTX administration, ABCC2 seem to be the main determinant in the elimination of MTX and its metabolite 7-OH-MTX [75].

rs717620

The rs717620 is a SNP characterized by a substitution of a G to an A in the 5´UTR [19]. Studies reporting the influence of this SNP in transporter function have associated A homozygotes with increased clearance [76]. Nevertheless, studies evaluating the impact of this polymorphism in low-

dose MTX therapeutic outcome have not shown any difference regarding clinical response [30, 40] or toxicity [68].

rs2273697

This SNP consists in a substitution of a G to an A at position 1249, which corresponds to a nonsynonymous change of a Val to an Ile at codon 417 [19]. Literature describes the effect of A homozygotes as conditioning an *in vitro* increased efflux [77]. Considering pharmacogenetic studies, Ranganathan *et al.* associated A homozygotes with increased risk for MTX-related GI disorders [68]. Nevertheless, other studies, both in RA and using DAS28 to define the clinical response to MTX, did not demonstrate associations of this SNP with low-dose MTX therapeutic outcome [40, 41]. Additionally, Ranganathan *et al.* collected toxicity information retrospectively and evaluated the influence in nine individual toxicities and in overall toxicity [68]; and, Stamp *et al.* applied a standardized questionnaire related to common MTX ADRs within the month preceding study entry was administered and, ADRs were grouped broadly into the follow categories: GI, CNS and others [41].

rs4148396

This SNP is characterized by a change of a T to a C in an intronic region (*ABCC2* IVS 23+56T>C) [19]. To the best of our knowledge there are not studies regarding the functional analysis of this polymorphism in ABCC2. However, studies analyzing its impact in low-dose MTX therapeutic outcome associated C homozygotes with increased risk for MTX-related skin and subcutaneous tissue disorders [68], GI disorders and overall toxicity [41]. Regarding clinical response, no associations were reported by Rotte *et al.* in JIA [30] and by Stamp *et al* in RA [41].

rs7080681

The rs7080681 SNP is characterized by an alteration of a G to an A at position 1058 (*ABCC2* G1058A) leading to a nonsynonymous amino acid substitution from Arg to His substitution at position 353 [19]. The impact of this SNP in ABCC2 function is currently unknown. Regarding pharmacogenetic studies, Ranganathan *et al.* observed that A homozygotes

were associated with an increased risk for MTX-related hepatobiliary disorders [68].

ABCC3

The ABCC3, also known as canalicular multispecific organic anion transporter 2 or multidrug resistance-associated protein 3 (MRP3), is encoded by *ABCC3* located on chromosome 17q22 [19]. It is constituted by seventeen transmembrane regions [61] and is found in basolateral plasma membranes of the liver and small intestine increasing MTX and 7-OH-MTX systemic levels and allowing an alternative route of elimination via the urine [78].

rs4793665

This SNP causes an alteration of a C to a T at 5´UTR region (*ABCC3* C211T) [19]. Literature associated T homozygotes for this SNP with lower ABCC3 transcript levels and a trend toward lower protein expression in human liver [79]. Considering pharmacogenetic studies, de Rotte *et al.* revealed that T homozygotes were associated with better response to MTX in JIA [30].

ABCC4

The ABCC4, also known as multidrug resistance protein 4 (MRP4), encoded by *ABCC4* and located on chromosome 13q32.1 [19], is ubiquitously expressed, with a high expression in kidney proximal tubules and in liver [48]. This transporter membrane location is apical in most tissues, but in hepatocytes is basolateral [10] and, consequently, can lead to higher systemic levels and/or to increased renal clearance. The ABCC4 has been described as implicated in the transport of several drugs such MTX, as well as endogenous molecules including folates [80]. Because of this, and since there are no studies analyzing the contribution of SNPs in *ABCC4* for low-dose MTX therapeutic outcome, it seems important to conduct studies concerning this issue.

ABCG2

The ABCG2, also known as multidrug resistance efflux transport ATP-binding or breast cancer resistance protein (BCRP), is expressed by *ABCG2* located on chromosome 4q22 [61]. This transporter has six transmembrane regions and is present in apical membranes of several tissues, including hepatocytes, intestine and kidneys [41, 48, 61], also in synovial tissue macrophages, at the intimal lining layer and synovial sublining, and on endothelial cells in RA patients [72]. Higher ABCG2 expression levels were associated with the persistence of infiltrated synovial tissue macrophages after treatment with MTX and a diminished response to DMARD therapy [72]. It seems that, when ABCC2 is absent, ABCG2 plays a significant role in the elimination of MTX and 7-OH-MTX [116]. Moreover, ABCG2 seems to transport not only MTX but also MTXPGs, particularly those with two and three glutamic acid residues [81].

rs2231142

This SNP is characterized by a change of a C to an A at position 421 (C421A), which corresponds to a substitution of a glutamine (Gln) to a lysine (Lys) at codon 141 [19]. Functional studies have associated A homozygotes with reduced efflux capability [82] and pharmacogenetic studies reported A homozygotes as associated with increased risk for MTX-related overall toxicity, but no associations were observed regarding clinical response to MTX [40, 41].

rs13120400

This polymorphism is characterized by a substitution of a T to a C in an intron region [19]. To the best of our knowledge there are no studies analyzing the functional impact of this SNP in ABCG2. Nevertheless, in a large population with psoriasis, Warren *et al.* associated C carriers with better response to MTX but no associations were observed regarding MTX-related toxicity [35].

rs17731538

This SNP is characterized by a change of a G to an A in an intronic region [19]. Similarly to the previous SNP, there are no studies regarding the

functional impact of this SNP in ABCG2. Nevertheless, Warren *et al.* associated A carriers with non-response to MTX [35], while Stamp *et al.* demonstrated no associations with either MTX clinical response or toxicity occurrence [41].

FUTURE PERSPECTIVES

There are many inconsistent results regarding the associations of SNPs in genes encoding for MTX membrane transport proteins (influx and/or efflux) with low-dose MTX therapeutic outcome. These somehow discrepant results could be explained by differences observed in: study design, sample sizes, ethnicity, disease models, treatment regiments, as well as in therapeutic outcome endpoints definition, such clinical response and toxicity, leading to difficult comparisons and conclusions. In order to reduce the inconsistency of results and provide more solid evidences, ideal future studies should consider several factors that can lead to MTX PK and/or PD reduced variability as follow: 1) existence of different MTX transporters, granting different flux directions, expressed simultaneously, in same cells and/or in different tissues; 2) presence of endogenous substances (e.g. folates) or other drugs that could interact with MTX and/or with MTX transporters; 3) pH differences that could alter MTX solubility and, thus, dissolution (crucial for absorption when MTX is administered by *per os* route); 4) alterations in polyglutamation processes, and thus, in MTX cellular retention, possibly by changing the functionality of FPGS and GGH; 5) existence of other polymorphisms that could alter transporter levels, function or expression; and 6) presence of polymorphisms in genes encoding for proteins involved in MTX action mechanism pathways [83]. Genome-wide association approaches and/or next generation sequencing could be applied to reveal additional genes/polymorphisms associated with MTX treatment outcome. In fact, and to the best of our knowledge, there are several unknown SNPs concerning to functional impact in transporter (*SLC16A7* rs10877333; *SLC19A1* rs7499, rs2838956 and rs3788200; *SLC22A11* rs11231809; *SLC46A1* rs2239907; *ABCC1* rs35592, rs246240, rs2074087 and rs3784864;

ABCC2 rs4148396 and rs7080681; and, *ABCG2* rs13120400 and rs17731538), concerning to influence on low-dose MTX therapeutic outcome (*SLC19A1* rs3788200; *SLC22A6* rs11568626; *SLC22A8* rs45566039; *SLC22A11* rs11231809; *SLCO1A2* rs10841795; and, *SLCO1B1* rs56387224) and unknown importance of genes/possible SNPs (*SLCO1B3*, *FOLR1* and *ABCC4*). Moreover, to improve the detection of more genetic factors responsible for interpatient variability in low-dose MTX therapeutic outcome, some important issues should be considered in future studies. Thus, studies should follow validated guidelines to define therapeutic outcomes, in order to homogenise definitions; study design should be prospective, due to the fewer potential sources of bias and confounding factors when compared to retrospective studies; studies should be larger and multicentric (a large sample size can greatly improve statistical power and multicentre international collaborations can rapidly generate large samples); and, studies should undertake genotype and haplotype based approaches, in order to clarify the influence in PK parameters and in predicting MTX therapeutic outcome.

CONCLUSIONS

Candidate polymorphisms in genes encoding proteins involved in MTX carrier-mediated transport systems have been successfully identified. Therefore, over the past decade, numerous pharmacogenetic studies have been undertaken to establish possible associations between these polymorphisms and MTX therapeutic outcome. Despite different results among published studies, there are not many doubts that this area is very promising for tailoring therapeutic outcome. Additionally, and considering the intra and inter variability among patients, there is an increasing need to establish strong basis for personalized medicine. Yet, and despite the recent advances in pharmacogenomics, a fairly long path has to be covered to reach to personalized medicine and to find the optimal genetic biomarkers of low-dose MTX therapeutic outcome.

EXECUTIVE SUMMARY

Introduction

- **Low-dose methotrexate (MTX) is used in several rheumatic and dermatological diseases.**
- **Significant intra and inter variability to MTX treatment is observed among patients.**

Methotrexate Pharmacogenomics: The Role of MTX-Transporters

- **Low-dose MTX membrane transport proteins (influx and/or efflux) can be major determinants of MTX therapeutic outcome.**
- **Several pharmacogenetic studies have been undertaken to decipher associations between SNPs in genes encoding for MTX membrane transport proteins and MTX therapeutic outcome but, the majority of these findings are still inconclusive and inconsistent.**

Conclusions & Future Perspectives

- **Methotrexate therapeutic outcome prediction will offer a powerful tool for the translational role of into clinical practice and will be essential to sustain a breakthrough in the field of personalized medicine.**
- **Developing effective biomarkers to help clinicians in the prediction of drug responses in routine clinical practice remains a big challenge.**

ABBREVIATIONS LIST

A: adenine; aa: amino acid; ABCB1: ATP-binding cassette sub-family B member 1; ABCC1: ATP-binding cassette sub-family C member 1; ABCC2: ATP-binding cassette sub-family C member 2; ABCC3: ATP-binding cassette sub-family C member 3; ABCC4: ATP-binding cassette sub-family C member 4; ABCG2: ATP-binding cassette sub-family G member 2; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reaction; Ala: alanine; ALT: alanine aminotransferase; Arg: arginine; AST: aspartate aminotransferase; AUC: area under the curve; BCRP: breast cancer resistance protein; C: cytosine; CD: cluster of differentiation; Chr: chromosome; CNS: central nervous system; CRP: C-reactive protein; CTCAE: Common Terminology Criteria for Adverse Events; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; EULAR: European League Against

Rheumatism; FBP: folate binding protein; FOLR1: folate receptor alpha; FOLT: folate transporter; FPGS: folylpolyglutamate synthetase; G: guanine; GI: gastrointestinal; GGH: gamma-glutamyl hydrolase; Gln: glutamine; Gly: glycine; HAQ: Health Assessment Questionnaire; HCP1: heme carrier protein 1; HCQ; hydroxychloroquine; His: histidine; ID: identification; Ile: isoleucine; IU: international units; JIA: Juvenile Idiopathic Arthritis; LD: linkage disequilibrium; Lys: lysine; LST: liver-specific transporter; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MMP-3: matrix metallopeptidase 3; mRNA: messenger ribonucleic acid; MRP: multidrug resistance-associated protein; MTX: methotrexate; MTXPG: methotrexate polyglutamate; n.a.: not assigned; NA: not analyzed; NS: not-statistically significant; OAT: organic anion transporter; OATP: organic anion transporting polypeptide; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; PD: pharmacodynamics; PK: pharmacokinetics; RA: rheumatoid arthritis; Ref: reference; RFC1: reduced folate carrier 1; S: statistically significant; Ser: serine; SLC16A7: solute carrier family 16 member 7; SLC19A1: solute carrier family 19 member 1; SLC22A6: solute carrier family 22 member 6; SLC22A8: solute carrier family 22 member 8; SLC22A11: solute carrier family 22 member 11; SCL46A1: solute carrier family 46 member 1; SLCO1B1: solute carrier organic anion transporter family member 1B1; SLCO1B3: solute carrier organic anion transporter family member 1B3; SLCO1A2: solute carrier organic anion transporter family member 1A2; SNP: single nucleotide polymorphism; SOC: System Organ Class; SSZ: sulfasalazine; T: thymine; Thr: threonine; TNF-α: tumor necrosis factor-alpha; UTR: untranslated region; UK: United Kingdom; US: United States; Val: valine; VAS: visual analog scale.

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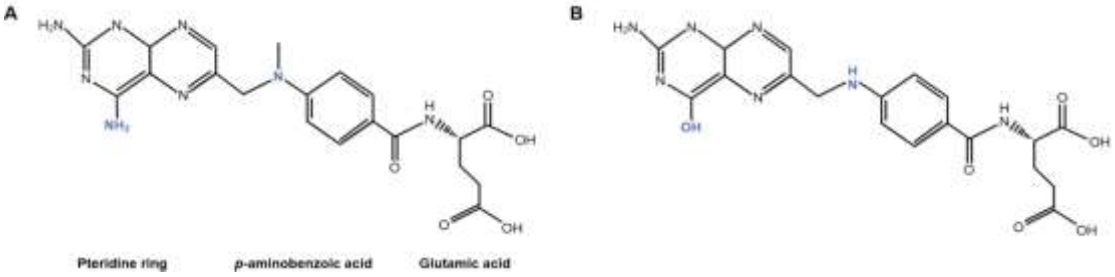


Figure 1. Chemical structures of Methotrexate (A) and Folic Acid (B). Methotrexate is composed by a pteridine ring, *p*-aminobenzoic acid plus glutamic acid. Methotrexate and Folic Acid chemical structures are similar, differing in the pteridine ring (by substitution of a hydroxyl group for an amine) and on the 10(th) nitrogen of *p*-aminobenzoic acid (by addition of a methyl group).

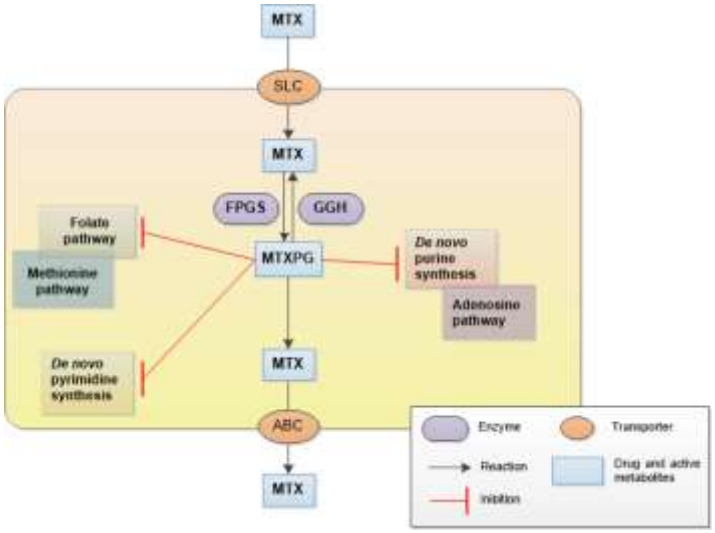


Figure 2. Methotrexate mechanism of action. Left panel represents the intervention of MTX in *de novo* pyrimidine synthesis, folate and methionine pathways. Right panel shows the effect of MTX in *de novo* purine synthesis and adenosine pathway. These action mechanisms contribute to MTX antiproliferative and anti-inflammatory effects.

ABC: adenosine triphosphate-binding cassette; FPGS: folylpolyglutamate synthetase; GGH: gamma-glutamyl hydrolase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SLC: solute carrier.

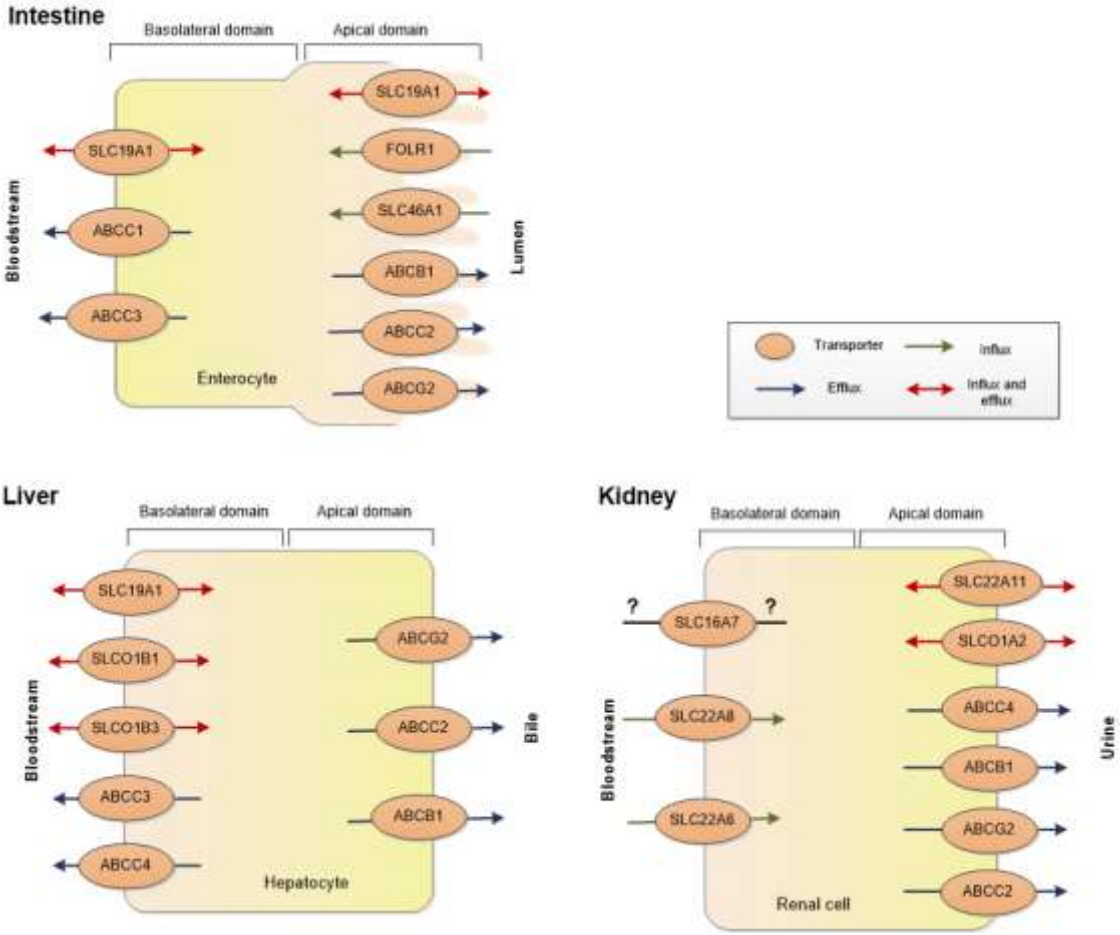


Figure 3. Methotrexate transporters cells location and representation of their influx and/or efflux function.

ABCB1: ATP-binding cassette sub-family B member 1; ABCC1: ATP-binding cassette sub-family C member 1; ABCC2: ATP-binding cassette sub-family C member 2; ABCC3: ATP-binding cassette sub-family C member 3; ABCC4: ATP-binding cassette sub-family C member 4; ABCG2: ATP-binding cassette sub-family G member 2; FOLR1: folate receptor alpha; SLC16A7: solute carrier family 16 member 7; SLC19A1: solute carrier family 19 member 1; SLC22A6: solute carrier family 22 member 6; SLC22A8: solute carrier family 22 member 8; SLC22A11: solute carrier family 22 member 11; SCL46A1: solute carrier family 46 member 1; SLCO1B1: solute carrier organic anion transporter family member 1B1; SLCO1B3: solute carrier organic anion transporter family member 1B3; SLCO1A2: solute carrier organic anion transporter family member 1A2.

Table 1. Characteristics and putative effects of SNPs in low-dose MTX transport function

Genes	Other names	Location	Chr	rs ID	Alleles	Region (aa change)	Putative effects in MTX transport function	Ref.
Solute carriers								
SLC16A7	*MCT2*	Kidney	12	rs3763980	A>T	Exon (Thr445Ser)	T carriers: ↓ influx	[17, 18]
				rs10877333	T>G	Intron	*	[19]
SLC19A1	*RFC1 FOLT*	Ubiquitous	21	rs7499	G>A	3´UTR	*	[20]
				rs1051266	G>A	Exon (Arg27His)	AA: ↑ influx	[24]
				rs1131596	T>C	5´UTR	CC: ↓ influx	[39]
				rs2838956	A>G	Intron	*	[19]
				rs3788200	G>A	Intron	*	[19]
SLC22A6	*OAT1*	Kidney	11	rs11568626	C>T	Exon (Arg50His)	TT: ↓ influx	[44, 46]
SLC22A8	*OAT3*	Kidney	11	rs45566039	C>A	Exon (Arg149Ser)	AA: ↓ influx	[47]
SLC22A11	*OAT4*	Kidney	11	rs11231809	T>A	Intron	AA: ↑ clearance	[49]
SLC46A1	*HCP1 PCFT SLC21A3*	Intestine	17	rs2239907	G>A	3´UTR	*	[19]
SLCO1A2		Kidney	12	rs10841795	T>C	Exon (Ile13Thr)	CC: ↑ influx	[50, 51]
SLCO1B1	*LST1 OATP1B1*	Liver	12	rs2306283	A>G	Exon (Asn130Asp)	GG: ↑ influx	[50, 54]
				rs4149056	T>C	Exon (Val174Ala)	CC: ↓ influx and clearance	[52-54, 56]
				rs56387224	A>G	Exon (Asn432Asp)	GG: ↓ influx	[52]
SLCO1B3	*LST3 OATP1B3*	Liver	12	*	n.a.	n.a.	n.a.	[50]
FOLR1	*FBP FOLR*	Ubiquitous	11	*	n.a.	n.a.	n.a.	[58]
ATP-binding cassette transporters								
ABCB1	*MDR P-GP ABC20 CD243*	Intestine Liver Kidney	7	rs1045642	C>T	Exon (Ile1145Ile)	TT: ↓ efflux	[64]
				rs1128503	C>T	Exon (Gly412Gly)	*	[19]
				rs2032582	G>A/T	Exon (Ala899Ser/Thr)	A/T alleles: ↓ efflux	[70]
ABCC1	*MRP1 ABC29*	Intestine	16	rs35592	T>C	Intron	*	[71]
				rs246240	A>G	Intron	*	[19]
				rs2074087	G>C	Intron	*	[19]
				rs2230671	C>G	Exon (Ser1334Ser)	GG: ↑ efflux	[74]
				rs3784864	G>A	Intron	*	[19]
ABCC2	*MRP2 ABC30*	Intestine Liver Kidney	10	rs717620	G>A	5´UTR	AA: ↑ clearance	[76]
				rs2273697	G>A	Exon (Val417Ile)	AA: ↑ efflux	[77]
				rs4148396	C>T	Intron	*	[19]
				rs7080681	G>A	Exon (Arg353His)	*	[19]
ABCC3	*MRP3 ABC31*	Intestine Liver	17	rs4793665	C>T	5´UTR	TT: ↓ efflux	[79]
ABCC4	*MRP4 ABC32*	Liver Kidney	13	*	n.a.	n.a.	n.a.	[48]
ABCG2	*BCRP ABC15 CD338*	Intestine	4	rs2231142	C>A	Exon (Gln141Lys)	AA: ↓ efflux	[82]
		Liver		rs13120400	T>C	Intron	*	[19]
		Kidney		rs17731538	G>A	Intron	*	[19]

↑: increased; ↓: decreased; *: unknown.
A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; BCRP: breast cancer resistance protein; C: cytosine; CD: cluster of differentiation; Chr: chromosome; FBP: folate binding protein; FOLR1: folate receptor alpha; FOLT: folate transporter; G: guanine; Gln: glutamine; Gly: glycine; HCP1: heme carrier protein 1; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST: liver-specific transporter; MCT2: monocarboxylate transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MTX: methotrexate; n.a.: not assigned; OAT: organic anion transporter; OATP: organic anion transporting polypeptide; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SLC: solute carrier; SNP: single nucleotide polymorphism; T: thymine; Thr: threonine; UTR: untranslated region; Val: valine.

Table 2. Relation of MTX influx transporters SNPs in low-dose MTX therapeutic outcome

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population /Ethnicity	Therapeutic	Judgment criteria¥		Findings
						Clinical Response	Toxicity	
SLC16A7 A>T (rs3763980)								
Moncrieffe *et al.* (2010) [18]	JIA	UK: 123 US: 143	Prospective	UK+US/ Caucasian	MTX	UK: ACRped70 (1a) US: Joints count (1b)	NA	UK/US - T allele: ↑ response
SLC16A7 T>G (rs10877333)								
Moncrieffe *et al.* (2010) [18]	JIA	UK: 123 US: 143	Prospective	UK+US/Ca ucasian	MTX	(1a) (1b)	NA	UK - G allele: ↑ response US - NS
SLC19A1 G>A (rs7499)								
Owen *et al.* (2013) [22]	RA	309	Retrospective	UK/ Caucasian	MTX	Physician statement of inefficacy + failure to reduce ESR and/or CRP by at least 20% (2)	Persistent or serious ADR leading to treatment cessation, resolved after treatment cessation and, in the case of GI disorders, recur after MTX re-challenge (6)	A allele: ↓ response NS for toxicity
SLC19A1 G>A (rs1051266)								
Dervieux *et al.* (2004) [26]	RA	108	Retrospective	US/-	MTX	Physician's global assessment of disease VAS score	NA	AA: ↑ response
Dervieux *et al.* (2006) [37]	RA	48	Prospective	US/-	MTX or MTX+HQC or MTX+SSZ	DAS28-CRP or DAS28-ESR (EULAR criteria - good responders were patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment)	Patients report by questionnaire, physician examination and laboratory measurements ADRs graded as mild, moderate, or severe.	NS for response NS for toxicity
Takatori *et al.* (2006) [36]	RA	124	Retrospective	Japan/ Asian	MTX or DMARDs	MTX+other	Last maintenance dosage of MTX >6mg/week or MTX substitution attending to mHAQ, CRP, ESR and MMP-3	Physician examination and laboratory measurements	NS for response NS for toxicity
Chatzikyriakidou *et al.* (2007) [39]	RA	106	Retrospective	Greece/ Caucasian	MTX or DMARDs	MTX+other	DAS28-ESR (EULAR criteria - good responders were patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment), ACR 20% and ACR 50% (3)	n.a.	NS for response NS for toxicity
Drozdzik *et al.* (2007) [27]	RA	174	Prospective	Poland/ Caucasian	MTX	ACR 20%	ALT and AST levels above 100 IU	AA: ↑ response NS for toxicity

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. ¥ Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under the curve; AST: aspartate aminotransferase; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; CTCAE: Common Terminology Criteria for Adverse Events; DAS28: disease activity in 28 joints; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; G: guanine; GI: gastrointestinal; HQC: hydroxychloroquine; IU: international units; JIA: Juvenile Idiopathic Arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; n.a.: not available; NA: not analyzed; NS: not statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SLC: solute carrier; SLCO: solute carrier organic anion; SNP: single nucleotide polymorphism; SSZ: sulfasalazine; T: thymine; TNF-α: tumor necrosis factor alpha; UK: United Kingdom; US: United States; VAS: visual analog scale.

Table 2. Relation of MTX influx transporters SNPs in low-dose MTX therapeutic outcome (cont.)

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population/ Ethnicity	Therapeutic	Judgment criteria¥		Findings
						Clinical Response	Toxicity	
SLC19A1 G>A (rs1051266)								
Bohanec Grabar *et al.* (2008) [33]	RA	213	Retrospective	Slovenia/ Caucasian	MTX or DMARDs	MTX+other	DAS28-ESR criteria - (EULAR good responders patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment)	Patients' files ADRs classified in SOC disorders and graded as mild, moderate and serious	NS for response A allele: ↓ toxicity
James *et al.* (2008) [29]	RA	98	Prospective	England/ Caucasian	MTX+SSZ+HQC		DAS28-ESR criteria - (EULAR good responders patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment; clinical remission at 12 months was defined as a DAS28 <2.6)	NA	A allele: ↑ response
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX		Psoriasis area and severity index	Clinical records ADRs classified in SOC disorders leading to MTX cessation or requiring treatment	NS for response A,A: ↑ toxicity
Hayashi *et al.* (2009) [28]	RA	87	Prospective	Japan/ Asian	MTX		AUC of CRP levels	NA	A,A: ↑ response
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/ Caucasian	MTX or DMARDs	MTX+other	DAS28-ESR criteria - (EULAR good responders patients with a reduction in DAS28 >1.2 during the treatment and low disease activity, defined as a DAS28 ≤3.2)	Questionnaire ADRs classified in categories (GI, CNS and others)	NS for response NS for toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands /Caucasian	MTX or DMARDs	MTX+other	ACRped70 and/or anti-TNF-□ use (4)	NA	A,A: ↓ response
Bohanec Grabar (2012) [34]	RA	212	Retrospective	Slovenia/ Caucasian	MTX or DMARDs	MTX+other	NA	ADRs classified in SOC disorders and graded as mild, moderate and serious (7)	A carrier: ↓ toxicity

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. ¥ Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under the curve; AST: aspartate aminotransferase; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; CTCAE: Common Terminology Criteria for Adverse Events; DAS28: disease activity in 28 joints; DMARD: disease modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; G: guanine; GI: gastrointestinal; HQC: hydroxychloroquine; IU: international units; JIA: juvenile idiopathic Arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; n.a.: not available; NA: not analyzed; NS: not statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SLC: solute carrier; SLCO: solute carrier organic anion; SNP: single nucleotide polymorphism; SSZ: sulfasalazine; T: thymine; TNF-α: tumor necrosis factor alpha; UK: United Kingdom; US: United States; VAS: visual analog scale.

Table 2. Relation of MTX influx transporters SNPs in low-dose MTX therapeutic outcome (cont.)

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population/ Ethnicity	Therapeutic	Judgment criteria¥		Findings
						Clinical Response	Toxicity	
SLC19A1 G>A (rs1051266)								
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/ Asian	MTX	DAS28-CRP (EULAR criteria – good responders were patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment) (5)	NA	NS for response
Plaza-Plaza *et al.* (2012) [38]	RA	52	Prospective	Spain/ Caucasian	MTX	NA	AST and ALT >3 times than reference limit, in the absence of potential false positive results	NS for toxicity
Owen *et al.* (2013) [22]	RA	309	Retrospective	UK/ Caucasian	MTX	(2)	(6)	NS for response NS for toxicity
Lima *et al.* (2014) [32]	RA	233	Retrospective	Portugal/ Caucasian	MTX or DMARDs	MTX+other NA	Patients report, physician examination and laboratory measurements ADR classified in SOC disorders (CTCAE)	AA: ↓ toxicity
SLC19A1 T>C (rs1131596)								
Chatzikyriakidou *et al.* (2007) [39]	RA	106	Retrospective	Greece/ Caucasian	MTX or DMARDs	MTX+other (3)	n.a.	NS for response NS for toxicity
Bohanec Grabar *et al.* (2012) [34]	RA	212	Retrospective	Slovenia/ Caucasian	MTX or DMARDs	MTX+other NA	(7)	C carrier: ↓ toxicity
SLC19A1 A>G (rs2838956)								
Bohanec Grabar *et al.* (2012) [34]	RA	212	Retrospective	Slovenia/ Caucasian	MTX or DMARDs	MTX+other NA	(7)	NS for response
Owen *et al.* (2013) [22]	RA	309	Retrospective	UK/ Caucasian	MTX	(2)	(6)	G allele: ↓ response NS for toxicity
SLC46A1 A>G (rs2239907)								
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands /Caucasian	MTX or DMARDs	MTX+other (4)	NA	NS for response
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/ Asian	MTX	(5)	NA	NS for response
SLCO1B1 A>G (rs2306283)								
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/ Asian	MTX	(5)	NA	NS for response
SLCO1B1 T>C (rs4149056)								
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/ Asian	MTX	(5)	NA	NS for response

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. ¥ Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under the curve; AST: aspartate aminotransferase; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; CTCAE: Common Terminology Criteria for Adverse Events; DAS28: disease activity in 28 joints; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; G: guanine; GI: gastrointestinal; HOC: hydroxychloroquine; IU: international units; JIA: Juvenile idiopathic Arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; n.a.: not available; NA: not analyzed; NS: not statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SLC: solute carrier; SLCO: solute carrier organic anion; SNP: single nucleotide polymorphism; SSZ: sulfasalazine; T: thymine; TNF-α: tumor necrosis factor alpha; UK: United Kingdom; US: United States; VAS: visual analog scale.

Table 3. Relation of MTX efflux transporters SNPs in low-dose MTX therapeutic outcome

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population/Ethnicity	Therapeutic	Judgment criteria¥		Findings
						Clinical Response	Toxicity	
ABCB1 C>T (rs1045642)								
Pawlik *et al.* (2004) [66]	RA	92	Retrospective	Poland/Caucasian	MTX	Swollen joints ≥3, morning stiffness >30 and ESR >25mm/h	NA	TT: ↑ response
Drozdzik *et al.* (2006) [65]	RA	174	Prospective	Poland/Caucasian	MTX + SSZ	ACR 20%	NA	TT: ↑ response
Takatori *et al.* (2006) [36]	RA	124	Retrospective	Japan/Asian	MTX or MTX+other DMARDs	Last maintenance dosage of MTX >6mg/week or MTX substitution attending to mHAQ, CRP, ESR and MMP-3	Physician examination and/or laboratory measurements	TT: ↓ response NS for toxicity
Ranganathan *et al.* (2007) [68]	RA	29+62	Retrospective	US/African American + Caucasian	MTX or MTX+other DMARDs	NA	9 individual disorders and overall toxicity (patients with ≥1 ADR) (6)	NS for toxicity
Bohanec Grabar *et al.* (2008) [33]	RA	213	Retrospective	Slovenia/Caucasian	MTX or MTX+other DMARDs	DAS28-ESR (EULAR criteria - good responders were patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment) (1)	Patients' files ADRs classified in SOC disorders and considered as mild, moderate and serious (7)	NS for response TT: ↑ toxicity
Kooloos *et al.* (2010) [67]	RA	205	Retrospective	Netherlands/Caucasian	MTX	DAS-ESR (Ritchie articular index - good responders were patients with DAS ≤2.4)	Patients' report, questionnaire, physician examination or laboratory measurements	NS for response T allele: ↑ toxicity
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/Caucasian	MTX or MTX+other DMARDs	DAS28-ESR (EULAR criteria - good responders were patients with a reduction in DAS28 >1.2 during the treatment and low disease activity, defined as a DAS28 ≤3.2) (2)	Questionnaire ADRs classified in categories (GI, CNS and others) (8)	NS for response NS for toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	ACRped70 and/or anti-TNF-□ use (3)	NA	TT: ↑ response
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	DAS28-CRP (EULAR criteria - good responders were patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment). (4)	NA	TT: ↑ response
Plaza-Plaza *et al.* (2012) [38]	RA	52	Prospective	Spain/Caucasian	MTX	NA	AST and ALT >3 times than reference limit, in the absence of potential false positive results	T allele: ↓ toxicity

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. ¥ Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under curve; AST: aspartate aminotransferase; ABC: ATP-binding cassette; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; DAS28: disease activity in 28 joints; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; G: guanine; GI: gastrointestinal; HQC: hydroxychloroquine; JIA: juvenile idiopathic arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; NA: not analyzed; NS: not-statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SSZ: sulfasalazine; T: thymine; TNF-α : tumor necrosis factor alpha; UK: United Kingdom; US: United States.

Table 3. Relation of MTX efflux transporters SNPs in low-dose MTX therapeutic outcome (cont.)

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population/Ethnicity	Therapeutic	Judgment criteria†			Findings
						Clinical Response		Toxicity	
ABCB1 C>T (rs1128503)									
Ranganathan *et al.* (2007) [68]	RA	18	Retrospective	US/African American	MTX or MTX+other DMARDs	NA	(6)		TT: ↑ toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA		NS for response
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	(4)	NA		NS for response
ABCB1 G>A/T (rs2032582)									
Bohanec Grabar *et al.* (2008) [33]	RA	213	Retrospective	Slovenia/Caucasian	MTX or MTX+other DMARDs	(1)	(7)		NS for response NS for toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA		NS for response
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	(4)	NA		AA/AT/TT: ↑ response
ABCC1 T>C (rs35592)									
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX	Psoriasis area and severity index (5)	Clinical records ADRs classified disorders leading cessation or treatment (9)	in SOC to MTX requiring	C carriers: ↓ response NS for toxicity
Stamp *et al.* (2010) [41]	RA	191		New Zealand/Caucasian	MTX or MTX+other DMARDs	(2)	(8)		NS for response NS for toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA		NS for response
ABCC1 A>G (rs246240)									
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX	(5)	(9)		NS for response G carriers: ↓ toxicity
ABCC1 C>G (rs2074087)									
Ranganathan *et al.* (2007) [68]	RA	29+62	Retrospective	US/African American + Caucasian	MTX or MTX+other DMARDs	NA	(6)		NS for toxicity
ABCC1 G>A (rs2230671)									
Ranganathan *et al.* (2007) [68]	RA	29+62	Retrospective	US/African American + Caucasian	MTX or MTX+other DMARDs	NA	(6)		NS for toxicity

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. †Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under curve; AST: aspartate aminotransferase; ABC: ATP-binding cassette; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; DAS28: disease activity in 28 joints; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; G: guanine; GI: gastrointestinal; HQC: hydroxychloroquine; JIA: juvenile idiopathic arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; NA: not analyzed; NS: not-statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SSZ: sulfasalazine; T: thymine; TNF-α: tumor necrosis factor alpha; UK: United Kingdom; US: United States.

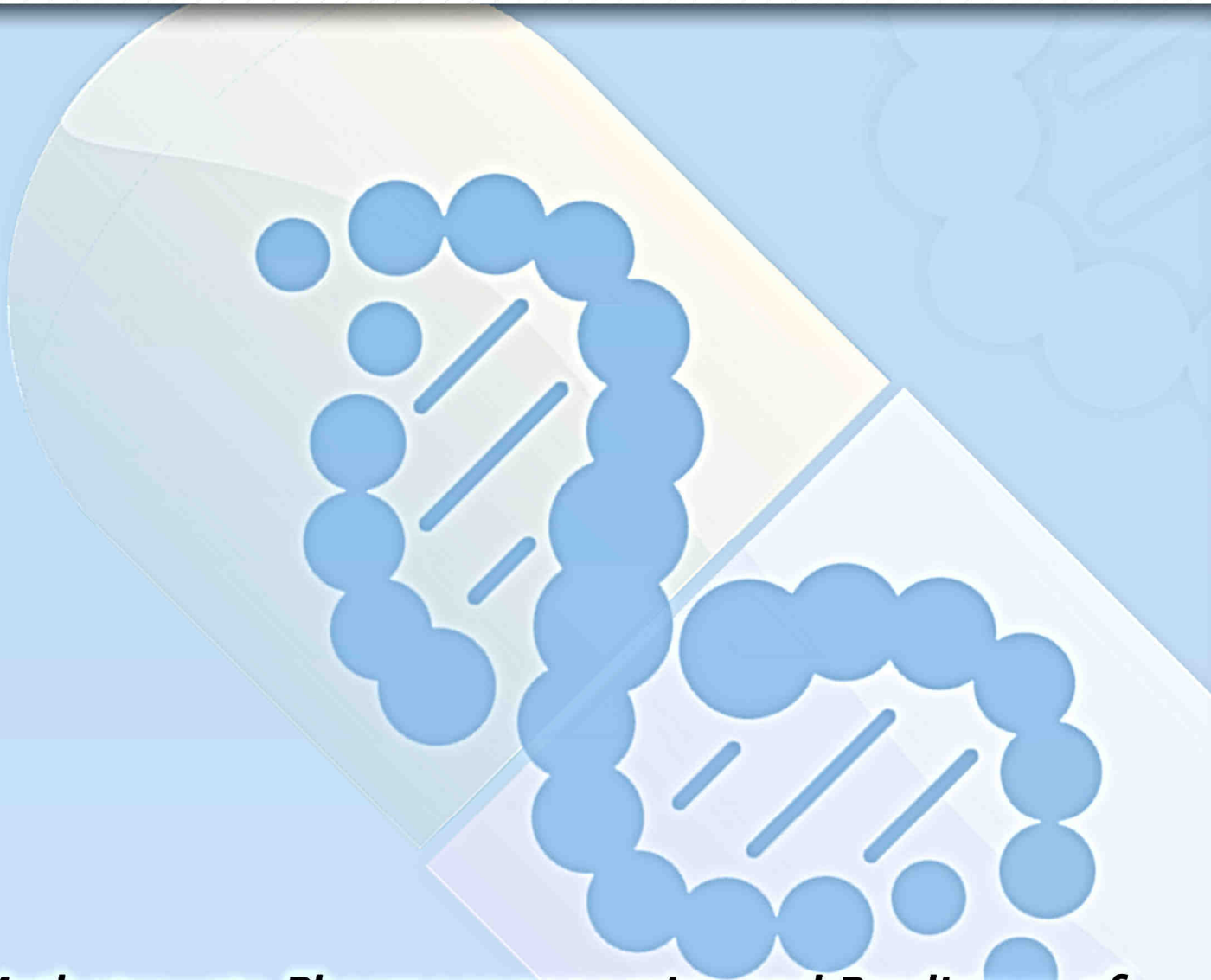
Table 3. Relation of MTX efflux transporters SNPs in low-dose MTX therapeutic outcome (cont.)

Author (year) [Ref.]	Disease Model	Patient's number	Study design*	Population/Ethnicity	Therapeutic	Judgment criteria†		Findings
						Clinical Response	Toxicity	
ABCC1 G>A (rs3784864)								
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX	(5)	(9)	NS for response A carriers: ↓ toxicity
ABCC2 C>T (rs717620)								
Ranganathan *et al.* (2007) [68]	RA	29+62	Retrospective	US/African American + Caucasian	MTX or MTX+other DMARDs	NA	(6)	NS for toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA	NS for response
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	(4)	NA	NS for response
ABCC2 G>A (rs2273697)								
Ranganathan *et al.* (2007) [68]	RA	29	Retrospective	US/African American	MTX or MTX+other DMARDs	NA	(6)	AA: ↑ toxicity
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/Caucasian	MTX or MTX+other DMARDs	(2)	(8)	NS for response NS for toxicity
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	(4)	NA	NS for response
ABCC2 T>C (rs4148396)								
Ranganathan *et al.* (2007) [68]	RA	41	Retrospective	US/Caucasian	MTX or MTX+other DMARDs	NA	(6)	CC: ↑ toxicity
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/Caucasian	MTX or MTX+other DMARDs	(2)	(8)	NS for response CC: ↑ toxicity
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA	NS for response
ABCC2 G>A (rs7080681)								
Ranganathan *et al.* (2007) [68]	RA	25	Retrospective	US/African American	MTX or MTX+other DMARDs	NA	(6)	AA: ↑ toxicity
ABCC3 C>T (rs4793665)								
de Rotte *et al.* (2012) [30]	JIA	287	Retrospective	Netherlands/Caucasian	MTX or MTX+other DMARDs	(3)	NA	TT: ↑ response
ABCG2 C>A (rs2231142)								
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/Caucasian	MTX or MTX+other DMARDs	(2)	(8)	NS for response AA: ↑ toxicity
Kato *et al.* (2012) [40]	RA	55	Retrospective	Japan/Asian	MTX	(4)	NA	NS for response
ABCG2 T>C (rs13120400)								
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX	(5)	(9)	C carriers: ↑ response NS for toxicity
ABCG2 G>A (rs17331538)								
Warren *et al.* (2008) [35]	Psoriasis	374	Retrospective	UK/-	MTX	(5)	(9)	A carriers: ↓ response
Stamp *et al.* (2010) [41]	RA	191	Prospective	New Zealand/Caucasian	MTX or MTX+other DMARDs	(2)	(8)	NS for response NS for toxicity

*The design of all studies was observational. ↑: increased; ↓: decreased; -: not assigned. †Judgment criterion for therapeutic outcome (clinical response and toxicity) for each study is described when the study is first referred and numbers are used thereafter to repeat previously described text.
A: adenine; ACRped70: American College of Rheumatology 70 pediatric criteria; ADR: adverse drug reactions; ALT: alanine aminotransferase; AUC: area under curve; AST: aspartate aminotransferase; ABC: ATP-binding cassette; C: cytosine; CRP: C-reactive protein; CNS: central nervous system; DAS28: disease activity in 28 joints; DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; G: guanine; GI: gastrointestinal; HQC: hydroxychloroquine; JIA: juvenile idiopathic arthritis; mHAQ: modified health assessment questionnaire; MMP-3: matrix metallopeptidase 3; MTX: methotrexate; NA: not analyzed; NS: not-statistically significant; RA: rheumatoid arthritis; Ref.: reference; S: statistically significant; SOC: system organ class; SSZ: sulfasalazine; T: thymine; TNF-α: tumor necrosis factor alpha; UK: United Kingdom; US: United States.

CHAPTER V

SLC19A1 80G ALLELE AS A BIOMARKER OF METHOTREXATE-RELATED GASTROINTESTINAL TOXICITY IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER V. *SLC19A1* 80G ALLELE AS A BIOMARKER OF METHOTREXATE-RELATED GASTROINTESTINAL TOXICITY IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS

This chapter presents an original work that elucidates the clinical relevance of the solute carrier 19 family 1 G80A polymorphism and a set of clinicopathological variables as putative biomarkers of Methotrexate-related toxicity in Portuguese Rheumatoid Arthritis patients. As previously demonstrated, genetic polymorphisms, in genes encoding for Methotrexate membrane transport proteins with functional significance, can be partly responsible for pharmacokinetic and pharmacodynamics interpatient variability. One of the most important Methotrexate transporters is the solute carrier 19 family 1, a cell surface transmembrane protein which gene is polymorphic, being the rs1051266 described in literature as the most implicated in Methotrexate therapeutic outcome.

RESEARCH ARTICLE

SLC19A1 80G allele as a biomarker of methotrexate-related gastrointestinal toxicity in Portuguese rheumatoid arthritis patients

Aim: The aim of our study was to characterize the association of clinicopathological variables and the *SLC19A1/RFC-1* G80A polymorphism in methotrexate (MTX)-related toxicity in Portuguese patients with rheumatoid arthritis. **Patients & methods:** The study included 233 consecutively recruited patients with rheumatoid arthritis under MTX treatment. The *SLC19A1* G80A polymorphism was evaluated by PCR-RFLP. **Results:** Statistical analysis revealed that *SLC19A1* 80G carriers had increased risk of gastrointestinal toxicity (odds ratio [OR]: 2.61, p = 0.019) and that regular folic acid supplementation was associated with both overall and gastrointestinal toxicity protection (OR: 0.15, p < 0.001 and OR: 0.19, p < 0.001, respectively). Multivariate analysis confirmed the association of *SLC19A1* 80G and regular folic acid supplementation to gastrointestinal toxicity (OR: 5.53 and 0.13, respectively). Moreover, a multivariate Cox regression model demonstrated a higher risk of earlier gastrointestinal toxicity in *SLC19A1* 80G carriers (hazard ratio: 3.63, p = 0.002). **Conclusion:** *SLC19A1* G80A genotyping may be a useful tool for dinicians to identify patients at higher risk for developing gastrointestinal toxicity related to MTX treatment.

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KEYWORDS: adverse drug reactions gastrointestinal toxicity methotrexate rheumatoid arthritis *SLC19A1/RFC-1* G80A polymorphism

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Rheumatoid arthritis (RA) is an autoimmune disease that results in a chronic and systemic inflammatory disorder that may affect many tissues and organs, especially synovial joints, leading to pain, stiffness and swelling of the involved joints [1]. Currently, the preferred disease-modifying antirheumatic drug (DMARD) for the treatment of RA is methotrexate (MTX) [2] but, despite its cost–effectiveness, the clinical response to MTX varies widely both in regards to ineffectiveness and/or toxicity. In fact, approximately 10–30% of patients have to discontinue MTX therapy because of the occurrence of adverse drug reactions (ADRs) [3,4]. Factors that possibly influence therapeutic outcome can be divided into patient-related (age, gender, ethnicity and comorbidities), disease-related (duration, activity, disability and biomarkers), treatment-related (compliance, dose and concomitant drugs used) and genetic factors (genetic polymorphisms implicated in MTX and its active metabolites [7-hydroxymethotrexate and MTX polyglutamates] bioavailability) [5–7].

Pharmacogenomics has led to great interest in the identification of novel biomarkers that may reliably predict therapeutic outcome. Several studies have demonstrated that the cause of these variations could be explained by alterations in the activity of MTX transporters [5,6]. One of the most important MTX transporters is SLC19A1, a cell surface transmembrane protein also known as the RFC-1. This bidirectional transporter is described as being expressed in the majority of tissues. Nevertheless, its expression level is different depending of the tissue and some authors suggest that it has a prominent role in the apical membrane of mucosa epithelial cells of small intestine, plasma membrane of hepatocytes and erythrocytes. [5,8,9].

A SNP of *SLC19A1*, characterized by a guanine transition to adenine at position 80 (G80A, rs1051266) of the transcription start region, has been reported [10]. This genetic alteration leads to an amino acid substitution of arginine to histidine at codon 27 (Arg27His, R27H) in the first transmembrane domain, which is considered crucial for carrier function since it is located near to the binding site on the external plasma membrane [11,12]. This polymorphism putatively causes an alteration in the transporter structure that can affect its function [11,12], modify MTX bioavailability and, consequently, influence the MTX therapeutic outcome. Several studies were conducted to elucidate this association. Some of them reported an association between *SLC19A1* 80G homozygotes and the occurrence of MTX-related toxicity and others did not find associations with toxicity [4,13–18]. Hence, the aim of this

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study was to elucidate the clinical relevance of the *SLC19A1* G80A polymorphism for a set of clinical and nongenetic variables as possible biomarkers for MTX-related toxicity in Portuguese RA patients.

Patients & methods

Study population, sample collection & processing

A retrospective study was performed between January 2009 and December 2012 at the São João Hospital Center (Porto, Portugal) in a cohort of consecutively recruited Caucasian patients (≥18 years) with active RA treated with MTX with genetic characterization analysis of the *SLC19A1* G80A polymorphism. Patients were diagnosed with RA, classified according the 1987 criteria of the American College of Rheumatology (ACR) and reclassified according the 2010 criteria of the ACR and the European League Against Rheumatism (EULAR) [19].

After RA diagnosis, all patients were treated with 10 mg *per os* (PO)/week of MTX in monotherapy. This dose was increased 5 mg every 3 weeks if the patient did not meet the EULAR criteria for response; in other words, whenever the patient presented a Disease Activity Score in 28 joints (DAS28) of >3.2. At 3 months, if the patient was still without response, the administration route was changed from PO to subcutaneous (SC) maintaining the MTX dose. Moreover, if patients presented gastrointestinal toxicity with PO route, independently of MTX dose, the administration route was also changed to SC with same MTX dose. If, after 3 months of using the SC administration route, MTX patients presented gastrointestinal toxicity or if, at the maximum tolerated doses, the patient did not meet the response criteria, MTX therapy was discontinued or was combined with leflunomide 20 mg/day. Exceptionally, in the case of RA patients without poor prognosis factors that did not respond to MTX in monotherapy, MTX was combined with sulfasalazine, hydroxychloroquine and/or cyclosporine before making the switch to leflunomide (classic DMARDs). After 3 more months, if patients continued without response in two successive evaluations and did not present any contraindication, therapy was changed by inclusion of a biological DMARD. The occurrence of MTX-related ADRs was registered. Owing to the well-known protective effect of folic acid supplementation for the prevention of toxicity occurrence, in particular for gastrointestinal disorders [20,21], this drug was prescribed to all patients and their regular compliance was registered. Patients were excluded from the study if they had a history of drug abuse, a recent pregnancy or a desire to become pregnant during the study.

Whole blood samples from each patient were obtained with a standard venipuncture technique using EDTA containing tubes, and genomic DNA was extracted using QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total genomic DNA was quantified and its purity and integrity were analyzed using the NanoDrop® 1000 Spectrophotometer v3.7 (Thermo Scientific, DE, USA).

Data collection & variable definition

The study was submitted to the local ethics committee (reference 33/2009) and procedures were considered to be according to the standards of the Declaration of Helsinki [22]. All patients provided written informed consent. Clinicopathological data was collected from individual clinical records by the patients' clinicians during their regular visits to the hospital.

The selection of clinical and nongenetic variables possibly influencing MTX toxicity was based on literature review and clinical significance [3,23–27]. These variables include patient-related variables: age, gender, menopause, BMI, smoking, number of pack years and comorbidities; disease-related variables: age at diagnosis, disease duration, rheumatoid factor, anti-CCP, antinuclear antibodies, DAS28, tender joints count (TJC), swollen joints count (SJC), erythrocyte sedimentation rate (ESR), patient's assessment to visual analog scale (VAS) and health assessment questionnaire; and treatment-related variables: symptomatic (corticosteroids and nonsteroidal anti-inflammatory drugs [NSAIDs]), supplements (folic acid) and other concomitant DMARDs. DAS28 was calculated as described by Prevoo *et al.* [28], according to four variables: TJC, SJC, VAS score of the patient's global health and the laboratory parameter ESR. Disease activity was defined as high when DAS28 >5.1; moderate for 3.2 < DAS28 ≤ 5.1; low for 2.6 < DA28 ≤ 3.2; and remission when DAS28 ≤2.6 [28]. Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia and/or cardiopathy beyond RA. Number of pack years was calculated by the formula: (number of cigarettes smoked per day × number of years smoking)/20. Daily corticosteroid therapy dose was considered in prednisolone equivalents.

The occurrence of MTX-related toxicity, defined as when patients presented any ADR related to MTX treatment, independently of administration route and MTX dose, was recorded at the time of each visit. The type of ADR was classified in System Organ Class (SOC) disorders, in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) published in 2010 by the US Department of Health and Human Services [31].

SLC19A1 G80A genotyping

The *SLC19A1* G80A polymorphism (rs1051266) was selected owing to its putative modification of MTX transporter structure and association with MTX therapeutic outcome [5,8,17,29,30]. This polymorphism was genotyped by PCR-RFLP as published previously [5]. PCR reaction was performed in a final volume of 50 µl containing 1× DreamTaq® Green master mix (Thermo Scientific), 0.3 µM of each primer (forward 5'-AGTGTCACCTTCGTCCCCTC-3' and reverse 5'-CTCCCGCGTGAAGTTCTT-3'), 1 unit of DreamTaq and 50–100 ng of genomic DNA. The PCR amplification consisted of initial denaturation at 95°C during 5 min followed by 40 cycles with denaturation for 15 s at 95°C, annealing/extension for 1 min at 58°C and a final extension at 72°C for 7 min. PCR product was digested by *Cfo*I (*Hha*I; Life Technologies, CA, USA) restriction endonuclease at 37°C for 1 h. Individuals with the *SLC19A1* 80GG genotype presented three fragments (125 bp, 68 bp and 37 bp), whereas individuals with the *SLC19A1* 80AA genotype presented two fragments (162 bp and 68 bp).

For quality control, 10% of the samples were randomly selected for a second analysis and 10% of all genotypes were confirmed by automated sequencing in a 3130×l Genetic Analyzer using the BigDye® Terminator v3.1 kit (Life Technologies). Results were 100% concordant.

Treating physicians and patients were blinded to the results of pharmacogenetic biomarker measurements during the entire study.

Statistical analysis

Statistical analyses were performed using the IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, NY, USA) considering a p-value of 5% or less as statistically significant. The χ^2 test was used to assess association between the groups and the different categorical variables. The odds ratio (OR) and the correspondent 95% CI were calculated as a measure of the association between the categorical variables. Genetic analysis was performed comparing the different genotypes *SLC19A1* 80GG, *SLC19A1* 80GA and *SLC19A1* 80AA. Hardy–Weinberg equilibrium (HWE) was calculated for *SLC19A1* G80A genotypes according to Rodriguez *et al.* [31]. For the comparison of quantitative variables between the studied MTX outcome groups, two-sample t-test for normally distributed variables and the nonparametric Mann–Whitney test for nonnormally distributed variables were applied. Multivariate analysis by binary logistic regression was used to identify which variables could predict the occurrence of MTX-related toxicity when the analysis was adjusted with the variables: *SLC19A1* G80A genotypes, age, gender, smoking, age at diagnosis, disease duration, folic acid supplementation, corticosteroid therapy, use of NSAIDs, other concomitant DMARDs used and MTX administration characteristics (dose, treatment duration and administration route). A Cox proportional hazard regression model was used to analyze the influence of *SLC19A1* G80A genotypes on the time to toxicity occurrence (determined as the interval of time elapsed from the beginning of MTX treatment to the clinician's visit where ADR was reported and registered) considering, as covariates, age, gender, smoking, age at diagnosis, disease duration, folic acid supplementation, corticosteroid therapy, use of NSAIDs, other concomitant DMARDs used and MTX administration characteristics (dose, treatment duration and administration route). Cox regression models were used to adjust for potential confounders.

Results

Characterization of studied population

In this study we have included follow-up data from 233 patients (196 females and 37 males), with a median age of 52 years old (range: 26.0–87.0) and median disease duration of 10.0 years (range: 0.3–51.0). Out of all the patients, 136 patients (58.4%) used MTX as the unique DMARD, while 97 (41.6%) were treated with MTX combined with other classic or biological DMARDs. MTX was administered PO in 210 (90.1%) and by the SC route in 23 patients (9.9%), and the median MTX treatment duration was 47.0 months (range: 1.0–240.0) with a median dose of 15.0 mg/week (range 2.5–25.0; Table 1).

A total of 77 patients (33.0%) developed MTX-related toxicity and the ADR types were as follows: 58 (75.3%) gastrointestinal disorders (abdominal distension, diarrhea, dyspepsia, nauseas, stomach pain and/or vomiting); nine (11.7%)

Table 1. Clinical and nongenetic variables of the population enrolled in the study.

Characteristic	Value
Patient related	
Female, n (%)	196 (84.1)
– Postmenopausal, n (%)	101 (51.5)
Male, n (%)	37 (15.9)
Age, median (IQR), years	52.0 (26.0–87.0)
BMI, median (IQR), kg/m²	26.2 (18.4–43.1)
Current smokers, n (%)	32 (13.7)
– NPY[†], median (IQR)	20.1 (0.8–120.0)
Comorbidity[*], n (%)	126 (54.1)
Disease related	
Diagnosis age, mean ± SD, years	40.3 ± 13.2
Disease duration, median (IQR), years	10.0 (0.3–51.0)
RF positive, n (%)	131 (56.2)
Anti-CCP positive, n (%)	175 (75.1)
ANAs positive, n (%)	66 (28.3)
DAS28, mean ± SD	4.2 ± 1.3
Disease activity – DAS28, n (%)	
– Remission (DAS28 ≤2.6)	46 (19.7)
– Low (2.6 < DAS28 ≤ 3.2)	37 (15.9)
– Moderate (3.2 < DAS28 ≤ 5.1)	114 (48.9)
– High (DAS28 >5.1)	36 (15.5)
Individual variables – DAS28	
– TJC (out of 28), median (IQR)	4.0 (0.0–27.0)
– SJC (out of 28), median (IQR)	3.0 (0.0–24.1)
– ESR, median (IQR), mm (1st hour)	18.0 (1.0–92.0)
– Global health on VAS, median (IQR)	48.0 (0.0–100.0)
HAQ score, median (IQR)	1.25 (0.0–2.9)
– HAQ ≤0.5, n (%)	39 (16.7)
Treatment related[‡]	
Symptomatic	
– Corticosteroids, n (%)	188 (80.7)
– Daily dose in prednisolone equivalents, median (IQR), mg	5.0 (0.0–20.0)
– NSAIDs, n (%)	170 (73.0)
Supplements	
– Folic acid[¶], n (%)	118 (50.6)
DMARDs	
– Methotrexate monotherapy, n (%)	136 (58.4)
– Combined methotrexate therapy – classic DMARDs, n (%)	44 (18.9)
– MTX + hydroxychloroquine	14 (31.8)
– MTX + sulfasalazine	15 (34.1)
– MTX + cyclosporine	2 (4.5)
– MTX + hydroxychloroquine + sulfasalazine	2 (4.5)
– MTX + hydroxychloroquine + cyclosporine	1 (2.3)
– MTX + hydroxychloroquine + sulfasalazine + cyclosporine	1 (2.3)
– MTX + leflunomide	9 (20.5)
– Combined methotrexate therapy – biological DMARDs, n (%)	53 (22.7)
– MTX + anti-TNFα[#]	40 (75.5)
– MTX + others[††]	13 (24.5)

[†]NPY = (number of cigarettes smoked per day × number of years smoking)/20.
[*]Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia and/or cardiopathy beyond rheumatoid arthritis.
[‡]Drugs coadministered with methotrexate when the toxicity occurred.
[¶]Patients with compliance to folic acid supplementation.
[#]Anti-TNFα drugs include adalimumab, etanercept and infliximab.
[††]Others biological DMARDs include anakinra, rituximab and tocilizumab.
ANA: Antinuclear antibody; DAS28: Disease activity score 28; DMARD: Disease-modifying antirheumatic drug; ESR: Erythrocyte sedimentation rate; HAQ: Health assessment questionnaire; IQR: Interquartile range; MTX: Methotrexate; NPY: Number of pack years; NSAID: Nonsteroidal anti-inflammatory drug; RF: Rheumatoid factor; SD: Standard deviation; SJC: Swollen joints count; TJC: Tender joints count; VAS: Visual analog scale.

Table 1. Clinical and nongenetic variables of the population enrolled in the study (cont.).

Characteristic	Value
Treatment related[1] (cont.)	
DMARDs (cont.)	
– Methotrexate administration characteristics:	
– Dose, median (IQR), mg/week	15.0 (2.5–25.0)
– Treatment duration, median (IQR), months	47.0 (1.0–240.0)
– *Per os* administration route, n (%)	210 (90.1)
– Subcutaneous administration route, n (%)	23 (9.9)

[1]NPY = (number of cigarettes smoked per day × number of years smoking)/20.
[2]Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia and/or cardiopathy beyond rheumatoid arthritis.
[3]Drugs coadministered with methotrexate when the toxicity occurred.
[4]Patients with compliance to folic acid supplementation.
[5]Anti-TNFα drugs include adalimumab, etanercept and infliximab.
[6]Others biological DMARDs include anakinra, rituximab and tocilizumab.
ANA: Antinuclear antibody; DAS28: Disease activity score 28; DMARD: Disease-modifying antirheumatic drug; ESR: Erythrocyte sedimentation rate; HAQ: Health assessment questionnaire; IQR: Interquartile range; MTX: Methotrexate; NPY: Number of pack years; NSAID: Nonsteroidal anti-inflammatory drug; RF: Rheumatoid factor; SD: Standard deviation; SJC: Swollen joints count; TJC: Tender joints count; VAS: Visual analog scale.

skin and subcutaneous tissue disorders (alopecia, rash maculopapular and rheumatoid nodulosis exacerbation); five (6.5%) hepatobiliary disorders (clinical or diagnostic observations, more precisely transaminases serum elevation); and five (6.5%) respiratory, thoracic and mediastinal disorders (hypersensitivity pneumonitis).

Owing to the severity of some observed ADRs, all patients that developed skin and subcutaneous tissue disorders; hepatobiliary disorders; and respiratory, thoracic and mediastinal disorders had discontinued MTX treatment. From those with MTX PO and that developed gastrointestinal disorders, 21 (36.2%) changed to the SC route of administration maintaining the MTX dose and 32 (55.2%) decreased MTX dose and combined MTX with another DMARD to ensure that EULAR criteria for response were met. From those patients using MTX via the SC route, three (5.2%) decreased MTX dose and two (3.4%) discontinued MTX because the used MTX dose was the minimum necessary to maintain EULAR criteria for response.

Since the number of cases with ADRs other than gastrointestinal disorders were few, the evaluation of the clinical relevance of the *SLC19A1* G80A polymorphism for a set of clinical and nongenetic variables as possible biomarkers for MTX-related toxicity in Portuguese RA patients was only performed for MTX-related overall toxicity and for MTX-related gastrointestinal toxicity.

Clinical & nongenetic variables & MTX-toxicity occurrence

Table 2 shows the association of clinical and nongenetic variables with the development of MTX-related overall toxicity and MTX-related gastrointestinal toxicity. Our results demonstrated that lower MTX treatment duration was significantly associated with toxicity occurrence (overall and gastrointestinal, both with $p < 0.001$) and regular folic acid supplementation use was demonstrated to be protective for toxicity occurrence (overall and gastrointestinal, both with $p < 0.001$). Additionally, higher ESR levels were significantly associated with MTX-related overall toxicity ($p = 0.036$). No associations were observed for the remaining variables studied.

SLC19A1 G80A genotypes & MTX-toxicity occurrence

The frequencies of *SLC19A1* G80A polymorphism alleles and genotypes were as follows: 80G allele 54.0% and 80A allele 46.0%; 80G homozygotes 34.3% (n = 80), 80GA heterozygotes 39.1% (n = 91) and 80A homozygotes 26.6% (n = 62). The genotype distribution of the *SLC19A1* G80A polymorphism was not in HWE ($p < 0.050$) in the studied population. The risk analysis demonstrated a statistically significant association of *SLC19A1* 80G carriers ($p = 0.019$; OR: 2.61, 95%CI: 1.15–5.94) with approximately threefold higher risk for MTX-related gastrointestinal toxicity when compared with *SLC19A1* 80A homozygotes. No statistically significant differences were observed regarding MTX-related overall toxicity and *SLC19A1* G80A genotypes (Table 3).

Multivariate analysis

The influence of the *SLC19A1* G80A polymorphism on MTX-related gastrointestinal toxicity occurrence was analyzed using a multivariate

Table 2. Relationship between clinical and nongenetic variables and methotrexate-related overall and gastrointestinal toxicity.

Characteristic	Nontoxicity (n = 156)	MTX overall toxicity (n = 77)	p-value	Nontoxicity (n = 156)	MTX gastrointestinal toxicity (n = 58)	p-value
Patient related						
Gender, n (%)						
– Female	129 (65.8)	67 (34.2)		129 (71.7)	51 (28.3)	
– Male	27 (73.0)	10 (27.0)	0.396	27 (79.4)	7 (20.6)	0.351
Postmenopausal, n (%)	60 (59.4)	41 (40.6)		60 (67.4)	29 (32.6)	
Nonpostmenopausal, n (%)	69 (72.6)	26 (27.4)	0.051	69 (75.8)	22 (24.2)	0.211
Age, median (IQR), years	52.5 (26.0–87.0)	51.0 (36.0–76.0)	0.533	52.5 (26.0–87.0)	50.0 (36.0–76.0)	0.329
BMI, median (IQR), kg/m²	26.2 (18.4–38.9)	26.3 (18.5–43.1)	0.926	26.2 (18.4–38.9)	26.2 (18.5–39.9)	0.543
Current smoker, n (%)	19 (79.4)	13 (40.6)		19 (67.9)	9 (32.1)	
Nonsmoker/ex-smoker, n (%)	137 (68.2)	64 (31.8)	0.327	137 (73.7)	49 (26.3)	0.520
– NPY*, median (IQR)	20.0 (0.8–120.0)	20.2 (3.0–43.8)	0.762	20.0 (0.8–120.0)	17.6 (3.0–43.8)	1.000
Comorbidity*, n (%)	82 (65.1)	44 (34.9)		82 (72.6)	31 (27.4)	
Noncomorbidity, n (%)	74 (69.2)	33 (30.8)	0.509	74 (73.3)	27 (26.7)	0.908
Disease related						
Diagnosis age, years	40.2 ± 13.3	40.6 ± 13.0	0.818	40.2 ± 13.3	39.1 ± 12.9	0.615
– Female	39.6 ± 13.0	39.4 ± 12.5	0.920	39.6 ± 13.0	37.8 ± 11.9	0.384
– Male	42.8 ± 14.8	48.4 ± 14.0	0.306	42.8 ± 14.8	49.6 ± 16.4	0.340
Disease duration, median (IQR), years	11.5 (1.0–46.0)	8.0 (0.3–51.0)	0.084	11.5 (1.0–46.0)	10.0 (0.5–51.0)	0.358
RF positive, n (%)	90 (68.7)	41 (31.3)		90 (73.2)	33 (26.8)	
RF negative, n (%)	66 (64.7)	36 (35.3)	0.520	66 (72.5)	25 (27.5)	0.917
Anti-CCP positive, n (%)	122 (69.7)	53 (30.3)		122 (75.3)	40 (24.7)	
Anti-CCP negative, n (%)	34 (58.6)	24 (41.4)	0.120	34 (65.4)	18 (34.6)	0.161
ANAs positive, n (%)	43 (65.2)	23 (34.8)		43 (69.4)	19 (30.6)	
ANAs negative, n (%)	113 (67.7)	54 (32.3)	0.713	113 (74.3)	39 (25.7)	0.457
DAS28	4.1 ± 1.3	4.4 ± 1.3	0.143	4.1 ± 1.3	4.3 ± 1.1	0.388
Individual variables – DAS28						
– TJC (out of 28), median (IQR)	4.0 (0.0–22.0)	4.0 (0.0–27.0)	0.691	4.0 (0.0–22.0)	4.0 (0.0–19.0)	0.836
– SJC (out of 28), median (IQR)	3.0 (0.0–23.0)	3.0 (0.0–24.0)	0.233	3.0 (0.0–23.0)	3.5 (0.0–15.0)	0.210
– ESR, median (IQR), mm (1st hour)	14.5 (1.0–92.0)	20.0 (1.0–88.0)	**0.036**	14.5 (1.0–92.0)	20.0 (2.0–88.0)	0.314
– Global health on VAS, median (IQR)	47.0 (0.0–100.0)	51.0 (3.0–85.0)	0.143	47.0 (0.0–100.0)	51.0 (15.0–85.0)	0.274
HAQ score, median (IQR)	1.2 (0.0–2.6)	1.4 (0.0–2.9)	0.178	1.2 (0.0–2.6)	1.4 (0.0–2.9)	0.359

Results are expressed as mean ± standard deviation unless stated otherwise. p-value <0.05 is considered to be of statistical significance (highlighted in bold).
*NPY = (number of cigarettes smoked per day ÷ number of years smoking)/20.
‡Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia and/or cardiopathy beyond rheumatoid arthritis.
§Drug discontinued from methotrexate when the toxicity occurred.
ANAs: Antinuclear antibody; DAS28: Disease activity score 28; ESR: Erythrocyte sedimentation rate; HAQ: Health assessment questionnaire; ICM: Intramuscular; IVPU: Intravenous; MTX: Methotrexate; NPY: Number of pack years; NSAID: Nonsteroidal anti-inflammatory drug; OR: Odds ratio; RF: Rheumatoid factor; SJC: Swollen joint count; TJC: Tender joint count; VAS: Visual analog scale.

Table 2. Relationship between clinical and nongenetic variables and methotrexate-related overall and gastrointestinal toxicity (cont.).

Characteristic	Nontoxicity (n = 156)	MTX overall toxicity (n = 77)	p-value	Nontoxicity (n = 156)	MTX gastrointestinal toxicity (n = 58)	p-value
Treatment related*						
Symptomatic, n (%)						
– Corticosteroids	128 (68.1)	60 (31.9)		128 (74.4)	44 (25.6)	
– Non-corticosteroids	28 (62.2)	17 (37.8)	0.453	28 (66.7)	14 (33.3)	0.311
– NSAIDs	120 (70.6)	50 (29.4)		120 (75.0)	40 (25.0)	
– Non-NSAIDs	36 (57.1)	27 (42.9)	0.053	36 (66.7)	18 (33.3)	0.234
Supplements, n (%)						
– Folic acid regular users	101 (85.6)	17 (14.4)		101 (87.1)	15 (12.9)	
– Folic acid nonregular users	55 (47.8)	60 (52.2)	**<0.001**‡	55 (56.1)	43 (43.9)	**<0.001**‡
Methotrexate administration characteristics						
– Dose, median (IQR), mg/week	17.0 (2.5–25.0)	15.0 (5.0–25.0)	0.119	15.0 (2.5–25.0)	15.0 (5.0–25.0)	0.080
– Treatment duration, median (IQR), months	60.0 (3.0–240.0)	19.0 (1.0–150.0)	**<0.001**	60.0 (3.0–240.0)	19.5 (1.0–150.0)	**<0.001**
– *Per os* administration route, n (%)	140 (66.7)	70 (33.3)		140 (72.5)	53 (27.5)	
– Subcutaneous administration route, n (%)	16 (69.6)	7 (30.4)	0.779	16 (76.2)	5 (23.8)	0.721

Results are expressed as mean ± standard deviation unless stated otherwise. *p-value <0.05 is considered to be of statistical significance (highlighted in bold).
‡NPY = (number of cigarettes smoked per day ÷ number of years smoking)/20.
*Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia and/or osteopathy beyond rheumatoid arthritis.
‖Drugs coadministered with methotrexate when the toxicity occurred.
¶OR: 0.15, 95% CI: 0.08–0.29.
#OR: 0.19, 95% CI: 0.10–0.37.
AHA: Anchorchar antibody; DAS28: Disease activity score 28; ESR: Erythrocyte sedimentation rate; HAQ: Health assessment questionnaire; IQR: Interquartile range; MTX: Methotrexate; NPY: Number of pack years; NSAID: Nonsteroidal anti-inflammatory drug; OR: Odds ratio; RF: Rheumatoid factor; SJC: Swollen joints count; TJC: Tender joints count; VAS: Visual analog scale.

Table 3. Genetic analysis of *SLC19A1* G80A polymorphism and methotrexate-related overall and gastrointestinal toxicity.

	Nontoxicity (n = 156)	MTX overall toxicity (n = 77)	p-value	OR (95% CI)	Nontoxicity (n = 156)	MTX gastrointestinal toxicity (n = 58)	p-value	OR (95% CI)
Genotype distribution								
80AA	46 (74.2)	16 (25.8)		Reference	46 (85.2)	8 (14.8)		Reference
80GA	58 (63.7)	33 (36.3)	0.174	1.64 (0.80–3.33)	58 (68.2)	27 (31.8)	**0.025**	2.68 (1.11–6.44)
80GG	52 (65.0)	28 (35.0)	0.240	1.55 (0.74–3.22)	52 (69.3)	23 (30.7)	**0.038**	2.54 (1.04–6.24)
Dominant model								
80AA	46 (74.2)	16 (25.8)		Reference	46 (85.2)	8 (14.8)		Reference
80G carrier	110 (64.3)	61 (35.7)	0.157	1.59 (0.83–3.05)	110 (68.8)	50 (31.2)	**0.019**	2.61 (1.15–5.94)
Recessive model								
80A carrier	104 (68.0)	49 (32.0)		Reference	104 (74.8)	35 (25.2)		Reference
80GG	52 (65.0)	28 (35.0)	0.647	1.14 (0.65–2.02)	52 (69.3)	23 (30.7)	0.389	1.31 (0.71–2.45)

Results are expressed as n (%). *p-value <0.05 is considered to be of statistical significance (highlighted in bold).
MTX: Methotrexate; OR: Odds ratio.

analysis, as shown in Table 4. The multivariate analysis was not performed in accordance with MTX-related overall toxicity since no statistically significant differences were observed in univariate analysis regarding MTX-related overall toxicity and *SLC19A1* G80A genotypes (Table 3).

Our results demonstrated that regular folic acid supplementation afforded protection against MTX-related gastrointestinal toxicity ($p < 0.001$; OR: 0.13; 95%CI: 0.05–0.32). Moreover, we observed that lower MTX treatment duration was significantly associated with MTX-related gastrointestinal toxicity ($p < 0.001$). Furthermore, *SLC19A1* 80G carriers had an approximately sixfold higher risk for MTX-related gastrointestinal toxicity when compared with *SLC19A1* 80A homozygotes ($p = 0.011$; OR: 5.53; 95%CI: 1.48–20.68). The remaining variables revealed no statistically significant associations.

Regarding the influence of *SLC19A1* genotypes on the onset time of MTX-related gastrointestinal toxicity, the multivariate Cox regression model demonstrated a higher risk of earlier MTX-related gastrointestinal toxicity in *SLC19A1* 80G carriers compared with *SLC19A1* 80A homozygotes ($p = 0.002$; hazard ratio: 3.63; 95%CI: 1.60–8.21; Figure 1).

Discussion

Despite gastrointestinal toxicity being the most common ADR related to MTX administration in this study (n = 58, 24.9%) and in other reported RA pharmacogenetic studies of *SLC19A1* G80A, controversial results have been published [4,13,15,32,35]. Several explanations may be proposed to clarify these reported differences. Thus, the majority of authors do not similarly define the concept of toxicity, thus influencing conclusions drawn and rendering comparisons difficult. In our study, we have defined ADR according to the International Committee on Harmonization as:

> *"...a response (mild, moderate or severe) to a drug that is noxious and unintended and occurs at doses normally used in humans for the prophylaxis, diagnosis and treatment of disease or for modification of physiological function"* [102].

In addition, most of the studies did not follow standard guidelines for ADR classification, such as the inclusion of transaminase serum elevation, which indicates hepatotoxicity, in gastrointestinal disorders group. Both reasons concur to the erratic conclusions observed and make the adequate comparison of studies difficult. In this study we have classified the ADRs in SOC disorders according to CTCAE [101]. Furthermore, another factor that must be highlighted is that reported studies relating overall toxicity with *SLC19A1* G80A polymorphisms are not entirely clear regarding the incidence of gastrointestinal ADR results. Since SLC19A1 carrier expression level is different depending on the tissue [9], different impacts on tissue toxicity are expected and so, considering the concept of overall toxicity can lead to difficulties in comparisons. Moreover, the SLC19A1 mechanisms of transport have been referred to as different depending on dose of MTX (low-doses implicate the transporters and high-doses implicate both transporters and passive diffusion [34]) and, consequently, this point is important to consider when reported studies are analyzed to evaluate the influence of *SLC19A1* G80A polymorphism on MTX bioavailability and on MTX-related toxicity. Furthermore, and owing to the complexity of the MTX mechanism of action, as previously reported by other studies [13,20,35–38], multiple factors could influence and/or be associated

Table 4. Multivariate logistic regression analysis and methotrexate-related gastrointestinal toxicity.

Variables	p-value†	OR (95% CI)
SLC19A1 G80A		
80AA		Reference
80G carriers	**0.011**	5.53 (1.48–20.68)
Folic acid supplementation		
Nonregular users		Reference
Regular users	**<0.001**	0.13 (0.05–0.31)

p-value <0.05 is considered to be of statistical significance (highlighted in bold).
†p-values correspond to multivariate logistic regression adjusted for SLC19A1 G80A genotypes, age, gender, smoking, age at diagnosis, disease duration, folic acid supplementation, corticosteroid therapy, use of NSAIDs, other concomitant DMARDs used, MTX administration characteristics (dose, treatment duration and administration route).
DMARD: Disease-modifying antirheumatic drug; MTX: Methotrexate; NSAID: Nonsteroidal anti-inflammatory drug; OR: Odds ratio.

with MTX-related toxicity occurrence, such as genetic polymorphisms in genes that code for other MTX transporters and for proteins involved in the folate pathway, such as MTHFR and DHFR, as well as gene–gene interactions.

The patient group studied is in accordance with the reported disease gender epidemiology, suggesting an occurrence of two- to four-times higher in women [39,40], and also in accordance with age of diagnosis range, spanning from the third to the fifth decade of life [41]. Regarding the *SLC19A1* G80A polymorphism, allele frequencies observed in our population (80G allele 54.0% and 80A allele 46.0%) were similar to other Caucasian populations [4,5,13–15] but genotype distribution was not in HWE and the observed genotype frequencies differ from those found in the National Center for Biotechnology Information (NCBI) database for Caucasians. Nevertheless, it is well known that the NCBI database does not consider all populations and there are no studies using this polymorphism in the Portuguese population to compare the obtained genotype distribution.

Clinical & nongenetic variables & MTX-toxicity occurrence

According to clinical and nongenetic variables, our results demonstrated statistically significant associations between lower MTX treatment duration and MTX-related toxicity occurrence (overall and gastrointestinal) in univariate analysis and with MTX-related gastrointestinal toxicity in multivariate analysis. Our results are in accordance with published literature, where the median time for MTX-related ADR occurrence was 36.5 months; the main ADRs were gastrointestinal disorders; hypersensitivity pneumonitis occured early in the course of MTX; and transaminase serum elevation occurred particularly during the first 4 years of treatment [42].

Both univariate and multivariate analysis confirmed the protective effect afforded when folic acid was regularly used. Nowadays concomitant use of folic acid and MTX is standard treatment. Although folic acid supplementation was prescribed to all patients, only 118 (50.6%) complied with the treatment. It is expected that folic acid supplements reduce the toxicity of low-dose MTX therapy [31,43–46]. Becker *et al.* reported an association between MTX-related toxicity and lower intracellular folates in low-dose MTX therapy use [47]. Furthermore, two meta-analyses support our findings by concluding that low dose folate supplementation has protective effects against MTX-related gastrointestinal toxicity [20,21]. Also, one study proved that gastrointestinal toxicity was the main reason for MTX withdrawal and was related to the absence of folate supplementation in RA patients [48]. Studies reported that MTX treatment leads to a folate deficiency status, given that mammalian cells cannot synthesize folates *de novo* [8]. Moreover, RA itself causes a diminution of folate levels [49] and, consequently, without the appropriate reposition of folates, several metabolic pathways, such as the biosynthesis of purines, pyrimidines and some amino acids (e.g., serine and methionine), processes that maintain normal cell growth and replication, will be compromised [8]. For this reason, rapidly dividing tissues, such as those of the gastrointestinal tract, are more susceptible to folate depletion and, therefore, supplementation with folic acid will help to protect from toxicity [8]. Other reasons contributing to the protective mechanism resulting from folic acid supplementation may be the interaction of folic acid with MTX. This interaction causes the blockage of MTX reabsorption at the renal distal tubule leading to lower MTX circulating levels, and as a consequence, lower toxicity [50].

Additionally, our results demonstrated an association between increased levels of ESR and overall toxicity but not with gastrointestinal toxicity. The DAS28 is widely used for the assessment of patients in the clinic to monitor disease activity of patients with RA [51–55] and is calculated from four components: TJC, SJC, VAS and ESR or CRP. Reasons for using CRP include not only its potential to be a more direct measure of inflammation, but also the increasing usage of B-cell directed therapies that may have differential effects on these inflammatory markers. However, recent data from two large observational studies suggested that DAS28 (CRP) scores tended to be lower than DAS28 (ESR) scores and Inoue *et al.* suggested potential new thresholds for disease activity categories for DAS28 (CRP) [56,57]. Since these thresholds are not defined, in this study we used the ESR for DAS28 calculation. The ESR measurement is a simple and inexpensive laboratory test for assessing inflammatory or acute response [55] with reduced sensitivity and specificity. Consequently, our results can be explained by the greater impact of the other observed ADRs in the inflammatory process, such as skin and subcutaneous tissue disorders; hepatobiliary disorders; and respiratory, thoracic and mediastinal disorders, when compared with gastrointestinal toxicity. In fact, other relevant studies have reported an increase of acute phase reactants in rheumatoid

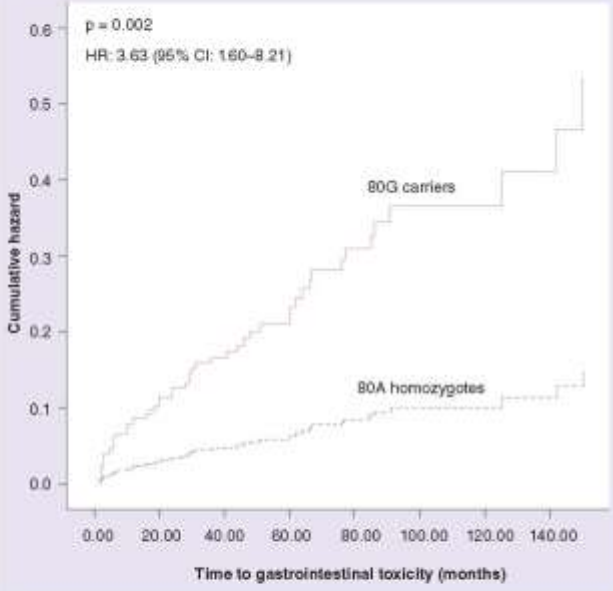

Figure 1. Cumulative hazard of methotrexate-related gastrointestinal toxicity according to *SLC19A1* G80A genotypes.
HR: Hazard ratio.

nodulosis [58,59], hepatotoxicity [60] and hypersensitivity pneumonitis [61], which were also observed as ADRs in our studied population.

SLC19A1 G80A genotypes & MTX-toxicity occurrence

Considering the influence of the *SLC19A1* G80A polymorphism in the development of MTX-related toxicity, no statistically significant differences were observed concerning overall toxicity but our results demonstrated that *SLC19A1* 80G carriers were at a significantly increased risk of MTX-related gastrointestinal toxicity when compared with *SLC19A1* 80A homozygotes (both in univariate and multivariate analyses). Moreover, we observed a higher risk of earlier MTX-related gastrointestinal toxicity in *SLC19A1* 80G carriers compared with *SLC19A1* 80A homozygotes. As is pointed out above, SLC19A1 carrier expression level is different depending on the tissue [9] and, consequently, different impacts on tissue toxicity are expected. This effect is consistent with previous *in vitro* results by Baslund *et al.*, demonstrating that the *SLC19A1* 80G allele is associated with lower uptake of MTX into lymphocytes owing to decreased SLC19A1 transport activity [62]. Indeed Dervieux *et al.* demonstrated an association of the *SLC19A1* 80G allele with reduced erythrocyte MTX polyglutamate levels [5]. Thus, it can be hypothesized that decreased intracellular levels (both lymphocytes and erythrocytes) leads to higher systemic levels and, consequently, to higher probability for toxicity, mainly in tissues where SLC19A1 is more highly expressed, such as the gastrointestinal cells. Considering that the SLC19A1 carrier has a higher affinity for MTX than for folic acid [8], it can be assumed that the presence of the *SLC19A1* 80G allele would impede the entrance of folic acid into cells causing a greater folate depletion in rapidly dividing tissues, such as the gastrointestinal tract and, therefore, rendering it more susceptible to this depletion, resulting in the observed toxicity. In fact, studies have associated *SLC19A1* 80G homozygotes with reduced cellular folate levels [63]. Despite Bohanec Grabar *et al.* previously reporting that *SLC19A1* 80G homozygotes had a higher risk for overall MTX toxicity compared with *SLC19A1* 80A carriers, they did not clearly describe the impact on gastrointestinal ADRs, and the majority of other studies did not describe associations with toxicity [4,13–18].

Controversial studies regarding *SLC19A1* polymorphisms and gastrointestinal ADRs have been published, prompting the need for more and larger studies necessary to support our results. In our study, we are aware of some potential weaknesses due to the relatively reduced population size and follow-up time and some limitations in the study design; however, our results showed that the study was not biased by genetic heterogeneity, and multivariate analysis was used to eliminate potential confounding variables. Nevertheless, one important consideration drawn from this work is that future studies need methodological standardization. Further comparisons are also required owing to ethnic variability and the existence of other polymorphisms in *SLC19A1* that could counterbalance the carrier functionality or interfere with its expression. The presence of other genetic polymorphisms that could alter the relationship of the *SLC19A1* G80A polymorphism with gastrointestinal toxicity should also be evaluated.

Conclusion & future perspective

To the best of our knowledge, this is the first report regarding the study of the association of the *SLC19A1* G80A polymorphism with MTX-related toxicity in Portuguese RA patients. This study concluded that lower MTX treatment duration was associated with toxicity occurrence; *SLC19A1* 80G carriers had an increased risk for

MTX-related gastrointestinal toxicity; a higher risk of earlier MTX-related gastrointestinal toxicity was observed in *SLC19A1* 80G carriers; and regular folic acid supplementation conferred protection against MTX-related toxicity. Thus, the *SLC19A1* G80A polymorphism may help clinicians to identify patients who will not benefit from MTX treatment owing to the potential occurrence of gastrointestinal ADRs. Our findings suggest that, for MTX-related gastrointestinal toxicity protection, the optimal combination seems to include genotype 80AA for the *SLC19A1* G80A polymorphism and regular folic acid supplementation.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Despite the cost-effectiveness of methotrexate (MTX) for rheumatoid arthritis treatment, adverse drug reactions related to MTX may occur.
- One of the most important MTX influx transporters is SLC19A1.
- A SNP in *SLC19A1*, G80A (rs1051266), has been associated with an alteration in the transporter structure and function, which influences MTX therapeutic outcomes.

Aim

- To analyze the association of the *SLC19A1* G80A polymorphism and a set of clinical and nongenetic variables with MTX-related toxicity in Portuguese rheumatoid arthritis patients.

Results

- In both univariate and multivariate analyses, lower MTX treatment duration was associated with toxicity occurrence.
- In both univariate and multivariate analyses, *SLC19A1* 80G carriers had an increased risk for MTX-related gastrointestinal toxicity.
- A higher risk of earlier MTX-related gastrointestinal toxicity was observed in *SLC19A1* 80G carriers.
- Regular folic acid supplementation conferred protection for MTX-related toxicity.

Conclusion

- This study suggests that *SLC19A1* G80A polymorphism could be a possible biomarker for MTX-related gastrointestinal toxicity.
- Further comparisons are required owing to ethnic variability and the existence of other polymorphisms in *SLC19A1* that could counterbalance the carrier functionality or interfere with its expression.

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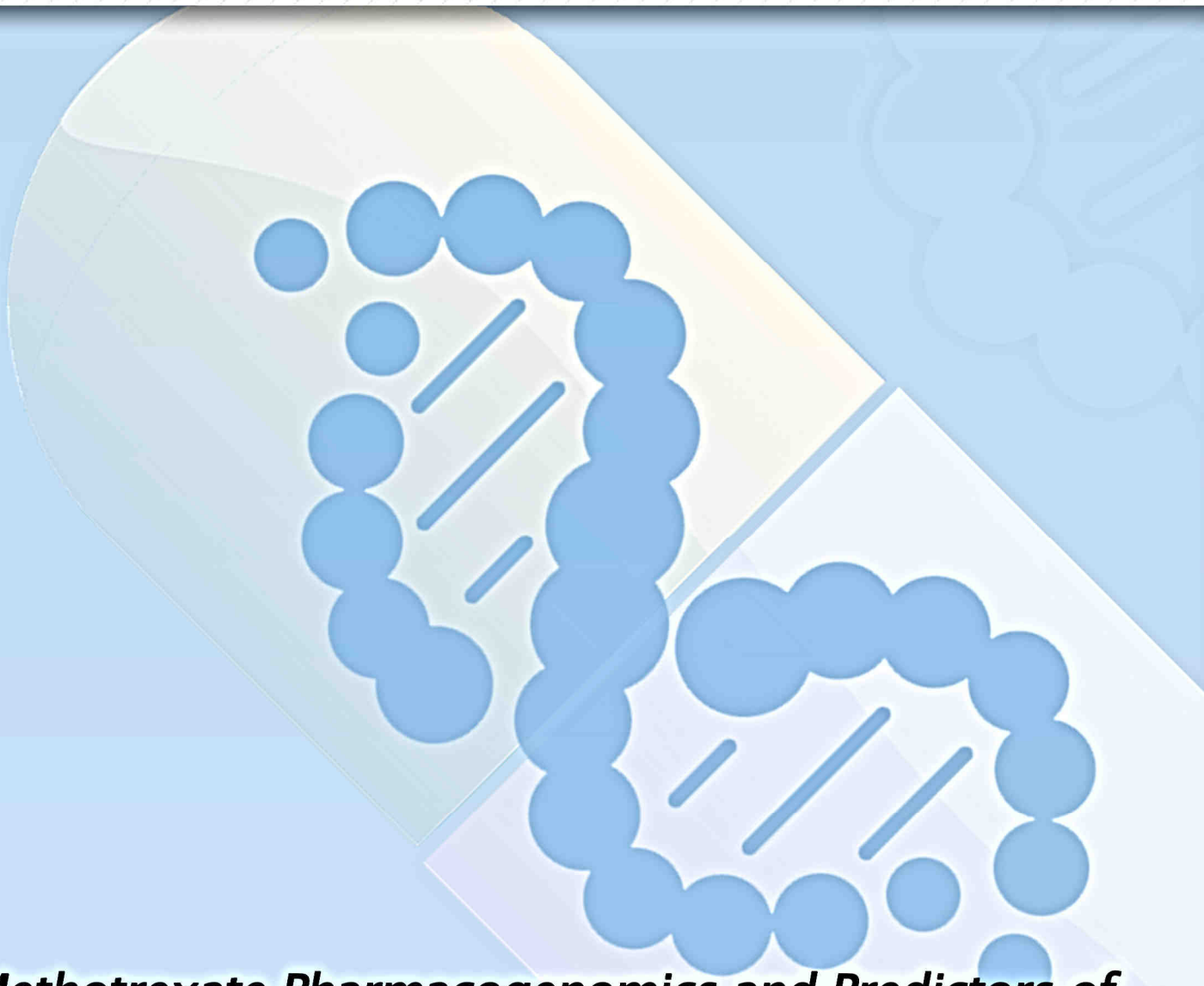
CHAPTER VI

SLC19A1, SLC46A1 AND SLCO1B1 POLYMORPHISMS AS PREDICTORS OF METHOTREXATE-RELATED TOXICITY IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER VI. *SLC19A1*, *SLC46A1* AND *SLCO1B1* POLYMORPHISMS AS PREDICTORS OF METHOTREXATE-RELATED TOXICITY IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS

This chapter elucidates the influence of single nucleotide polymorphisms in genes encoding for Methotrexate membrane transporter proteins on the occurrence of Methotrexate-related toxicity in Portuguese Rheumatoid Arthritis patients. The work was performed in a relatively large number of single nucleotide polymorphisms (twenty-three) and in different genes (ten), because it was previously accepted that Methotrexate could enter cells by solute carrier 19 family 1 and, the presence of other influx and efflux transporters was not thoroughly explored concerning their functional polymorphisms impact in Methotrexate therapeutic outcome. In addition, many of the included single nucleotide polymorphisms had never been studied before, in both Rheumatoid Arthritis and Caucasian populations, concerning Methotrexate therapeutic outcome influence.

SLC19A1, *SLC46A1* and *SLCO1B1* polymorphisms as predictors of Methotrexate-related Toxicity in Portuguese Rheumatoid Arthritis patients

RUNNING TITLE

MTX transporters SNPs & MTX-related toxicity

ABSTRACT

Methotrexate (MTX) is used for rheumatoid arthritis (RA) treatment showing a wide toxicity profile. This study aims to evaluate the influence of single nucleotide polymorphisms (SNP) in genes that codify for MTX transporters with the occurrence of MTX-related toxicity (overall and gastrointestinal). A total of 233 Portuguese RA patients were genotyped for 23 SNPs. Haplotype analyses were performed and a toxicogenetic risk index (TRI) was created for those SNPs revealed to be statistically significant. Regarding MTX overall toxicity, an increased risk was associated to *SLC19A1* rs7499 G carriers (*P*=0.017), *SLC46A1* rs2239907 GG (*P*=0.030) and *SLCO1B1* rs4149056 T carriers (*P*=0.040) and TT (*P*=0.019). TRI revealed that patients with index 3 were 18-fold more likely to present an adverse drug reaction when compared to those with index 1 (*P*=0.001). For MTX gastrointestinal toxicity, results demonstrated an increased risk associated with *SLC19A1* rs7499 G carriers (*P*=0.012) and GG (*P*=0.045), *SLC19A1* rs1051266 G carriers (*P*=0.034), *SLC19A1* rs2838956 A carriers (*P*=0.049) and, *SLCO1B1* rs4149056 T carriers (*P*=0.042) and TT (*P*=0.025). Haplotype analysis showed association between GGAG haplotype for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200 with MTX gastrointestinal toxicity (*P*=0.029). TRI revealed that patients with index 4 were 9-fold more likely to present a gastrointestinal disorder when compared to those with index 1 (*P*=0.020). This study revealed that *SLC19A1*, *SLC46A1* and *SLCO1B1* genotypes may help to identify patients with increased risk of MTX-related overall toxicity and that *SLC19A1* and *SLCO1B1* genotypes, and *SLC19A1* haplotypes may help to identify patients with increased risk of MTX-related gastrointestinal toxicity.

Keywords: Methotrexate; Polymorphisms; Rheumatoid arthritis; Transporters; Toxicity; Toxicogenetic risk index.

INTRODUCTION

Rheumatoid Arthritis (RA) is a complex, systemic autoimmune disease, characterized by a chronic inflammation of multiple peripheral joints (Smith *et al.*, 2011), with a worldwide prevalence of 0.3% to 1.1% and incidence of 9 to 60 cases *per* 100.000 inhabitants (Silman and Pearson, 2002). In Portugal, the prevalence of this disease is 0.36% and the incidence is between 20 and 40 cases *per* 100 000 inhabitants (Branco and Canhao, 2011; Dias, 2001).

Methotrexate (MTX) is currently the most widely used disease-modifying antirheumatic drug (DMARD) for RA treatment (Benucci *et al.*, 2011) in doses up to 25mg per week. Nevertheless, treatment with MTX is not devoid of drawbacks and significant adverse drug reactions (ADRs) can occur due to interpatient variability (Benucci, *et al.*, 2011; Kremer, 2004). This variability can be consequence of MTX pharmacokinetics (PK) changes partly due to single nucleotide polymorphisms (SNPs) in genes that codify for MTX membrane transporter proteins - influx and/or efflux (Bohanec Grabar *et al.*, 2012; Bohanec Grabar *et al.*, 2008; Chatzikyriakidou *et al.*, 2007; Moncrieffe *et al.*, 2010; Plaza-Plaza *et al.*, 2012; Trevino *et al.*, 2009). MTX transporters are expressed in several tissues (International Transporter *et al.*, 2010; Mikkelsen *et al.*, 2011; Qiu *et al.*, 2006) which can affect absorption, distribution and/or elimination. In gastrointestinal tract, at apical membrane of enterocytes, MTX is absorbed through active transport mediated by solute carrier (SLC) family 19 member 1 (SLC19A1) and possibly by SLC family 46 member 1 (SLC46A1) (Qiu, *et al.*, 2006; Swierkot and Szechinski, 2006; Tian and Cronstein, 2007). As SLC19A1-mediated transport is saturable, MTX may also be transported by folate receptor alpha (FOLR1) (Spinella *et al.*, 1995; Tian and Cronstein, 2007). Moreover, MTX effluxes from enterocytes to intestinal tract lumen can be mediated by ATP-binding cassette transporters (ABCs), i.e., by ABC sub-family C member 2 (ABCC2), ABC sub-family B member 1 (ABCB1) and ABC sub-family G member 2 (ABCG2), or to

bloodstream by ABC sub-family C member 1 (ABCC1) and ABC sub-family C member 3 (ABCC3) (Mikkelsen, *et al.*, 2011). MTX hepatic uptake involves SLC19A1, SLC organic anion transporter family member 1B1 (SLCO1B1) and SLCO family member 1B3 (SLCO1B3) (Mikkelsen, *et al.*, 2011); and, most of the MTX in hepatocytes reenters in bloodstream by ABCC3 and ABC sub-family C member 4 (ABCC4) and only a small portion is excreted into the bile duct by ABCC2, ABCB1 and ABCG2 (International Transporter, *et al.*, 2010; Mikkelsen, *et al.*, 2011). MTX clearance is mainly through renal glomerular filtration and active secretion over the proximal tubular cells (Mikkelsen, *et al.*, 2011). Several renal transporters have an affinity for MTX, allowing MTX influx into renal cells by SLC family 22 member 6 (SLC22A6) and SLC family 22 member 8 (SLC22A8), in basolateral membrane, and by SLC family 22 member 11 (SLC22A11) and SLCO family member 1A2 (SLCO1A2), in apical membrane (Inoue and Yuasa, 2014; Mikkelsen, *et al.*, 2011). Moreover, SLC family 16 member 7 (SLC16A7) has been described as having a moderate to low expression in plasma membrane of tubular cells, but its function on MTX transport remains unclear (Halestrap, 2013). Furthermore, MTX excretion through urinary tract can be mediated by ABCB1, ABCC2, ABCC4 and ABCG2 (Benucci, *et al.*, 2011; Swierkot and Szechinski, 2006) (Figure 1). Since MTX therapeutic outcome can be conditioned by PK changes we aimed to elucidate the influence of SNPs in genes that codify for MTX membrane transporter proteins on the occurrence of MTX-related toxicity in Portuguese RA patients.

MATERIALS AND METHODS

Study design

A retrospective study was performed between January 2009 and December 2012 at São João Hospital Center (Porto, Portugal) in a cohort of consecutive Portuguese Caucasian patients with RA treated with MTX. All patients (≥ 18 years) had to meet the 2010 revised classification criteria of American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) (Aletaha *et al.*, 2010) and received MTX for at least one month. Other

concomitant drugs, such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and other DMARDs were allowed during the study. Patients were excluded from the study if had drug abuse history, recent pregnancy or desire to become pregnant during the study. The study was approved by the local research ethics committee (reference 33/2009) and informed written consent was obtained from all patients according to the standards of the Helsinki Declaration.

Clinical assessments

Patient demographics, clinicopathological and treatment characteristics were collected from clinical records. Clinical efficacy was assessed using the Disease Activity Score in 28 joints (DAS28) as described by Prevoo *et al.* (Prevoo *et al.*, 1995). MTX-related toxicity was defined when patients presented any ADR related to MTX. At the time of each visit, physician directly asked the patient whether MTX-related ADRs were currently occurring and ADRs were recorded. The type of ADR was classified in System Organ Class (SOC) disorders, in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) (U.S.department of health and human services, 2010). Due to the well-known protective effect of folic acid supplementation for the prevention of toxicity occurrence (Lima *et al.*, 2013b; Ortiz *et al.*, 2000), this drug was prescribed to all patients and their regular compliance was registered.

Sample processing

Whole blood samples were obtained with standard venipuncture technique in ethylenediaminetetraacetic acid (EDTA) containing tubes. Genomic deoxyribonucleic acid (DNA) was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer instructions, and quantified using the NanoDrop® 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA).

SNPs selection and genotyping procedures

A total of twenty-three SNPs in ten genes that codify for MTX membrane transporter proteins were selected based on their putative effects on MTX transport function and/or MTX-related toxicity (Table 1). Sequenom® Assay Design 3.1 software was used to design the primers and genotyping was performed according to standard Sequenom® iPLEX protocol (Bradic *et al.*, 2011). Genomic sequence was amplified by multiplex polymerase chain reaction (PCR), the amplified product was treated with Shrimp alkaline phosphatase and used for allele specific primer extension (iPLEX) reaction (Sequenom®, Sand Diego, CA, USA). Reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the matrix-assisted laser desorption/ionization time-of-fight mass spectrometry (MALDI-TOF MS). Genotypes were assigned based on the presence of mass peaks by the MassARRAY Typer v4.0 software (Sequenom®, San Diego, CA, USA) (Bradic*, et al.*, 2011). Results were manually inspected and verified, using the MassARRAY Typer Analyzer v4.0 software (Sequenom®, San Diego, CA, USA). For quality control, 10% of the samples were randomly selected for a second analysis and results were 100% concordant.

Statistical analysis

Statistical analyses were performed with either IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA) and SNPStats software (Sole *et al.*, 2006). Genotype frequencies were assessed and tested for Hardy-Weinberg equilibrium (HWE). SNPs were excluded from analysis when genotyping call rates were less than 95% and when minor allele frequency was less than 10.0%. Binary logistic regression analysis was used to identify which genotypes were associated with MTX-related toxicity. Analysis was performed adjusting to potential confounders, i.e. clinicopathological variables possibly influencing MTX-related toxicity, selected based on literature review and clinical significance (Halilova *et al.*, 2012; Lima*, et al.*, 2013b; Morel and Combe, 2005). These variables included: 1) patient-related: gender, age, smoking and renal function (estimated glomerular filtration rate

– eGFR – and serum creatinine – SCr); 2) disease-related: diagnosis age and disease duration; and, 3) treatment-related: folic acid, corticosteroids, NSAIDs, other DMARDs and MTX administration characteristics (dose, treatment duration and administration route). Haplotype analysis was performed to assess possible consequences on the phenotype by the co-presence of several variants of the same gene. Linkage disequilibrium (LD) between SNPs in the same gene was estimated and expressed as *D´* coefficients. The measure was interpretable as the proportion of the maximum possible level of association between two *loci*, given the allele frequencies, ranging from 0 (linkage equilibrium) to 1 (complete LD) (Schaid *et al.*, 2002). Haplotype frequencies were estimated by SNPStats software. Possible haplotypes were tested for association with MTX-related toxicity by taking the most frequent haplotype as reference. Rare haplotypes (estimated haplotype frequency <2.0%) were excluded. A toxicogenetic risk index (TRI) was created for each patient by the sum of risk genotypes from the SNPs that revealed to be statistically significant when associated with MTX-related toxicity. Results were expressed as odds ratios (OR) with 95% confidence intervals (CI) and considering a probability (*P*) value of 5% or less as statistically significant.

RESULTS

Patients' characteristics

This study included follow-up data from 233 patients, 196 (84.1%) females and 37 (15.9%) males, with a median age of 52.0 (26.0-87.0) years old, of which 32 (13.7%) were smokers. In this population, the median of SCr was 8.20 mg/L (4.00-19.80), the median of eGFR was 82.0 ml/min/1.73m^2 (29.00-186.00) and 30 patients (12.9%) presented chronic renal insufficiency (eGFR <60 ml/min/1.73m^2). Considering disease-related variables, the mean diagnosis age was 40.3 ± 13.2 years old and the median disease duration was 10.0 years (0.3-51.0). Only 136 patients (58.4%) used MTX as unique DMARD, while 97 patients (41.6%) were treated with MTX combined with other synthetic or biological DMARDs. MTX was administered by *per os* (PO)

in 210 (90.1%) and by subcutaneous route (SC) in 23 (9.9%) of the patients, and the median MTX treatment duration was 47.0 months (1.0-240.0) with a median dose of 15.0 mg/week (2.5-25.0). Regarding concomitant drugs, other than MTX, 188 (80.7%) patients were under corticosteroids therapy, 170 patients (73.0%) used NSAIDs and 118 patients (50.6%) complied regularly with a folic acid supplementation. MTX-related toxicity was registered in 77 (33.0%) patients. Figure 2 represents the observed ADRs classified in SOCs disorders according to CTCAE.

MTX transporters SNPs: Genotypes and Haplotypes characteristics

Genotypes distribution of studied SNPs is represented in Table 2. *SLC22A6* rs11568626 and *ABCG2* rs2231142 were excluded from analysis since minor allele frequency was less than 10.0% and *ABCC1* rs2230671 was excluded from analysis because genotyping call rates were less than 95%. Taking this into account, twenty SNPs were considered. For *ABCB1* rs2032582, 3 patients (1.3%) presented AG genotype and 3 patients (1.3%) presented AT genotype. Given the low frequency, these 6 patients were excluded from the analysis. Genotypes distribution was in HWE (*P* >0.050) except for the *SLC19A1* G>A, rs1051266. Figure 3 demonstrates the estimated *D´* coefficients of the studied SNPs for the possible haplotypes in the same gene. *SLC16A7*, *SLC19A1*, *ABCB1*, *ABCC1*, *ABCC2* and *ABCG2* SNPs were in LD, except for *ABCC1* rs246240, and *ABCC1* rs2074087.

MTX transporters SNPs: Genotypes & MTX-related toxicity

Table 3 and Table 4 represent the relation between MTX-related toxicity and SNPs in *SLCs* and *ABCs*, respectively. Regarding MTX overall toxicity, our results demonstrated that for SNPs in *SLCs, SLC19A1* rs7499 G carriers (*P*=0.017, OR=3.72), *SLC46A1* rs2239907 G homozygotes (*P*=0.030, OR=2.32) and *SLCO1B1* rs4149056 T carriers and T homozygotes (*P*=0.040, OR=2.78; and *P*=0.019, OR=2.82; respectively) were associated with an increased risk for MTX-related overall toxicity. Considering the MTX gastrointestinal toxicity, similar results were shown for SNPs in *SLCs. SLC19A1* rs7499 G carriers and G homozygotes (*P*=0.012, OR=5.64; and *P*=0.045, OR=2.39;

respectively), *SLC19A1* rs1051266 G carriers (*P*=0.034, OR=3.07), *SLC19A1* rs2838956 (*P*=0.049, OR=3.21) and *SLCO1B1* rs4149056 T carriers and T homozygotes (*P*=0.042, OR=3.09; and *P*=0.025, OR=2.92, respectively) were associated with MTX-related gastrointestinal toxicity. No statistically significant differences were observed in relation to the SNPs in *ABCs* for MTX-related overall and/or gastrointestinal toxicity.

MTX transporters SNPs: Haplotypes & MTX-related toxicity

Table 5 represents the relation between MTX transporters genes haplotypes and MTX-related toxicity. Since *ABCC1* rs246240 and rs2074087 were not in LD, analyses were performed considering the following combinations: 1) *ABCC1* rs35592, rs246240 and rs3784864; and 2) *ABCC1* rs35592, rs2074087 and rs3784864. Results showed that no statistically significant differences were observed in relation to MTX transporters genes haplotypes and MTX-related overall toxicity. Nevertheless, for MTX gastrointestinal toxicity, our results demonstrated that GGAG haplotype (constituted by ancestral alleles) for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200, respectively, was associated with MTX-related gastrointestinal toxicity when compared to AAGA haplotype (constituted by minor alleles) (*P*=0.029). No statistically significant differences were observed for the remaining haplotypes.

MTX transporters SNPs: Toxicogenetic risk index for MTX

A TRI for MTX-related toxicity was created for each patient by the sum of risk genotypes from the SNPs that revealed to be statistically significant when associated with MTX-related toxicity (see Table 3 and Table 4). The TRI was adjusted for potential confounders as described in the Methods section. Regarding MTX overall toxicity, the risk genotypes were as follow: *SLC19A1* rs7499 G carriers, *SLC46A1* rs2239907 G homozygotes and *SLCO1B1* rs4149056 T carriers. Figure 4 represents the contribution of the TRI to the occurrence of MTX-related overall toxicity in RA patients treated with MTX. The number (%) of patients is given for each incremental unit of the index. The TRI ranged from 0 to 3. An increased TRI value was associated with an

increased incidence of ADRs (*P*=0.020). Patients with index 2 were 3.06 times (95% CI: 1.04-8.99) more likely to present a ADR when compared to those with index 1 (*P*=0.042), patients with index 3 were 3.52 times (95% CI: 1.37-9.03) more likely to present a ADR when compared to those with index 2 (*P*=0.009) and patients with index 3 were 18.79 times (95% CI: 3.39-104.12) more likely to present a ADR when compared to those with index 1 (*P*=0.001). Regarding MTX gastrointestinal toxicity, the risk genotypes were as follow: *SLC19A1* rs7499 G carriers, *SLC19A1* rs1051266 G carriers, *SLC19A1* rs2838956 A carriers and *SLCO1B1* rs4149056 T carriers. Figure 5 represents the contribution of the TRI to the occurrence of MTX-related gastrointestinal toxicity in RA patients under MTX. The number (%) of patients is given for each incremental unit of the index. The TRI ranged from 0 to 4 and an increased TRI value was associated with an increased incidence of gastrointestinal disorders (*P*=0.010). No statistically significant differences were observed between patients with index 1 and patients with index 2 (*P*=1.000) and between patients with index 2 and patients exhibiting index 3 (*P*=0.429). Patients with index 4 were 5.11 times (95% CI: 1.56-16.70) more likely to present gastrointestinal disorders compared to those with index 3 (*P*=0.007) and patients with index 4 were 9.50 times (95% CI: 1.43-62.95) more likely to present gastrointestinal disorders when compared to those with index 1 (*P*=0.020).

DISCUSSION

MTX therapeutic outcome can be altered by several factors such as SNPs in genes that codify for MTX membrane transporter proteins. This study evaluated the influence of 23 SNPs in *SLCs* and *ABCs* MTX transporters as predictors of MTX-related toxicity in Portuguese RA patients. Hence, we have performed genotype and haplotype based approaches, considering a toxicogenetic risk index, with multivariate logistic regression analysis adjusted to potential confounders. Genotypes distribution of studied SNPs were in HWE and were similar to those previously described for Caucasian populations (de Rotte *et al.*, 2012; Moncrieffe, *et al.*, 2010; Owen *et al.*,

2013) and in the National Center for Biotechnology Information (NCBI) database, except for the *SLC19A1* rs1051266. Regarding the observed ADRs, results were in accordance with literature and associated with weekly administration of MTX in low-dose, since the most frequent ADRs were gastrointestinal disorders (Bohanec Grabar*, et al.*, 2012; Kremer, 2004).

Genotypes & MTX-related toxicity

SLC19A1 is a bidirectional transporter, described as being expressed in the majority of tissues, with relevance in enterocytes and hepatocytes (Hinken*, et al.*, 2011; Qiu*, et al.*, 2006). Accordingly to genotype analysis, our results demonstrated that *SLC19A1* rs7499 G carriers were associated with an increased risk for MTX-related toxicity (overall and gastrointestinal). This SNP occurs in the 3´-untranslated region (UTR) in a region thought to be important for messenger ribonucleic acid (mRNA) stability, localization and translational efficiency (Lynch *et al.*, 2005) and, therefore, important to membrane transporter expression. Nevertheless, its effect in this bidirectional transporter function is unknown. We propose that GG genotype could provide an increased influx capability leading to higher bioavailability, increased MTX tissues exposure and, consequently, to toxicity. In literature, only one study in Caucasian RA patients dealt with the influence of this SNP in MTX-related toxicity, but no association was found (Owen*, et al.*, 2013). Regarding *SLC19A1* rs1051266, our results showed that G carriers were associated with MTX-related gastrointestinal toxicity but no associations were observed in accordance to MTX overall toxicity. Bohanec Grabar *et al.* have previously reported that G homozygotes had a higher risk for MTX overall toxicity (Bohanec Grabar*, et al.*, 2012; Bohanec Grabar*, et al.*, 2008) but did not clearly described the impact on gastrointestinal ADRs, and the majority of other studies did not have associations with toxicity (Chatzikyriakidou*, et al.*, 2007; Owen*, et al.*, 2013; Plaza-Plaza*, et al.*, 2012). Thus, these controversial studies regarding to this SNP and MTX-related gastrointestinal toxicity need further clarification. We hypothesize that GG genotype could provide an increased influx capability leading to higher

bioavailability, increased MTX tissues exposure, mainly in tissues where SLC19A1 is highly expressed and, consequently, to gastrointestinal toxicity. Furthermore, is important to consider if enterohepatic recirculation contributes to major differences in bioavailability, reinforcing the importance of understanding the role of transporters in this pathway. In addition, A carriers for *SLC19A1* rs2838956, had a borderline trend towards significance for MTX-related toxicity (overall and gastrointestinal). This is possibly caused by an increased influx capability of A carriers, which consequently, leads to greater MTX tissues exposure and toxicity. Our results are in accordance with a previously report by Bohanec Grabar *et al.* that demonstrated a borderline significant trend towards MTX-related overall toxicity for A carriers, particularly for skin and subcutaneous tissue disorders (Bohanec Grabar, *et al.*, 2012), yet other study reported no statistically significant association (Owen, *et al.*, 2013). The impact of this variant in SLC19A1 is currently unknown and functional studies are essential since intronic SNPs can potentially influence RNA splicing, which may affect transporter structure and function (Wang and Cooper, 2007).

In relation to *SLC46A1* rs2239907, G homozygotes revealed association with MTX overall toxicity. This SNP is also located in a 3´UTR region, which is thought to be important in mRNA stability, localization and translational efficiency (Lynch, *et al.*, 2005) and then considered as a potential functional SNP. However, the effect of this SNP in the transporter function is currently unknown. SLC46A1 is mostly expressed in apical membrane of enterocytes but also can be found in other cells (Qiu, *et al.*, 2006). Thus, we hypothesized that GG genotype could provide an increased influx leading to higher bioavailability and, consequently, to higher MTX tissues exposure and toxicity. Nevertheless, the complexity of these mechanisms and the presence of other factors that could also play a role, such as the putative contribution of enterohepatic recirculation as well as its possible influence in renal function deserves further consideration. To our best knowledge this is the first report to analyze the effect of this SNP with MTX-related toxicity in RA.

Regarding *SLCO1B1* rs4149056, T carriers were associated with MTX-related toxicity (overall and gastrointestinal). T carriers have been associated with an increased membrane expression of SLCO1B1 and higher MTX influx and clearance (Trevino*, et al.*, 2009). Despite the SLCO1B1 transporter is mainly expressed on basolateral membrane of hepatocytes (Konig*, et al.*, 2000), its mRNA also has been detected in other tissues, including small intestinal enterocytes (Glaeser *et al.*, 2007), which can explain the MTX intracellular retention (gastrointestinal and hepatic) leading to cytotoxicity. The association of T allele with MTX-related toxicity has been previously described for high-dose MTX (Trevino*, et al.*, 2009) but this is the first report to analyze the influence of *SLCO1B1* rs4149056 with MTX-related toxicity in RA.

Accordingly to *ABCG2* rs13120400, C carriers had a borderline trend towards significance for MTX-related toxicity. ABCG2 transporter, located in apical membranes of enterocytes, hepatocytes and kidney tubular cells, is responsible for MTX efflux from the enterocytes to intestinal tract lumen and MTX excretion into bile and urine (Mikkelsen*, et al.*, 2011). Then, it is plausible to explain our results as follows: C carriers should cause a reduced efflux capability, which is translated, in less MTX elimination and higher MTX bioavailability, thus leading to toxicity. This is the first report to associate this SNP with MTX-related toxicity in RA patients.

Haplotypes & MTX-related toxicity

Haplotypes may have a particular significance in regard to functionality or as genetic markers for unknown functional variants, claiming for full haplotypic information to be encompassed into studies in order to better characterize the role of a candidate gene (Hodge *et al.*, 1999; Lima *et al.*, 2013a). In fact, haplotypes constituted by SNPs with both unknown and known impact in transporter functions could provide a putative association of these, yet unknown variants, towards depicting the role of transporters function in toxicity development. Therefore, haplotype analysis was performed, to assess of possible consequences on the phenotype in the co-presence of

several variants of the same gene. From haplotypes analysis, our results showed that GGAG haplotype for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200, was associated with MTX-related gastrointestinal toxicity when compared to AAGA haplotype. The association of GGAG haplotype with gastrointestinal toxicity was expected from the genotype analyses obtained results for those SNPs in *SLC19A1*. Considering this, we can hypothesize that GGAG haplotype could provide an increased MTX influx capability, leading to higher MTX intracellular levels, mainly in tissues where SLC19A1 is highly expressed (Qiu*, et al.*, 2006) and, thus, have an increased risk for gastrointestinal toxicity development.

Toxicogenetic risk index for MTX

To an improved characterization of the impact of studied SNPs that were statistically significant associated with MTX-related toxicity, a TRI was created, both for overall and gastrointestinal toxicity. Accordingly to MTX overall toxicity, an increased TRI value was associated with an increased incidence of ADRs. Our results demonstrated that patients with index 3 were 18-fold times more likely to present an ADR when compared to those with index 1. Regarding to the occurrence of gastrointestinal disorders, the TRI demonstrated that patients with index 4, when compared to those with index 3 and 1, were 5-fold and 9-fold, respectively, more likely to present gastrointestinal disorders. This highlights the importance of genotyping patients and the urgency of developing the field of therapy personalization for the prediction of MTX-related toxicity development.

Besides the potential importance of our results, we are aware of possible study limitations such the sample size and the study design. Despite this, our data are supported by the fact that: 1) our population is relatively homogenous regarding ethnic origin (all Caucasians from the North region of Portugal) with a prevalence of RA similar to other countries; 2) studied patient group characteristics were in accordance with other reported studies in regard to disease gender epidemiology (Gibofsky, 2012) and to diagnosis age range (Rindfleisch and Muller, 2005); 3) statistical analyses were

performed attending to potential confounder variables limiting the selection bias; and 4) having studied twenty-three SNPs in genes that codify for MTX membrane transporter proteins, many of which had never been studied before, in both RA and Caucasian populations. Moreover, and due the low frequency of SOC disorders, other than gastrointestinal disorders in our population, the influence of the studied SNPs in there occurrence could not be performed. Knowing that MTX transporters are expressed in different tissues, this line of investigation could be proved of remarkable relevance since it would enable the prediction of toxicity in each tissue and help to guide therapeutic choices. Moreover, the influence of SNPs in MTX transporters genes in MTX circulating levels should be performed in order to elucidate SNPs impact in transport function and toxicity development. Despite this, we have to consider that MTX retention is also dependent of MTX polyglutamation levels and, thus, genetic polymorphisms in enzymes involved in MTX polyglutamation process should also be evaluated.

Other studies with similar approach demonstrated irrelevant results concerning the associations of discussed SNPs with MTX-related toxicity. Several explanations may be proposed to clarify these discrepancies as follow: differences in genotyping methodologies, ethnic origins, study population size and possible confounding variables not considered in studies; majority of authors do not define similarly the concept of toxicity; and, most of studies did not follow standard guidelines for ADRs classification. Those reasons concur to some erratic conclusions to be taken and render adequate comparison between studies difficult to pursuit. Therefore, and due to the lack of SNPs combined studies using both functional and/or associated with MTX-related toxicity, further evidence is necessary to support the interpretation of our results and elucidate previous inconsistent results.

Final conclusions

From this study, we can conclude that of all studied SNPs in *ABCs*, only *ABCG2* rs13120400 seems to be associated with MTX-related toxicity

occurrence. Interestingly, it also reveals that SNPs in *SLCs* should be helpful to elucidate which patients will benefit from MTX treatment since *SLC19A1* rs7499, *SLC46A1* rs2239907 and *SLCO1B1* rs4149056, appeared to be associated with increased risk for MTX overall toxicity and *SLC19A1* rs7499, *SLC19A1* rs1051266, *SLC19A1* rs2838956 and *SLCO1B1* rs4149056, showed association with MTX gastrointestinal toxicity. Furthermore, *SLC19A1* haplotypes may help to identify patients with increased risk of developing MTX gastrointestinal toxicity. Additionally, the proposed toxicogenetic risk index highlights the importance of genotyping patients and the urgency of developing the field of therapy personalization for the prediction of MTX-related toxicity development.

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ABBREVIATIONS LIST

A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; ACR: American College of Rheumatology; ADR: adverse drug reaction; BCRP: breast cancer resistance protein; C: cytosine; Chr: chromosome; CI: confidence interval; CTCAE: Common Terminology Criteria for Adverse Events; DAS28: Disease Activity Score in 28 joints; DMARDs: disease modifying anti-rheumatic drugs; EDTA: ethylenediaminetetraacetic acid; eGFR: estimated glomerular filtration rate; EULAR: European League Against Rheumatism; FOLT: folate transporter; G: guanine; Gln: glutamine; Gly: glycine; HCP1: heme carrier protein 1; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid – transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MTX: methotrexate; NSAIDs: non-steroidal anti-inflammatory drugs; OAT: organic anion transporter; OR: odds ratio; P-GP: P-glycoprotein; PCFT: proton-

coupled folate transporter; PK: pharmacokinetics; RA: rheumatoid arthritis; Ref: reference; RFC1: reduced folate carrier 1; SCr: serum creatinine; Ser: serine; SNP: single nucleotide polymorphisms; SLC: solute carrier; SOC: System Organ Class; T: thymine; Thr: threonine; TRI: toxicogenetic risk index; UTR: untranslated region; Val: valine.

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FIGURES

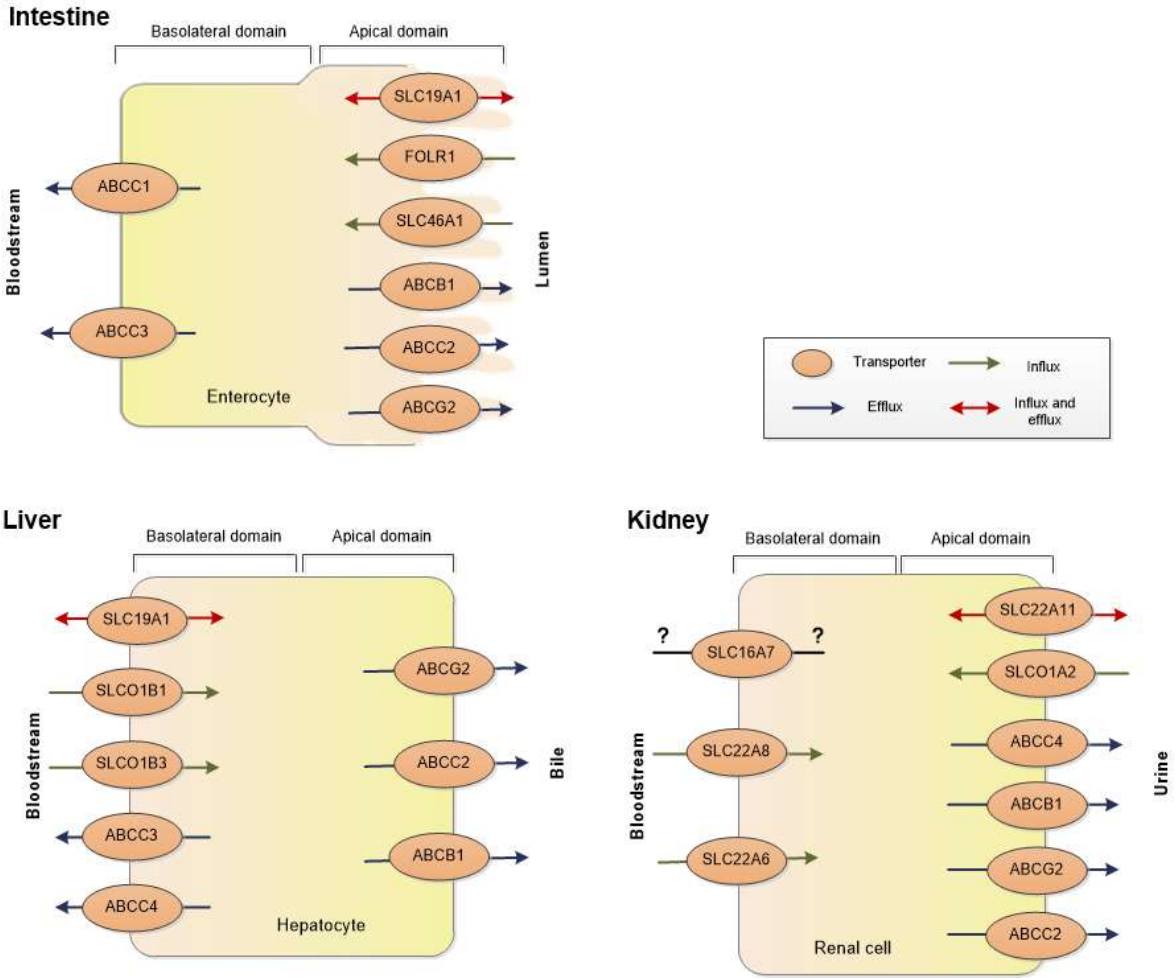

Figure 1. Methotrexate membrane transporter proteins location and representation of their influx and/or efflux function.

ABCB1: ATP-binding cassette sub-family B member 1; ABCC1: ATP-binding cassette sub-family C member 1; ABCC2: ATP-binding cassette sub-family C member 2; ABCC3: ATP-binding cassette sub-family C member 3; ABCC4: ATP-binding cassette sub-family C member 4; ABCG2: ATP-binding cassette sub-family G member 2; FOLR1: folate receptor alpha; SLC16A7: solute carrier family 16 member 7; SLC19A1: solute carrier family 19 member 1; SLC22A6: solute carrier family 22 member 6; SLC22A8: solute carrier family 22 member 8; SLC22A11: solute carrier family 22 member 11; SCL46A1: solute carrier family 46 member 1; SLCO1A2: solute carrier organic anion transporter family member 1A2; SLCO1B1: solute carrier organic anion transporter family member 1B1; SLCO1B3: solute carrier organic anion transporter family member 1B3.

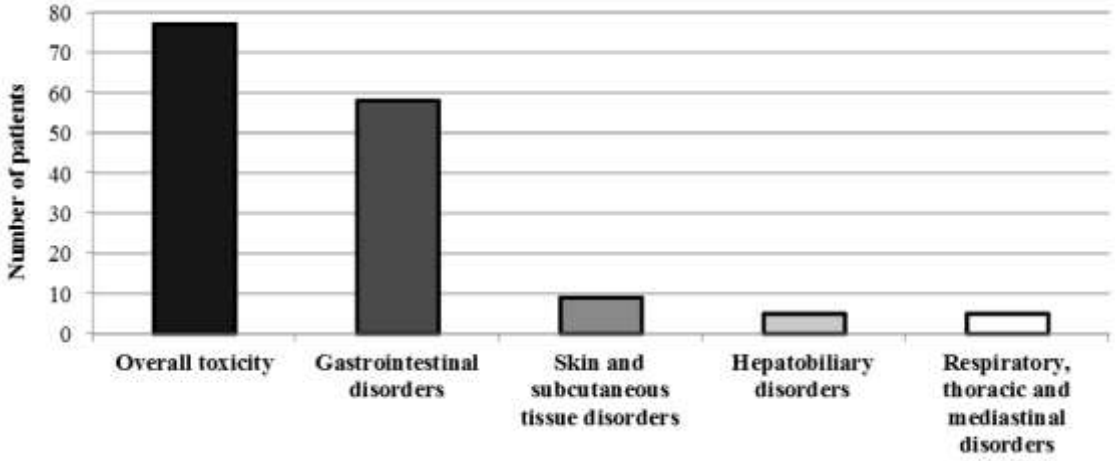

Figure 2. Observed MTX-related ADRs by SOCs disorders.

Gastrointestinal disorders included anorexia, abdominal distension, diarrhea, dyspepsia, nauseas, stomach pain and/or vomiting. Skin and subcutaneous tissue disorders included alopecia, rash maculo-papular and rheumatoid nodulosis exacerbation. Hepatobiliary disorders included transaminases serum elevation. Respiratory, thoracic and mediastinal disorders included hypersensitivity pneumonitis.

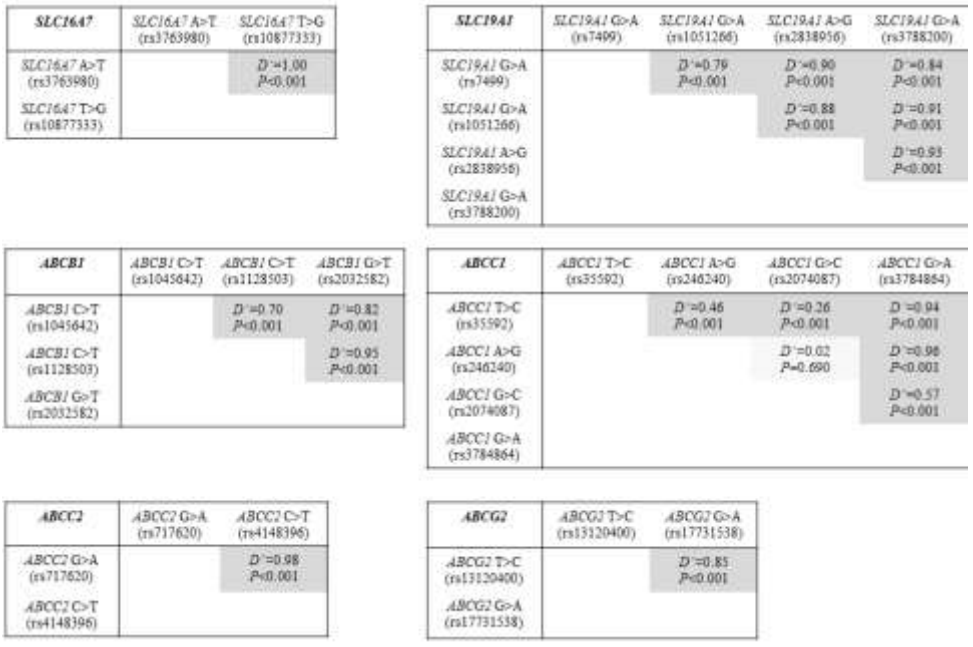

Figure 3. Estimated *D´* coefficients and *P* values for possible haplotypes.

Dark gray boxes represent the single nucleotide polymorphisms that were in linkage disequilibrium.

A: adenine; ABCB1: ATP-binding cassette sub-family B member 1; ABCC1: ATP-binding cassette sub-family C member 1; ABCC2: ATP-binding cassette sub-family C member 2; ABCG2: ATP-binding cassette sub-family G member 2; C: cytosine; G: guanine; SLC16A7: solute carrier family 16 member 7; SLC19A1: solute carrier family 19 member 1; T: thymine.

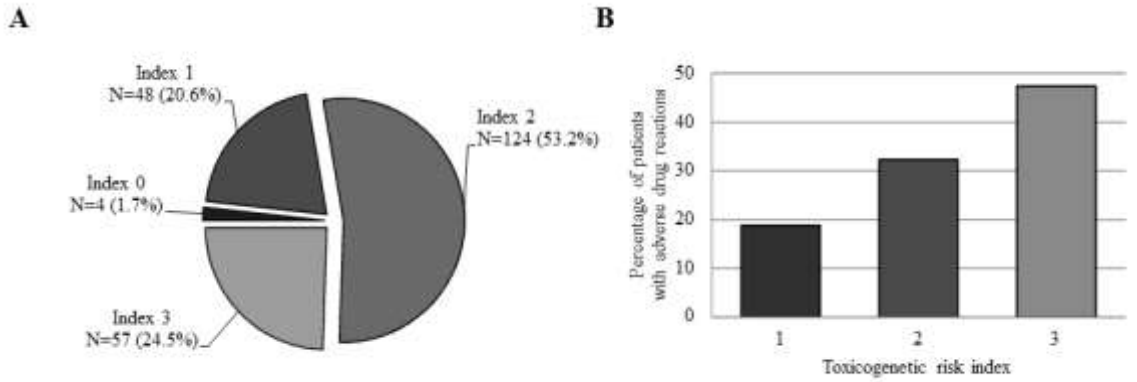

Figure 4. A. Contribution of the toxicogenetic risk index in MTX-related overall toxicity occurrence. B. Percentage of RA patients with MTX-related ADRs in relation to toxicogenetic risk index for overall toxicity.

Index 0 included *SLC19A1* rs7499 A homozygotes + *SLC46A1* rs2239907 T carriers + *SLCO1B1* rs4149056 C homozygotes. Index 1 included *SLC19A1* rs7499 G carriers, *SLC46A1* rs2239907 G homozygotes or *SLCO1B1* rs4149056 T carriers. Index 2 included *SLC19A1* rs7499 G carriers + *SLC46A1* rs2239907 G homozygotes; *SLC19A1* rs7499 G carriers + *SLCO1B1* rs4149056 T carriers; and, *SLC46A1* rs2239907 G homozygotes + *SLCO1B1* rs4149056 T carriers. Index 3 included *SLC19A1* rs7499 G carriers + *SLC46A1* rs2239907 G homozygotes + *SLCO1B1* rs4149056 T carriers.

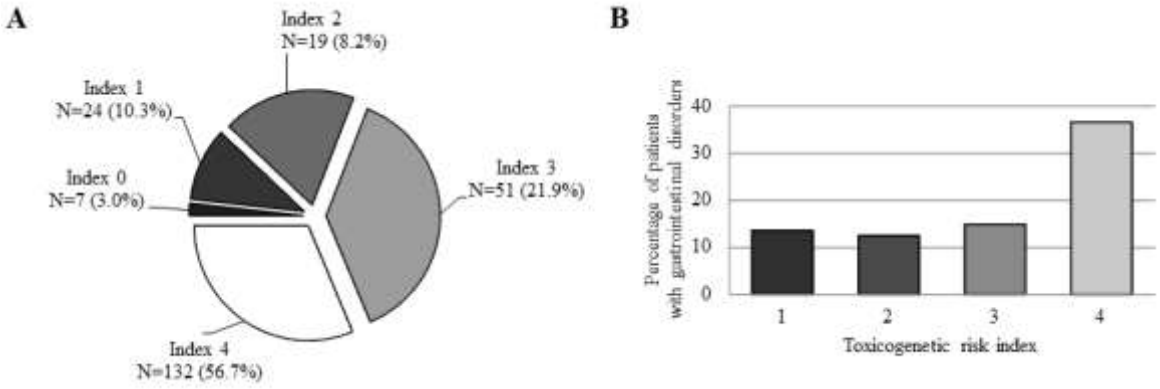

Figure 5. A. Contribution of the toxicogenetic risk index in MTX-related gastrointestinal toxicity occurrence. B. Percentage of RA patients with MTX-related gastrointestinal disorders in relation to toxicogenetic risk index for gastrointestinal toxicity.

Index 0 included *SLC19A1* rs7499 A homozygotes + *SLC19A1* rs1051266 A homozygotes + *SLC19A1* rs2838956 G homozygotes + *SLCO1B1* rs4149056 C homozygotes. Index 1 included *SLC19A1* rs7499 G carriers, *SLC19A1* rs1051266 G carriers, *SLC19A1* rs2838956 A carriers or *SLCO1B1* rs4149056 T carriers. Index 2 included *SLC19A1* rs7499 G carriers + *SLC19A1* rs1051266 G carriers; *SLC19A1* rs7499 G carriers + *SLC19A1* rs2838956 A carriers; *SLC19A1* rs7499 G carriers + *SLCO1B1* rs4149056 T carriers; *SLC19A1* rs1051266 G carriers + *SLC19A1* rs2838956 A carriers; *SLC19A1* rs1051266 G carriers + *SLCO1B1* rs4149056 T carriers; and, *SLC19A1* rs2838956 A carriers + *SLCO1B1* rs4149056 T carriers. Index 3 included *SLC19A1* rs7499 G carriers + *SLC19A1* rs1051266 G carriers + *SLC19A1* rs2838956 A carriers; *SLC19A1* rs7499 G carriers + *SLC19A1* rs1051266 G carriers + *SLCO1B1* rs4149056 T carriers; *SLC19A1* rs7499 G carriers + *SLC19A1* rs2838956 A carriers + *SLCO1B1* rs4149056 T carriers; and, *SLC19A1* rs1051266 G carriers + *SLC19A1* rs2838956 A carriers + *SLCO1B1* rs4149056 T carriers. Index 4 included *SLC19A1* rs7499 G carriers + *SLC19A1* rs1051266 G carriers + *SLC19A1* rs2838956 A carriers + *SLCO1B1* rs4149056 T carriers.

TABLES

Table 1. Characteristics and putative effects of 23 SNPs in MTX transport function and related toxicity

Genes	Protein name	Chr	rs ID	Alleles	Region (aa change)	Putative effects		Ref.
						MTX Transport Function	MTX-related Toxicity	
Solute carriers								
SLC16A7	MCT2	12	rs3763980	A>T	Exon (Thr445Ser)	T allele: ↑ influx	-	(Halestrap, 2013)
			rs10877333	T>G	Intron	-	-	-
SLC19A1	RFC1 FOLT	21	rs7499	G>A	3´UTR	-	n.a.	(Hinken *et al.*, 2011; Owen *et al.*, 2012)
			rs1051266	G>A	Exon (Arg27His)	AA: ↑ influx	A carriers: ↓ toxicity	(Baslund *et al.*, 2008; Bohanec Grabar, *et al.*, 2012; Bohanec Grabar, *et al.*, 2008; Chatzikyriakidou, *et al.*, 2007; Lima, *et al.*, 2013b)
			rs2838956	A>G	Intron	-	A carriers: ↑ toxicity	(Bohanec Grabar, *et al.*, 2012)
			rs3788200	G>A	Intron	AA: ↓ influx	-	(O'Byrne *et al.*, 2010)
SLC22A6	OAT1	11	rs11568626	C>T	Exon (Arg50His)	TT: ↓ influx	-	(Bleasby *et al.*, 2005; Sun *et al.*, 2001)
SLC22A11	OAT4	11	rs11231809	T>A	Intron	AA: ↓ clearance	-	(Vormfelde *et al.*, 2006)
SLC46A1	HCP1 PCFT	17	rs2239907	G>A	3´UTR	-	-	-
SLCO1B1	LST1 OATP1B1	12	rs4149056	T>C	Exon (Val174Ala)	CC: ↓ influx and clearance	-	(Konig *et al.*, 2000; Trevino, *et al.*, 2009)
ATP-binding cassette transporters								
ABCB1	MDR P-GP	7	rs1045642	C>T	Exon (Ile1145Ile)	TT: ↓ efflux	TT: ↑ toxicity	(Bohanec Grabar, *et al.*, 2008; Kooloos *et al.*, 2010; Wang *et al.*, 2005)
			rs1128503	C>T	Exon (Gly412Gly)	-	TT: ↑ toxicity	(Ranganathan *et al.*, 2008)
			rs2032582	G>A/T	Exon (Ala899Ser/Thr)	A/T alleles: ↓ efflux	n.a.	(Bohanec Grabar, *et al.*, 2008)
ABCC1	MRP1	16	rs35592	T>C	Intron	-	n.a.	(Berggren *et al.*, 2007; Stamp *et al.*, 2010)
			rs246240	A>G	Intron	-	-	-
			rs2074087	G>C	Intron	-	n.a.	(Ranganathan, *et al.*, 2008)
			rs2230671	C>G	Exon (Ser1334Ser)	GG: ↑ efflux	n.a.	(Ranganathan, *et al.*, 2008)
			rs3784864	G>A	Intron	-	-	-
ABCC2	MRP2	10	rs717620	G>A	5´UTR	AA: ↑ clearance	n.a.	(Ranganathan, *et al.*, 2008; Simon *et al.*, 2013)
			rs4148396	C>T	Intron	-	CC: ↑ toxicity	(Ranganathan, *et al.*, 2008; Stamp, *et al.*, 2010)
ABCG2	BCRP	4	rs2231142	C>A	Exon (Gln141Lys)	AA: ↓ efflux	AA: ↑ toxicity	(Stamp, *et al.*, 2010; Zhang *et al.*, 2013)
			rs13120400	T>C	Intron	-	-	-
			rs17731538	G>A	Intron	-	n.a.	(Stamp, *et al.*, 2010)

-: Unknown; n.a.: not associated.
A: adenine; aa: amino acid; Ala: alanine; Arg: arginine; ABC: ATP-binding cassette; BCRP: breast cancer resistance protein; C: cytosine; Chr: chromosome; FOLT: folate transporter; G: guanine; Gln: glutamine; Gly: glycine; HCP1: heme carrier protein 1; His: histidine; ID: identification; Ile: isoleucine; Lys: lysine; LST1: liver-specific transporter 1; MCT2: monocarboxylic acid – transporter 2; MDR: multidrug resistance protein; MRP: multidrug resistance-associated protein; MTX: methotrexate; OAT: organic anion transporter; PCFT: proton-coupled folate transporter; P-GP: P-glycoprotein; Ref: reference; RFC1: reduced folate carrier 1; Ser: serine; SNP: single nucleotide polymorphism; SLC: solute carrier T: thymine; Thr: threonine; UTR: untranslated region; Val: valine.

Table 2. Genotype distribution for the 23 studied SNPs

Genes	rs ID	Alleles	Genotype frequency		
			Ancestral allele homozygotes	Heterozygotes	Minor allele homozygotes
Solute carriers					
SLC16A7	rs3763980	A>T	119 (51.1)	92 (39.5)	22 (9.4)
	rs10877333	T>G	161 (69.1)	68 (29.2)	4 (1.7)
SLC19A1	rs7499	G>A	91 (39.1)	104 (44.6)	38 (16.3)
	rs1051266	G>A	80 (34.3)	91 (39.1)	62 (26.6)
	rs2838956	A>G	82 (35.2)	109 (46.8)	42 (18.0)
	rs3788200	G>A	82 (35.2)	109 (46.8)	42 (18.0)
SLC22A6	rs11568626#	C>T	232 (99.6)	1 (0.4)	0 (0.0)
SLC22A11	rs11231809	T>A	80 (34.3)	120 (51.5)	33 (14.2)
SLC46A1	rs2239907	G>A	87 (37.3)	99 (42.5)	47 (20.2)
SLCO1B1	rs4149056	T>C	169 (72.5)	16 (6.9)	48 (20.6)
ATP-binding cassette transporters					
ABCB1	rs1045642	C>T	63 (27.0)	109 (46.8)	61 (26.2)
	rs1128503	C>T	77 (33.0)	107 (46.0)	49 (21.0)
	rs2032582*	G>A/T	81 (34.8)	99 (42.5)	47 (20.1)
ABCC1	rs35592	T>C	124 (53.2)	86 (36.9)	23 (9.9)
	rs246240	A>G	159 (68.3)	66 (28.3)	8 (3.4)
	rs2074087	G>C	146 (62.7)	76 (32.6)	11 (4.7)
	rs2230671#	C>G	117 (92.9)	9 (7.1)	0 (0.0)
	rs3784864	G>A	67 (28.8)	111 (47.6)	55 (23.6)
ABCC2	rs717620	G>A	135 (57.9)	91 (39.1)	7 (3.0)
	rs4148396	C>T	75 (32.2)	109 (46.8)	49 (21.0)
ABCG2	rs2231142#	C>A	200 (85.8)	33 (14.2)	0 (0.0)
	rs13120400	T>C	116 (49.8)	96 (41.2)	21 (9.0)
	rs17731538	G>A	137 (58.8)	87 (37.3)	9 (3.9)

SNPs were excluded from analysis when genotyping call rates were less than 95% and when minor allele frequency was less than 10.0%.
* 6 patients were excluded from the analyses since 3 (1.3%) presented AG genotype and 3 (1.3%) presented AT genotype.
A: adenine; ABC: ATP-binding cassette; C: cytosine; G: guanine; SLC: solute carrier; SLCO: solute carrier organic anion transporter; SNP: single nucleotide polymorphism; T: thymine.

Table 3. Relation between SNPs in *solute carriers* and MTX-related toxicity

	Model	Alleles	MTX overall toxicity		MTX gastrointestinal toxicity	
			P	OR (95% CI)	*P*	OR (95% CI)
SLC16A7 A>T (rs3763980)	Dominant	A carriers		Reference		Reference
		TT	0.581	0.72 (0.22-2.35)	0.590	0.70 (0.19-2.55)
	Recessive	AA		Reference		Reference
		T carriers	0.447	1.34 (0.63-2.85)	0.539	1.29 (0.57-2.91)
SLC16A7 T>G (rs10877333)	Dominant	T carriers		Reference		Reference
		GG	0.636	0.53 (0.04-7.31)	0.843	0.77 (0.06-10.00)
	Recessive	TT		Reference		Reference
		G carriers	0.761	1.23 (0.52-2.42)	0.842	1.09 (0.48-2.43)
SLC19A1 G>A (rs7499)	Dominant	G carriers		Reference		Reference
		AA	0.017	0.27 (0.09-0.79) [a)]	0.012	0.18 (0.05-0.69) [b)]
	Recessive	GG		Reference		Reference
		A carriers	0.109	0.54 (0.25-1.15)	0.045	0.43 (0.19-0.98) [c)]
SLC19A1 G>A (rs1051266)	Dominant	G carriers		Reference		Reference
		AA	0.150	0.53 (0.23-1.26)	0.034	0.33 (0.12-0.92) [d)]
	Recessive	GG		Reference		Reference
		A carriers	0.690	0.86 (0.40-1.82)	0.512	0.77 (0.35-1.70)
SLC19A1 A>G (rs2838956)	Dominant	A carriers		Reference		Reference
		GG	0.055	0.37 (0.14-1.02)	0.049	0.31 (0.10-1.00) [e)]
	Recessive	AA		Reference		Reference
		G carriers	0.170	0.60 (0.28-1.25)	0.126	0.54 (0.24-1.19)
SLC19A1 G>A (rs3788200)	Dominant	G carriers		Reference		Reference
		AA	0.140	0.48 (0.18-1.27)	0.078	0.36 (0.12-1.12)
	Recessive	GG		Reference		Reference
		A carriers	0.158	0.58 (0.28-1.23)	0.113	0.53 (0.24-1.16)
SLC22A11 T>A (rs11231809)	Dominant	T carriers		Reference		Reference
		AA	0.806	0.89 (0.34-2.33)	0.506	0.70 (0.24-2.02)
	Recessive	TT		Reference		Reference
		A carriers	0.906	0.95 (0.43-2.09)	0.716	1.17 (0.50-2.78)
SLC46A1 G>A (rs2239907)	Dominant	G carriers		Reference		Reference
		AA	0.605	0.79 (0.32-1.94)	0.859	0.92 (0.36-2.33)
	Recessive	GG		Reference		Reference
		A carriers	0.030	0.43 (0.20-0.92) [f)]	0.155	0.55 (0.24-1.25)
SLCO1B1 T>C (rs4149056)	Dominant	T carriers		Reference		Reference
		CC	0.040	0.36 (0.14-0.96) [g)]	0.042	0.32 (0.11-0.96) [h)]
	Recessive	TT		Reference		Reference
		C carriers	0.019	0.36 (0.15-0.84) [i)]	0.025	0.34 (0.13-0.88) [j)]

***P* value <0.05 is considered to be of statistically significance (highlighted in bold).**

***P* value, odds ratio (OR) and 95% confidence intervals (CI) corresponds to multivariate logistic regression adjusted to patient-related variables (gender, age, smoking, eGFR and SCr), disease-related variables (diagnosis age and disease duration), and treatment-related variables (folic acid, corticosteroids, NSAIDs, other concomitant DMARDs and MTX administration characteristics such as dose, treatment duration and administration route).**

[a)]When reference was AA genotype: OR=3.72, 95% CI: 1.26-10.93. [b)]When reference was AA genotype: OR=5.64, 95% CI: 1.45-21.86. [c)]When reference was A carriers: OR=2.39, 95% CI: 1.06-5.38. [d)]When reference was AA genotype: OR=3.07, 95%CI: 1.09-8.65. [e)]When reference was GG genotype: OR=3.21, 95%CI: 1.00-10.25. [f)]When reference was A carriers: OR=2.32, 95% CI: 1.08-4.97. [g)]When reference was CC genotype: OR=2.78, 95% CI: 1.05-7.39. [h)]When reference was CC genotype: OR=3.09, 95% CI: 1.04-9.17. [i)]When reference was C carriers: OR=2.82, 95% CI: 1.18-6.72. [j)]When reference was C carriers: OR=2.92, 95% CI: 1.14-7.46.

A: adenine; C: cytosine; CI: confidence interval; eGFR: estimated glomerular filtration rate; G: guanine; MTX: methotrexate; OR: odds ratio; SCr: serum creatinine; SLC: solute carrier; SLCO: solute carrier organic anion transporter; SNP: single nucleotide polymorphism; T: thymine.

Table 4. Relation between SNPs in *ATP-binding cassette transporters* and MTX-related toxicity

	Model	Alleles	MTX overall toxicity		MTX gastrointestinal toxicity	
			P	OR (95% CI)	*P*	OR (95% CI)
ABCB1 C>T	Dominant	C carriers		Reference		Reference
(rs1045642)		TT	0.515	0.76 (0.33-1.75)	0.475	0.72 (0.30-1.77)
	Recessive	CC		Reference		Reference
		T carriers	0.794	0.90 (0.41-1.98)	0.540	0.77 (0.34-1.77)
ABCB1 C>T	Dominant	C carriers		Reference		Reference
(rs1128503)		TT	0.481	1.36 (0.58-3.15)	0.777	1.14 (0.45-2.89)
	Recessive	CC		Reference		Reference
		T carriers	0.269	1.55 (0.71-3.37)	0.426	1.39 (0.62-3.14)
ABCB1 G>A/T	Dominant	G carriers		Reference		Reference
(rs2032582)		TT	0.833	0.91 (0.38-2.18)	0.652	0.80 (0.30-2.10)
	Recessive	GG		Reference		Reference
		T carriers	0.167	1.71 (0.80-3.66)	0.346	1.47 (0.66-3.25)
ABCC1 T>C	Dominant	T carriers		Reference		Reference
(rs35592)		CC	0.734	1.21 (0.40-3.73)	0.367	1.68 (0.55-5.15)
	Recessive	TT		Reference		Reference
		C carriers	0.349	1.40 (0.69-2.85)	0.141	1.79 (0.82-3.88)
ABCC1 A>G	Dominant	A carriers		Reference		Reference
(rs246240)		GG	0.978	0.97 (0.14-6.64)	0.356	0.28 (0.02-4.13)
	Recessive	AA		Reference		Reference
		G carriers	0.148	1.78 (0.82-3.86)	0.174	1.78 (0.77-4.11)
ABCC1 G>C	Dominant	G carriers		Reference		Reference
(rs2074087)		CC	0.864	1.15 (0.24-5.46)	0.716	1.34 (0.28-6.40)
	Recessive	GG		Reference		Reference
		C carriers	0.210	0.62 (0.29-1.31)	0.103	0.50 (0.22-1.15)
ABCC1 G>A	Dominant	G carriers		Reference		Reference
(rs3784864)		AA	0.733	1.16 (0.50-2.70)	0.975	0.99 (0.40-2.46)
	Recessive	GG		Reference		Reference
		A carriers	0.328	0.67 (0.30-1.49)	0.325	0.66 (0.28-1.52)
ABCC2 G>A	Dominant	G carriers		Reference		Reference
(rs717620)		AA	0.556	1.74 (0.28-10.84)	0.701	1.50 (0.19-11.89)
	Recessive	GG		Reference		Reference
		A carriers	0.722	0.88 (0.42-1.81)	0.672	0.84 (0.38-1.85)
ABCC2 C>T	Dominant	C carriers		Reference		Reference
(rs4148396)		TT	0.953	1.03 (0.43-2.48)	0.894	1.07 (0.41-2.78)
	Recessive	CC		Reference		Reference
		T carriers	0.995	1.00 (0.45-2.21)	0.562	1.29 (0.54-3.05)
ABCG2 T>C	Dominant	T carriers		Reference		Reference
(rs13120400)		CC	0.457	0.62 (0.18-2.18)	0.354	0.51 (0.12-2.12)
	Recessive	TT		Reference		Reference
		C carriers	0.069	1.99 (0.95-4.16)	0.052	2.21 (0.94-4.92)
ABCG2 G>A	Dominant	G carriers		Reference		Reference
(rs17731538)		AA	0.459	0.52 (0.10-2.89)	0.455	0.48 (0.07-3.31)
	Recessive	GG		Reference		Reference
		A carriers	0.510	0.78 (0.37-1.65)	0.557	0.78 (0.35-1.76)

***P* value <0.05 is considered to be of statistically significance (highlighted in bold).**
***P* value, odds ratio (OR) and 95% confidence intervals (CI) corresponds to multivariate logistic regression adjusted to patient-related variables (gender, age, smoking, eGFR and SCr), disease-related variables (diagnosis age and disease duration), and treatment-related variables (folic acid, corticosteroids, NSAIDs, other concomitant DMARDs and MTX administration characteristics such as dose, treatment duration and administration route).**
A: adenine; ABC: ATP-binding cassette; C: cytosine; CI: confidence interval; eGFR: estimated glomerular filtration rate; G: guanine; MTX: methotrexate; OR: odds ratio; SCr: serum creatinine; SNP: single nucleotide polymorphism; T: thymine.

Table 5. Relation between MTX transporters genes haplotypes and MTX-related toxicity

Haplotype				Estimated frequency (%)	MTX overall toxicity		MTX gastrointestinal toxicity	
					P	OR (95% CI)	*P*	OR (95% CI)
SLC16A7 A>T (rs3763980)	*SLC16A7* T>G (rs10877333)							
A	T			54.5		Reference		Reference
T	T			29.2	0.780	1.10 (0.58-2.08)	0.940	1.03 (0.51-2.05)
A	G			16.3	0.630	1.22 (0.55-2.70)	0.670	1.20 (0.53-2.72)
SLC19A1 G>A (rs7499)	*SLC19A1* G>A (rs1051266)	*SLC19A1* A>G (rs2838956)	*SLC19A1* G>A (rs3788200)					
G	G	A	G	48.7		Reference		Reference
A	A	G	A	33.6	0.089	0.58 (0.31-1.08)	0.029	0.46 (0.23-0.92)
G	A	A	G	5.8	0.920	1.07 (0.25-4.52)	0.550	0.58 (0.09-3.55)
G	A	G	A	4.4	0.590	1.50 (0.34-6.56)	0.580	1.66 (0.28-10.02)
A	G	A	G	2.0	0.920	1.10 (0.19-6.27)	0.610	0.59 (0.07-4.64)
ABCB1 C>T (rs1045642)	*ABCB1* C>T (rs1128503)	*ABCB1* G>A/T (rs2032582)						
C	C	G		43.7		Reference		Reference
T	T	T		37.5	0.530	1.21 (0.66-2.23)	0.740	1.12 (0.58-2.15)
T	C	G		10.7	0.084	0.33 (0.10-1.15)	0.100	0.35 (0.10-1.22)
C	T	T		3.9	0.670	1.40 (0.30-6.62)	0.720	1.35 (0.27-6.75)
C	T	G		2.7	0.920	0.91 (0.15-5.57)	0.770	0.75 (0.11-5.24)
ABCC1 T>C (rs35592)	*ABCC1* G>C (rs2074087)	*ABCC1* G>A (rs3784864)						
T	G	A		42.6		Reference		Reference
C	G	G		18.1	0.640	1.21 (0.54-2.69)	0.300	1.57 (0.68-3.62)
T	G	G		17.7	0.200	1.83 (0.73-4.56)	0.220	1.85 (0.69-4.94)
C	C	G		9.4	0.370	1.63 (0.56-4.74)	0.320	1.78 (0.57-5.48)
T	C	G		7.4	0.140	0.23 (0.03-1.62)	0.120	0.18 (0.02-1.51)
T	C	A		4.0	0.560	0.47 (0.04-6.06)	0.700	0.60 (0.05-7.75)
ABCC1 T>C (rs35592)	*ABCC1* A>G (rs246240)	*ABCC1* G>A (rs3784864)						
T	A	A		46.3		Reference		Reference
T	A	G		18.9	0.890	1.06 (0.44-2.54)	0.710	1.20 (0.46-3.09)
C	A	G		16.4	0.710	1.18 (0.52-2.62)	0.510	1.34 (0.56-3.18)
C	G	G		11.1	0.200	1.91 (0.71-5.16)	0.140	2.19 (0.78-6.12)
T	G	G		6.2	0.290	2.00 (0.56-7.11)	0.810	1.21 (0.25-5.96)
ABCC2 G>A (rs717620)	*ABCC2* C>T (rs4148396)							
G	C			55.3		Reference		Reference
A	T			22.2	0.240	1.60 (0.74-3.48)	0.110	1.98 (0.85-4.59)
G	T			22.2	0.700	1.14 (0.60-2.17)	0.800	1.10 (0.55-2.20)
ABCG2 T>C (rs13120400)	*ABCG2* G>A (rs17731538)							
T	G			48.6		Reference		Reference
C	G			28.7	0.900	1.04 (0.52-2.11)	0.840	1.08 (0.52-2.22)
T	A			21.7	0.210	0.58 (0.25-1.35)	0.290	0.65 (0.29-1.44)

***P* value < 0.05 is considered to be of statistically significance (highlighted in bold).**
***P* value, odds ratio (OR) and 95% confidence intervals (CI) corresponds to multivariate logistic regression adjusted to patient-related variables (gender, age, smoking, eGFR and SCr), disease-related variables (diagnosis age and disease duration), and treatment-related variables (folic acid, corticosteroids, NSAIDs, other concomitant DMARDs and MTX administration characteristics such as dose, treatment duration and administration route).**
A: adenine; ABC: ATP-binding cassette; C: cytosine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; eGFR: estimated glomerular filtration rate; G: guanine; MTX: methotrexate; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; SCr: serum creatinine; SLC: solute carrier; T: thymine.

CHAPTER VII

PREDICTION OF METHOTREXATE CLINICAL RESPONSE IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS: IMPLICATION OF MTHFR rs1801133 AND ATIC rs4673993 POLYMORPHISMS

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER VII. PREDICTION OF METHOTREXATE CLINICAL RESPONSE IN PORTUGUESE RHEUMATOID ARTHRITIS PATIENTS: IMPLICATION OF *MTHFR* rs1801133 AND *ATIC* rs4673993 POLYMORPHISMS

Besides the influence of Methotrexate-carrier mediated system in therapeutic outcome, functional polymorphisms in genes encoding for target-proteins involved on Methotrexate action mechanism (such as folate and de novo nucleotides synthesis pathways) could also determine Methotrexate pharmacodynamics. Thus, this chapter elucidates the influence of clinicopathological variables on clinical response to Methotrexate, as well of methylenetetrahydrofolate reductase C677T and aminoimidazole carboxamide adenosine ribonucleotide transformylase T675C polymorphisms, in Portuguese Rheumatoid Arthritis patients. Methylenetetrahydrofolate reductase, an enzyme involved in folate pathway, despite not directly inhibited by Methotrexate or by its polyglutamate forms, its expression levels seemed play a role in Methotrexate effect by modifying the folate status. Additionally, it is known that aminoimidazole carboxamide adenosine ribonucleotide transformylase, an enzyme involved in the de novo purine synthesis pathway, is directly inhibited by Methotrexate polyglutamate forms, leading to the release of adenosine, a potent anti-inflammatory agent. Therefore, these enzymes seem to be key targets for analyzing potential functional polymorphisms impact in Methotrexate therapeutic outcome.

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Research Article

Prediction of Methotrexate Clinical Response in Portuguese Rheumatoid Arthritis Patients: Implication of *MTHFR* rs1801133 and *ATIC* rs4673993 Polymorphisms

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Objective. Methotrexate (MTX), the most used drug in rheumatoid arthritis (RA) treatment, showing variability in clinical response, is often associated with genetic polymorphisms. This study aimed to elucidate the role of methylenetetrahydrofolate reductase (*MTHFR*) C677T and aminoimidazole carboxamide adenosine ribonucleotide transformylase (*ATIC*) T675C polymorphisms and clinicopathological variables in clinical response to MTX in Portuguese RA patients. *Methods.* Study included 233 RA patients treated with MTX for at least six months. *MTHFR* C677T and *ATIC* T675C polymorphisms were genotyped and clinicopathological variables were collected. Statistical analyses were performed and binary logistic regression method adjusted to possible confounding variables. *Results.* Multivariate analyses demonstrated that *MTHFR* 677TT (OR = 4.63; $P = 0.013$) and *ATIC* 675T carriers (OR = 5.16; $P = 0.013$) were associated with over 4-fold increased risk for nonresponse. For clinicopathological variables, noncurrent smokers (OR = 7.98; $P = 0.001$), patients positive to anti-cyclic citrullinated peptide (OR = 3.53; $P = 0.004$) and antinuclear antibodies (OR = 2.28; $P = 0.045$), with higher health assessment questionnaire score (OR = 2.42; $P = 0.007$), and nonsteroidal anti-inflammatory drug users (OR = 2.77; $P = 0.018$) were also associated with nonresponse. Contrarily, subcutaneous administration route (OR = 0.11; $P < 0.001$) was associated with response. *Conclusion.* Our study suggests that *MTHFR* C677T and *ATIC* T675C genotyping combined with clinicopathological data may help to identify patients whom will not benefit from MTX treatment and, therefore, assist clinicians in personalizing RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by an inflammation of the joints with an autoimmune profile and the most widely used disease modifying antirheumatic drug (DMARD) for RA treatment is methotrexate (MTX) [1]. Despite MTX cost-effectiveness, clinical response to MTX varies widely [2]. The factors that are possibly influencing

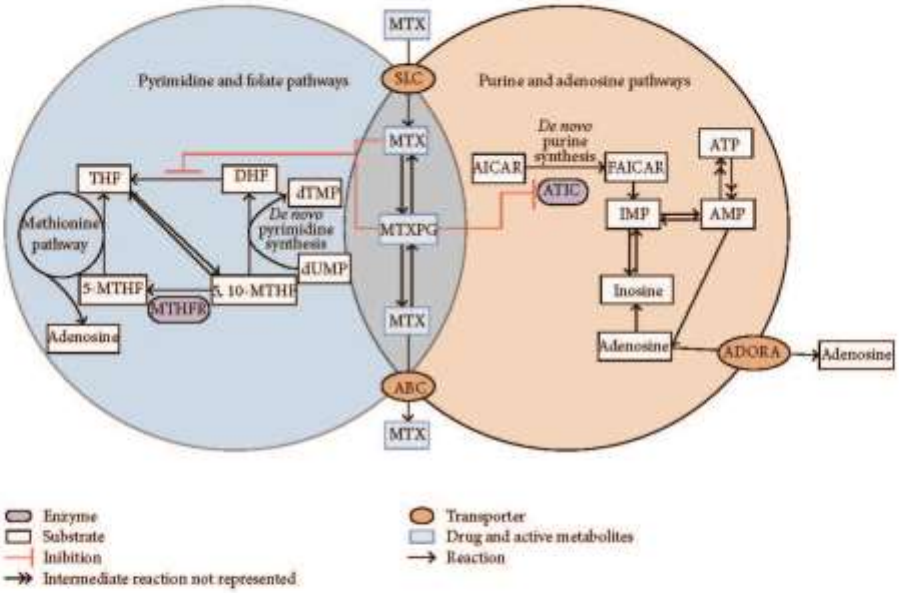

FIGURE 1: Methotrexate action mechanism. Left panel represents the intervention of MTX in *de novo* pyrimidine synthesis, folate, and methionine pathways by the inhibition of key enzymes. Right panel shows the effect of MTX in *de novo* purine synthesis and adenosine pathway by ATIC inhibition. 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; ABC: ATP-binding cassette; ADORA: adenosine receptor; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; AMP: adenosine monophosphate; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; ATP: adenosine triphosphate; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FAICAR: 5-formamidoimidazole-4-carboxamide ribonucleotide; IMP: inosine monophosphate; MTHFR: methylenetetrahydrofolate reductase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; SLC: solute carrier; THF: tetrahydrofolate.

disease course and therapeutic outcome can be classified into (1) clinicopathological variables, which can be divided into patient-related variables (age, gender, ethnicity, and comorbidities), disease-related variables (duration, activity, disability, and biomarkers), and treatment-related variables (compliance, dose, and previous drugs used) [3–9], and (2) genetic factors, such as genetic polymorphisms implicated in key MTX pathway genes [2, 10–15]. Several studies have been performed in order to evaluate the influence of clinicopathological variables in clinical response to MTX [3, 5, 7, 16, 17]; nevertheless, there is no consensus on which factors can be used as predictors [18]. Pharmacogenomics has raised great interest and, in fact, some studies have attempted to clarify the influence of genetic variations on clinical response to MTX [19].

MTX is an antifolate drug, with antiproliferative and anti-inflammatory effects, by inhibition of folate and adenosine pathways and also inhibition of purines and pyrimidines synthesis (Figure 1) [16, 20, 21]. Methylenetetra- hydrofolate reductase (MTHFR), an enzyme involved in folate pathway, is responsible for the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF) that acts as a carbon donor for the remethylation of homocysteine into methionine [22]. On the other hand, methionine can be transformed into S-adenosyl methionine (SAM) and then to S-adenosyl homocysteine (SAH), which can be reversibly hydrolyzed into adenosine and homocysteine [23]. Despite the fact that MTHFR is not directly inhibited by MTX or by its polyglutamated forms (MTXPG), its expression levels seem to influence MTX effect by modifying the folate *status* [16]. Additionally, it is known that aminoimidazole carboxamide adenosine ribonucleotide (AICAR) transformylase (ATIC), an enzyme involved in the *de novo* purine synthesis pathway responsible for the conversion of AICAR into formyl-AICAR (FAICAR), is directly inhibited by MTXPG, causing intracellular accumulation of AICAR [16]. AICAR and its metabolites can then inhibit two enzymes, adenosine deaminase (ADA) and adenosine monophosphate deaminase 1 (AMPD1), which are involved in adenosine metabolism, thus leading to increased intracellular concentrations of adenosine and its consequent release to the extracellular space [21]. This release contributes to the anti-inflammatory effects of MTX since adenosine is a potent anti-inflammatory agent [21].

Several studies have demonstrated that the occurrence of variations on clinical response to MTX could be explained by genetic polymorphisms in *MTHFR* and *ATIC* genes [11, 13–16, 24–28]. The most studied polymorphism in *MTHFR* is C677T

(rs1801133), which is responsible for a substitution of an alanine to a valine, leading to a thermolabile form of MTHFR with reduced activity [29]. In fact, it has been suggested that *MTHFR* 677T allele is related to MTX nonresponse in RA [13, 24]. Similar to MTHFR, some authors have studied the role of the T675C (rs4673993) polymorphism in *ATIC*, of which the *ATIC* 675C allele has been associated with improved clinical *status* and, consequently, with clinical response to MTX [14, 26].

The pattern of MTX therapeutic outcome is considered to be a major factor for the motivation of researchers and clinicians to enroll patients in pharmacogenetic studies, mainly by comparative studies within different populations. Therefore, the aim of this study was to elucidate the association of clinical response to MTX with *MTHFR* C677T and *ATIC* T675C polymorphisms, in Portuguese RA patients.

2. Methods

2.1. Characterization of the Studied Population. This study was developed as a retrospective study in a cohort of consecutive Caucasian patients (≥18 years) with RA treated with MTX for at least six months and was conducted between January 2009 and December 2012 at São João Hospital Center (Porto, Portugal). After diagnosis, patients were classified according to the 1987 criteria of the American College of Rheumatology (ACR) and reclassified according to the 2010 criteria of ACR and the European League Against Rheumatism (EULAR) [30]. All patients were initially treated with 10 mg *per os* (PO)/week of MTX in monotherapy. This dose was increased 5 mg at each three weeks if patients did not meet EULAR criteria for response, that is, if presenting a disease activity score in 28 joints (DAS28) > 3.2. At three months, if patients were still without response, the administration route was changed from PO to subcutaneous (SC) maintaining the MTX dose. If within three months, using SC at the maximum tolerable doses, patients did not meet the response criteria, MTX therapy was associated with other synthetic DMARDs. After three more months, if patients continued without response in two successive evaluations and did not present any contraindication, MTX therapy was discontinued or associated with biological DMARDs. The adjustment of MTX therapy also occurred when patients developed MTX-related toxicity. Due to the well-known protective effect of folic acid supplementation for the prevention of toxicity occurrence, in particular for gastrointestinal disorders [31–33], this drug was prescribed once a week to all patients and their regular compliance was registered.

Patients were excluded from the study if not treated with MTX for at least six months and if there was history of drug abuse, recent pregnancy, or desire to become pregnant. The study procedures were considered according to the ethical standards of the Helsinki Declaration by the local Ethical Committee (reference 33/2009) and all patients provided a signed informed written consent.

2.2. Data Collection and Variable Definition. Clinicopathological data were collected from individual clinical records by clinicians during patients' regular hospital visits and include variables possibly influencing disease state and clinical response to MTX, which were selected based on either the literature review and/or the clinical significance [3, 5, 7, 16, 17, 33]. These variables included (1) patient-related variables: age, gender, menopause, body mass index (BMI), smoking, number of pack years (NPY), and comorbidities; (2) disease-related variables: diagnosis age, duration, rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), antinuclear antibodies (ANAs), DAS28, and health assessment questionnaire (HAQ); and (3) treatment-related variables: symptomatic (corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), supplements (folic acid), other concomitant DMARDs, and MTX administration characteristics (dose, treatment duration, and administration route).

NPY was calculated by the formula: (number of cigarettes smoked per day × number of years smoking)/20. Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia, and/or cardiac disorders beyond RA. DAS28 was calculated as described by Prevoo et al. [34]. Daily corticosteroid therapy dose was considered in prednisolone equivalents.

MTX clinical response was recorded at the time of each visit. Nonresponse was defined if patients presented a DAS28 > 3.2 in two consecutive evaluations despite the use of MTX either in monotherapy or combined with other DMARDs. Therefore, at least six months of MTX therapy was required to define which patients had nonresponse to MTX. Response to MTX was defined when patients presented a DAS28 ≤ 3.2.

2.3. Sample Collection and Processing. Whole blood samples were obtained with standard venipuncture technique using ethylenediaminetetraacetic acid (EDTA) containing tubes and genomic deoxyribonucleic acid (DNA) extracted with QIAamp DNA Blood Mini Kit according to the manufacturer instructions (QIAGEN, Hilden, Germany). Total genomic DNA was quantified and its purity and integrity were analyzed using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington, DE, USA).

2.4. MTHFR C677T and ATIC T675C Genotyping. *MTHFR* C677T and *ATIC* T675C polymorphisms were selected based on the role of MTHFR and ATIC in MTX action pathway, upon the putative alteration of these proteins levels and the consequent implication in MTX clinical response [13, 14, 24, 26, 29].

Genotyping protocols were adjusted from those proposed by Sadananda Adiga et al. [35] for *MTHFR* C677T and Hinks et al. [27] for *ATIC* T675C.

MTHFR C677T polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. PCR amplification was performed for a final volume of 50 μL containing 0.3 μM of each primer (forward: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′; reverse: 5′-AGG ACG GTG CGG TGA GAG TG-3′), 1x DreamTaq Green master mix (Thermo Scientific, Vilnius, Lithuania), and 50–100 ng of genomic DNA. The PCR conditions consisted of initial denaturation at 94°C

during 5 minutes followed by 30 cycles with denaturation for 1 minute at 94°C, annealing for 1 minute at 57°C, extension for 15 seconds at 72°C, and a final extension at 72°C during 10 minutes. RFLP was performed at 37°C, overnight, using HinfI (Thermo Scientific, Vilnius, Lithuania). Individuals with the CC genotype presented 1 fragment with 198 base pairs (bp), whereas individuals with the TT genotype presented 1 fragment with 175 bp.

ATIC T675C polymorphism was genotyped using TaqMan SNP Genotyping Assay (C_362264_10) from Applied Biosystems (Foster City, CA, USA) with fluorogenic binding probes. Reactions were performed on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with a 5 μL final volume mixture containing 1x TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of each primer, 200 nM of probes labeled with either FAM or VIC, and 10 ng of extracted DNA. Thermal cycling conditions were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Allelic discrimination was performed by measuring endpoint fluorescence using ABI PRISM Sequence Detection System (Version 1.2.3, Applied Biosystems, Foster City, CA, USA).

For quality control, 10% of the samples were randomly selected for a second analysis and 10% percent of cases were confirmed by automated sequencing in a 3130xl Genetic Analyzer using the Kit BigDye Terminator v3.1 (Life Technologies, Foster City, CA, USA). Results were 100% concordant.

2.5. Statistical Analysis. Statistical analyses were performed using the IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA), considering a statistically significant probability (P) value of 5% or less. The chi-square test was used to assess the association between the groups (response *versus* nonresponse) and the different categorical variables. Odds ratio (OR) and the correspondent 95% confidence intervals (CI) were calculated as a measure of the association between the categorical variables. For the comparison of quantitative variables two sample t-tests and nonparametric Mann-Whitney U tests were applied.

Multivariate analysis with binary logistic regression was used to identify which genetic variables (*MTHFR* C677T and *ATIC* T675C genotypes) and clinicopathological variables could predict risk for occurrence of nonresponse to MTX. This analysis was performed adjusting to potential confounding variables in three steps: (1) patient-related variables; (2) patient- and disease-related variables; and (3) patient-, disease-, and treatment-related variables.

3. Results

3.1. Characterization of the Studied Population. Table 1 reports the clinicopathological variables of population enrolled in the study, that includes follow-up data from a total of 233 patients (196 females and 37 males), with a mean age of 52 ± 11.9 and disease duration of 8.0 (range: 0.5–53.0) years. Considering MTX therapy, the median treatment duration was 28.0 (range: 6.0–230.0) months with a median dose of 15.0 (range: 2.5–25.0) mg/week. Furthermore, 201 patients (86.3%) administered MTX by PO administration route and 32 (13.7%) by SC administration route. Nonresponse to MTX was observed in 128 (54.9%) patients and the mean for DAS28 was 4.2 ± 1.3.

3.2. Clinicopathological Variables and Clinical Response to MTX. Table 2 represents the relation between clinicopathological variables and clinical response to MTX. In accordance with patient-related variables, our results showed that early age of diagnosis ($P < 0.001$) and noncurrent smokers (OR = 0.32; $P = 0.004$) were statistically significant associated with nonresponse to MTX. Concerning disease-related variables, our results demonstrated that positivity to anti-CCP (OR = 2.28; $P = 0.007$) and ANAs (OR = 1.98; $P = 0.024$) was statistically significant associated with nonresponse to MTX. Additionally, higher number of tender joints count (TJC) ($P = 0.007$) and swollen joints count (SJC) ($P = 0.008$) and higher health assessment questionnaire (HAQ) score ($P = 0.006$) were statistically significant associated with nonresponse to MTX. Considering the treatment-related variables, our results revealed that NSAIDs users (OR = 3.09; $P < 0.001$) were associated with nonresponse to MTX. In addition, attending to MTX administration characteristics, higher MTX doses ($P < 0.001$) were associated with nonresponse to MTX, while SC administration route (OR = 0.32; $P = 0.004$) was statistically significant associated with response to MTX.

3.3. MTHFR C677T and ATIC T675C and Clinical Response to MTX. The frequencies of *MTHFR* C677T (rs1801133) genotypes were 105 CC (45.1%), 99 CT (42.5%), and 29 TT (12.4%), while for *ATIC* T675C (rs4673993) they were 110 TT (47.2%), 99 TC (42.5%), and 24 CC (10.3%). In our population, the minor allele for *MTHFR* C677T was T and for *ATIC* T675C was C (see Figure S1 in Supplementary Materials available online at http://dx.doi.org/10.1155/2014/368681). Considering distribution between responders and nonresponders, results showed significant differences for *MTHFR* C667T ($P = 0.049$) and *ATIC* T675C ($P = 0.025$) genotypes.

Table 3 and Figures S2 and S3 represent the relation between genetic variables and clinical response to MTX. In accordance with *MTHFR* C677T polymorphism, our results showed that *MTHFR* 677TT was statistically significant associated with about 3-fold increased risk for nonresponse to MTX when compared to *MTHFR* 677CC (OR = 3.08; $P = 0.015$) and *MTHFR* 677C carriers (OR = 2.91; $P = 0.015$). Regarding *ATIC* T675C polymorphism, we observed that *ATIC* 675CC was associated with response to MTX when compared to *ATIC* 675TT (OR = 0.32; $P = 0.016$) and *ATIC* 675T carriers (OR = 0.30; $P = 0.007$).

3.4. Multivariate Analysis and Clinical Response to MTX. Multivariate analysis with binary logistic regression was used to identify which clinicopathological and genetic variables (*MTHFR* C677T and *ATIC* T675C genotypes) could predict risk for the occurrence of nonresponse to MTX (Table 4).

Table I: Clinicopathological variables of population enrolled in the study.

	Value
Patient-related	
Male, *n* (%)	37 (15.9)
Female, *n* (%)	196 (84.1)
Postmenopausal, *n* (%)	96 (49.0)
Current smokers, *n* (%)	32 (13.7)
NPY*, median (IQR)	19.5 (0.8–120.0)
Comorbidity**, *n* (%)	126 (54.1)
Disease-related	
Diagnosis age, mean ± SD, years	40.3 ± 13.2
Disease duration, median (IQR), years	8.0 (0.5–53.0)
RF positive, *n* (%)	131 (56.2)
Anti-CCP positive, *n* (%)	175 (75.1)
ANAs positive, *n* (%)	66 (28.3)
DAS28, mean ± SD	4.2 ± 1.3
Individual variables—DAS28	
TJC (out of 28), median (IQR)	4.0 (0.0–27.0)
SJC (out of 28), median (IQR)	3.0 (0.0–24.1)
ESR, median (IQR), minutes (1st hour)	18.0 (1.0–92.0)
Global health on VAS, median (IQR)	48.0 (0.0–100.0)
HAQ score, median (IQR)	1.25 (0.0–2.9)
HAQ ≤ 0.5, *n* (%)	39 (16.7)
Treatment-related †	
Symptomatic	
Corticosteroids, *n* (%)	188 (80.7)
Daily dose in prednisolone equivalents, median (IQR), mg	5.0 (0.0–20.0)
NSAIDs, *n* (%)	170 (73.0)
Supplements	
Folic acid‡, *n* (%)	118 (50.6)
DMARDs	
Methotrexate monotherapy, *n* (%)	146 (62.7)
Combined methotrexate therapy—synthetic DMARDs, *n* (%)	59 (25.3)
Combined methotrexate therapy—biological DMARDs, *n* (%)	28 (12.0)
Methotrexate administration characteristics	
Dose, median (IQR), mg/week	15.0 (2.5–25.0)
Treatment duration, median (IQR), months	28.0 (6.0–230.0)
Per os administration route, *n* (%)	201 (86.3)
Subcutaneous administration route, *n* (%)	32 (13.7)

*NPY = (number of cigarettes smoked per day × number of years smoking)/20.
**Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia, and/or cardiac disorders beyond rheumatoid arthritis.
†Drugs coadministered with methotrexate when clinical response to methotrexate was recorded.
‡Patients in compliance with folic acid supplementation.
ANAs: antinuclear antibodies; Anti-CCP: anti-cyclic citrullinated peptide; BMI: body mass index; DAS28: disease activity score 28; DMARDs: disease modifying antirheumatic drugs; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; IQR: interquartile range; NPY: number of pack years; NSAIDs: nonsteroidal anti-inflammatory drugs; RF: rheumatoid factor; SD: standard deviation; SJC: swollen joints count; TJC: tender joints count; VAS: visual analog scale.

This analysis was performed in three steps adjusting to potential confounding variables. In the first step, patient-related variables were considered and our results demonstrated that *MTHFR* 677TT (OR = 2.64; $P = 0.040$) and *ATIC* 675T carriers (OR = 3.20; $P = 0.022$) were associated with about 3-fold increased risk for nonresponse to MTX. In a second step, beyond patient-related variables, disease-related variables were added and results confirmed that *MTHFR* 677TT (OR = 3.23; $P = 0.025$) and *ATIC* 675T carriers (OR = 4.63; $P = 0.007$) were associated with nonresponse to MTX. In a third step, beyond patient- and disease-related variables, treatment-related variables were added and the obtained

Table 2: Relation between clinicopathological variables and clinical response to methotrexate.

Characteristic	Response ($n = 105$)	Nonresponse ($n = 128$)	P value
Patient-related			
Male, n (%)	19 (51.4)	18 (48.6)	Reference
Female, n (%)	86 (43.9)	110 (56.1)	0.402
Premenopausal, n (%)	39 (39.0)	61 (61.0)	Reference
Postmenopausal, n (%)	47 (49.0)	49 (51.0)	0.160
Age, mean ± SD, years	55.1 ± 11.6	49.3 ± 11.5	**<0.001**
BMI, median (IQR), Kg/m^2	26.2 (18.5–43.1)	26.3 (18.4–38.9)	0.574
Noncurrent smoker*, n (%)	83 (41.3)	118 (58.7)	Reference
Current smoker, n (%)	22 (68.8)	10 (31.2)	**0.004**[a]
NPY**, median (IQR)	20.1 (1.5–120.0)	14.0 (0.8–40.0)	0.269
Noncomorbidity, n (%)	51 (47.7)	56 (52.3)	Reference
Comorbidity***, n (%)	54 (42.9)	72 (57.1)	0.462
Disease-related			
Diagnosis age, mean ± SD, years	42.1 ± 13.3	39.1 ± 12.8	0.081
Disease duration, median (IQR), years	8.0 (1.0–53.0)	8.0 (0.5–38.0)	0.164
RF negative, n (%)	42 (41.2)	60 (58.8)	Reference
RF positive, n (%)	63 (48.1)	68 (51.9)	0.293
Anti-CCP negative, n (%)	35 (60.3)	23 (39.7)	Reference
Anti-CCP positive, n (%)	70 (40.0)	105 (60.0)	**0.007**[b]
ANAs negative, n (%)	83 (49.7)	84 (50.3)	Reference
ANAs positive, n (%)	22 (33.3)	44 (66.7)	**0.024**[c]
DAS28, mean ± SD	4.0 ± 1.5	4.3 ± 1.2	0.089
Individual variables—DAS28			
TJC (out of 28), median (IQR)	3.0 (0.0–27.0)	5.0 (0.0–20.0)	**0.007**
SJC (out of 28), median (IQR)	2.0 (0.0–24.0)	4.0 (0.0–23.0)	**0.008**
ESR, median (IQR), minutes (1st hour)	19.0 (1.0–88.0)	17.0 (1.0–92.0)	0.509
Global health on VAS, median (IQR)	47.0 (0.0–100.0)	49.0 (0.0–100.0)	0.516
HAQ score, median (IQR)	1.1 (0.0–2.9)	1.5 (0.0–2.6)	**0.006**
Treatment-related[§]			
Symptomatic			
Noncorticosteroids, n (%)	21 (46.7)	24 (53.3)	Reference
Corticosteroids, n (%)	84 (44.7)	104 (55.3)	0.810
Non-NSAIDs, n (%)	41 (65.1)	22 (34.9)	Reference
NSAIDs, n (%)	64 (37.6)	106 (62.4)	**<0.001**[d]
Supplements			
Folic acid nonregular users, n (%)	52 (45.2)	63 (54.8)	Reference
Folic acid regular users, n (%)	53 (44.9)	65 (55.1)	0.963
Methotrexate administration characteristics			
Dose, median (IQR), mg/week	15.0 (2.5–25.0)	20.0 (7.5–25.0)	**<0.001**
Treatment duration, median (IQR), months	28.0 (6.0–230.0)	29.0 (6.0–209.0)	0.204
Per os administration route, n (%)	83 (41.3)	118 (58.7)	Reference
Subcutaneous administration route, n (%)	22 (68.8)	10 (31.2)	**0.004**[e]

*Noncurrent smokers include the never smokers and the ex-smokers.
**NPY = (number of cigarettes smoked per day × number of years smoking)/20.
***Comorbidity was defined as the presence of diabetes mellitus, hypertension, dyslipidemia, and/or cardiac disorders beyond rheumatoid arthritis.
§Drugs coadministered with methotrexate when clinical response to methotrexate was recorded.
P value < 0.05 is considered to be of statistical significance (highlighted in bold).
[a]OR = 0.32, 95% CI: 0.14–0.71. [b]OR = 2.28, 95% CI: 1.24–4.19. [c]OR = 1.98, 95% CI: 1.09–3.58. [d]OR = 3.09, 95% CI: 1.69–5.65. [e]OR = 0.32, 95% CI: 0.14–0.71.
ANAs: antinuclear antibodies; anti-CCP: anti-cyclic citrullinated peptide; BMI: body mass index; DAS28: disease activity score 28; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; IQR: interquartile range; NPY: number of pack years; NSAIDs: nonsteroidal anti-inflammatory drugs; RF: rheumatoid factor; SD: standard deviation; SJC: swollen joints count; TJC: tender joints count; VAS: visual analog scale.

Table 3: Relation between genetic variables and clinical response to methotrexate.

	Response (n = 105)	Nonresponse (n = 128)	P value	OR (95% CI)
MTHFR C677T, rs1801133				
CC	52 (49.5)	53 (50.5)		Reference
CT	46 (46.5)	53 (53.5)	0.662	1.13 (0.65–1.96)
TT	7 (24.1)	22 (75.9)	**0.015**	3.08 (1.21–7.84)
CC	52 (49.5)	53 (50.5)		Reference
T carrier	53 (41.4)	75 (58.6)	0.215	1.39 (0.83–2.33)
C carrier	98 (48.0)	106 (52.0)		Reference
TT	7 (24.1)	22 (75.9)	**0.015**	2.91 (1.19–7.10)
ATIC T675C, rs4673993				
TT	48 (43.6)	62 (56.4)		Reference
TC	40 (40.4)	59 (59.6)	0.637	1.14 (0.66–1.98)
CC	17 (70.8)	7 (29.2)	0.016	0.32 (0.12–0.83)
TT	48 (43.6)	62 (56.4)		Reference
C carrier	57 (46.3)	66 (53.7)	0.679	0.90 (0.53–1.50)
T carrier	88 (42.1)	121 (57.9)		Reference
CC	17 (70.8)	7 (29.2)	0.007	0.30 (0.12–0.75)

Results are expressed in n (%).
P value < 0.05 is considered to be of statistical significance (highlighted in bold).
ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; C: cytosine; CI: confidence interval; MTHFR: methylenetetrahydrofolate reductase; OR: odds ratio; T: thymine.

Table 4: Multivariate logistic regression analysis and clinical response to methotrexate.

Genetic variables	Adjusted variables					
	Patient-related		Patient-related + disease-related		Patient-related + disease-related + treatment-related	
	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)
MTHFR C677T, rs1801133						
C carriers		Reference		Reference		Reference
TT	**0.040**	2.64 (1.04–6.67)	**0.025**	3.23 (1.16–9.02)	**0.013**	4.63 (1.37–15.60)
ATIC T675C, rs4673993						
CC		Reference		Reference		Reference
T carriers	**0.022**	3.20 (1.18–8.66)	**0.007**	4.63 (1.51–14.12)	**0.013**	5.16 (1.42–18.76)

P value < 0.05 is considered to be of statistical significance (highlighted in bold).
Adjusted variables include (1) patient related variables (age, gender, and smoking), (2) disease-related variables (diagnosis age, disease duration, anti CCPs, ANAs, TJC, SJC, and HAQ), and (3) treatment-related variables (folic acid supplementation, corticosteroids therapy, use of NSAIDs, other concomitant DMARDs used and MTX administration characteristics such as dose, treatment duration, and administration route). Genetic variables include *MTHFR* C677T and *ATIC* T675C polymorphisms.
ANAs: antinuclear antibodies; anti-CCP: anti-cyclic citrullinated peptide; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; C: cytosine; CI: confidence interval; HAQ: health assessment questionnaire; MTHFR: methylenetetrahydrofolate reductase; NSAIDs: nonsteroidal anti-inflammatory drugs; OR: odds ratio; SJC: swollen joints count; T: thymine; TJC: tender joints count.

results showed that *MTHFR* 677TT carriers (OR = 4.63; P = 0.013) were statistically significant associated with more than 4-fold increased risk for nonresponse to MTX when compared to *MTHFR* 677C carriers. Additionally, *ATIC* 675T carriers (OR = 5.16; P = 0.013) were statistically significant associated with more than 5-fold increased risk for nonresponse to MTX when compared to *ATIC* 675CC.

Furthermore, considering clinicopathological variables, we observed that noncurrent smokers (OR = 7.98; P = 0.001), positivity to anti-CCP (OR = 3.53; P = 0.004) and ANAs (OR = 2.28; P = 0.045), higher HAQ (OR = 2.42; P = 0.007), and NSAIDs users (OR = 2.77; P = 0.018) were statistically significant associated with nonresponse to MTX. Moreover, SC administration route (OR = 0.11; P < 0.001) was statistically significant associated with response to MTX.

4. Discussion

Despite the fact that MTX is extensively used in RA treatment, the individual clinical response to MTX is variable and, therefore, additional DMARDs are often required to achieve a low disease activity profile or even remission [2].

Previous studies revealed controversial results when clinicopathological variables were associated with *MTHFR*

C677T and *ATIC* T675C polymorphisms for clinical response to MTX. Several explanations can be proposed for such observed discrepancies, such as bias related to study design and settings, sample size/power, ethnicity, the population disease duration (early or established RA), changes in folate *status*, influence of less common single nucleotide polymorphisms (SNPs) in *MTHFR* and *ATIC*, polymorphisms in genes encoding to other intervenient proteins in folate, purine, pyrimidine, adenosine, and methionine pathways, and also differences in the definition of MTX clinical response [28].

Besides the potential importance of our results, we are aware of possible limitations, especially the sample size. Despite this, patient characteristics are similar to those reported in the literature [36, 37]. Our case series is a representative clinical practice cohort of established and well-defined RA patients [25, 38] and the genotypes distribution of *MTHFR* C677T and *ATIC* T675C polymorphisms is in accordance with the published literature for other Caucasian population [13, 14, 24–26, 39].

4.1. MTHFR C677T and ATIC T675C and Clinical Response to MTX. Regarding *MTHFR* C677T polymorphism, our results demonstrated a statistically significant association between *MTHFR* 677TT and nonresponse to MTX, which is in accordance with previously reported studies [13, 24]. Although MTHFR is not directly inhibited by MTX or MTXPG, its expression levels may play an important role in MTX overall effect by modifying the folate *status* of the cell [16]. Literature describes *MTHFR* 677TT as responsible for a reduction of MTHFR activity [29], leading to reduced 5-MTHF and other folate cofactors levels and, consequently, to decreased adenosine release [22, 23, 40], which can partially explain MTX nonresponse.

Regarding *ATIC* T675C polymorphism, our results indicate that *ATIC* 675T carriers presented an increased risk for nonresponse to MTX, as previously reported [14, 26]. To the best of our knowledge, there are no functional studies reporting the effect of this polymorphism in ATIC activity. Nevertheless, it can be hypothesized that the presence of *ATIC* 675T allele will lead to MTX nonresponse due to the increased conversion of AICAR to FAICAR (Figure 1), causing adenosine degradation and its nonrelease, hindering MTX anti-inflammatory effects. Additionally, *ATIC* 675T allele seems to contribute to the decrease of MTX antiproliferative effect [41]. Moreover, this polymorphism seems to be in linkage disequilibrium with *ATIC* C347G (rs2372536), of which *ATIC* 347G carriers (minor allele) have been reported as related to better response [16, 26, 42, 43]. Hence, results are consistent with ours reporting an association between *ATIC* 675CC (minor allele) and clinical response to MTX.

4.2. Clinicopathological Variables and Clinical Response to MTX. According to patient-related variables, multivariate analysis results demonstrated that noncurrent smokers were associated with nonresponse to MTX. Literature describes the association between smoking and decreased folate levels which, in fact, enhance the antifolate effect of MTX and, therefore, improve clinical response to MTX [44–46]. Furthermore, cigarette nicotine seems to potentiate the immunosuppressive and anti-inflammatory effects by acting on the immunological system [47, 48]. Although some studies have demonstrated that smokers had worst response to MTX, presenting a higher disease activity and severity [6, 49], others were able to demonstrate that tobacco exposure reduced radiographic progression and favored a better functional score [50, 51]. Considering disease-related variables, our results demonstrated an association of more than 2-fold higher risk between anti-CCP and ANAs positivity and nonresponse to MTX. Anti-CCP and ANAs are autoantibodies found in RA that are strongly correlated with erosive disease, worse functional *status*, and higher disease activity [1, 9, 52–55] associated with nonresponse. Other studies have shown a relation between anti-CCP positivity and MTX response or presented no associations in early RA patients [56, 57]; nevertheless, our results may be explained by the fact that our series was constituted mainly by patients with established disease. To the best of our knowledge there are no studies in RA associating ANAs and MTX response. Additionally, higher HAQ was associated with more than 2-fold increased risk for nonresponse to MTX. Since higher HAQ score represents an increased disease activity it was expected, as reported by others, that these patients have worst response [56, 58]. In accordance with treatment-related variables, the concomitant use of NSAIDs was correlated with nonresponse to MTX. These results could be explained by the existence of drug-drug interactions since NSAIDs are known to alter MTX and 7-hydroxymethotrexate binding to plasmatic proteins and to impair MTX hepatic metabolism [41]. This translates into low amount of free MTX and lesser formation of active MTX metabolites in hepatocytes. Due to the importance of NSAIDs as symptomatic therapy in RA and due to contradictory results reported, further studies are required to clarify this association [56, 59]. In addition, SC administration route was statistically significant associated with MTX response. This result can be explained by the higher MTX bioavailability associated with SC administration route [60]. Consequently, this will lead to a greater tissues exposure to MTX, higher cellular polyglutamation and retention, and better response to MTX.

5. Conclusions

Our results suggested that noncurrent smoking, anti-CCP and ANAs positivity, higher HAQ, NSAIDs utilization, PO administration route, T homozygosity for *MTHFR* C677T, and T allele carrying for *ATIC* T675C can be possible predictive factors of nonresponse to MTX. Thus, the inclusion of these polymorphisms in combination with clinicopathological variables may add valuable information that may help to identify patients who will benefit from MTX treatment and assist clinicians to make better treatment decisions. Despite the potential of these findings, translation into clinical practice requires larger and multicentric studies in order to clearly endorse the importance of these polymorphisms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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CHAPTER VIII

CURRENT APPROACHES ON THYMIDYLATE SYNTHASE POLYMORPHISMS AND ITS IMPORTANCE ON MOLECULAR EPIDEMIOLOGY AND PHARMACOGENETICS

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER VIII. CURRENT APPROACHES ON *THYMIDYLATE SYNTHASE* POLYMORPHISMS AND ITS IMPORTANCE ON MOLECULAR EPIDEMIOLOGY AND PHARMACOGENETICS

This chapter presents a review about thymidylate synthase biological and pharmacological role and its genetic polymorphisms association with disease susceptibility, Methotrexate therapeutic outcome (clinical response and toxicity occurrence) and patients' survival. This work was motivated by thymidylate synthase potential as an important target for several drugs, such as Methotrexate, and also to be a critical target for providing the needed nucleotide precursors to maintain deoxyribonucleic acid synthesis and repair. Since thymidylate synthase is involved in folate modulation, and several diseases have frequent genetic alterations in folate metabolism associated genes, it seems important to analyze the association between thymidylate synthase genetic polymorphisms and the diseases susceptibility and prognosis. The most studied thymidylate synthase polymorphisms (rs34743033, rs2853542 and rs34489327) are located on untranslated regions and seem to influence thymidylate synthase protein expression levels.

REVIEW

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Current approaches for *TYMS* polymorphisms and their importance in molecular epidemiology and pharmacogenetics

TS is critical for providing the requisite nucleotide precursors in order to maintain DNA synthesis and repair. Furthermore, it is an important target for several drugs such as 5-fluorouracil and methotrexate. However, several mechanisms of resistance to TS inhibitors have been explained as linked to *TYMS* overexpression. Some authors have described the relationship between genetic polymorphisms on *TYMS*, in particular rs34743033, rs2853542 and rs34489327, with the development of several diseases and with the clinical response to drug therapy and/or survival. Nevertheless, the obtained results described in the literature are controversial, which has lead to a search strategy to understand the impact of these polymorphisms on molecular epidemiology and pharmacogenetics. With the progress of these scientific areas, early identification of individuals at risk of disease along with improvement in the prediction of patients' outcome will offer a powerful tool for the translation of *TYMS* polymorphisms into clinical practice and individualization of treatments.

KEYWORDS: molecular epidemiology pharmacogenetics polymorphisms thymidylate synthase *TYMS*

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Biological role

TYMS, located on chromosome 18p11.32, is composed of six introns with sizes ranging between 507 and 6271 bp and seven exons with sizes ranging between 72 and 250 bp [1–3]. *TYMS* codes for TS (EC 2.1.1.45, OMIM# 188350), a folate-dependent protein involved in *de novo* pyrimidine synthesis with important roles in DNA synthesis and repair, and, consequently, in cellular replication [4].

Structurally, TS is a symmetrical dimer of identical subunits of 35 kDa, each one composed of seven α-helices and ten β-strands set in a three-layer domain anchored by a large mixed β-sheet (FIGURE 1) [4,5,101]. The arrangement of the large β-sheets from the monomers is crucial for the dimer interface [5]. An extended structure anchors the phosphate of deoxyuridine monophosphate (dUMP) and controls the orientation of a sulfhydryl group on a conserved cysteine residue in the active site [4].

TS is responsible for the reductive methylation of dUMP to deoxythymidine monophosphate (dTMP) using the oxidation of 5,10-methylenetetrahydrofolate (5,10-MTHF) to dihydrofolate [4]. This enzyme is the only source of cellular dTMP, which is subsequently phosphorylated to deoxythymidine triphosphate and used for DNA synthesis and repair [4,6]. Moreover, 5,10-MTHF is also important for the formation of 5-methyltetrahydrofolate, by MTHFR, which is then used to convert homocysteine to methionine by MS [7]. This reaction only occurs in the presence of vitamin B12 and is important for:

- Regeneration of tetrahydrofolate (THF);
- Progression of the methionine cycle, maintaining DNA methylation by the formation of *S*-adenosyl methionine in an ATP-dependent reaction catalyzed by MAT [7].

THF can also be regenerated by the conversion of dihydrofolate by DHFR [8], and this constant THF regeneration increases the formation of 5,10-MTHF providing the necessary conditions for TS activity (FIGURE 2) [7].

Several reports have described TS expression as increased in highly proliferative cells [9,10] and, in fact, TS is recognized as important for the maintenance of dTMP levels required for DNA synthesis and therefore for cellular replication. The cell cycle is regulated by four checkpoints that control the sequential phases of the cell cycle [11]:

- G1 phase, the 'growing' stage when cells start to produce molecules that will help to start the proliferation;
- S phase, the DNA replication stage;
- G2 phase, the preparation for cell division;
- M phase, when cells begin to divide.

During the cell cycle, TS levels are regulated according to cellular necessities and, in fact,

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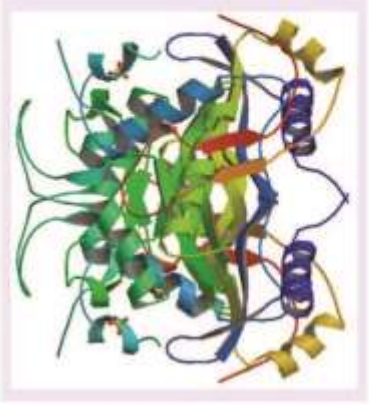

Figure 1. TS protein structure.
Adapted with permission from [101].

TYMS was one of the first eukaryotic genes shown to be autoregulated at the level of translation [12]. In the G1 phase there is no translation of TS due to the absence of dUMP and 5,10-MTHF. So, TS binds to two different *cis*-acting sequences on its mRNA that will consequently avoid its translation (FIGURE 3A) [12,13]. The first *cis*-acting sequence is 30 bp long and is located in the translational start site (ATG) within the stem-loop structure and the second element is a 70 bp sequence located in the coding region, from nucleotide 480 to 550, which has been reported to be sufficient to confer a translational signal independent of the first element [12]. However, when the cell enters S phase, TS suffers a conformational change towards its binding substrates (dUMP and 5,10-MTHF) and is no longer able to bind to the *cis*-acting sequences on its mRNA and, therefore, translation of protein will occur (FIGURE 3B) [13]. This mechanism works close with other regulatory events (genetic, transcriptional, post-transcriptional and post-translational) to assure the required levels of dTMPs for adequate cellular activity [12]. Moreover, this function is also critical to maintain DNA integrity by preventing an excess of uracil incorporation during replication [4,8,14].

Although TS levels are under strict control, its expression can be modified by other factors, including genetic variations in the *TYMS* gene. Thus, considering TS function and its involvement in the folate pathway, the decreased activity of this enzyme could be responsible for reduced DNA repair capability, lower folate levels and, consequently, contribute towards cancer risk [14–16].

Pharmacologic role

As TS is indispensable for DNA synthesis and repair, clinicians have used TS inhibition as an attractive tool to diminish cancer cell proliferation [12,17]. Additionally, this antiproliferation strategy contributes to other therapeutic outcomes beyond cancer, where the inhibition of the rapidly dividing cells is important for the disease outcome [6]. The inhibition of TS is achieved with the use of fluoropyrimidines or folate analogue compounds (antifolates) [4].

Fluoropyrimidines

Fluoropyrimidines are antimetabolite drugs, which include capecitabine, floxuridine and 5-fluorouracil (5-FU), widely used in monotherapy or in combination, for the treatment of cancer [13,18,19]. Since 5-FU is the most frequently used fluoropyrimidine, in this review it will be described as the example of a fluoropyrimidine-associated TS inhibition.

5-FU is an inactive uracil analogue that enters the cell via the solute carriers and leaves by ATP-binding cassette transporters. Inside cells, 5-FU is converted into the active metabolite 5-fluorodeoxyuridylate monophosphate. The binding of 5-fluorodeoxyuridylate monophosphate to the dUMP binding site of TS is achieved by a tight binding covalent complex that, in the presence of 5,10-MTHF, forms an inhibitory ternary complex [6,20,21]. This complex is very stable and, therefore, TS inhibition is so prolonged that dTMP levels will be efficiently depleted and cells will undergo death [10]. Additionally, 5-FU can be metabolized into 5-fluorouridine-5′-triphosphate, which is then incorporated into RNA and causes the inhibition of RNA processing and mRNA translation (FIGURE 2) [4,17].

Frequently, the potentiation of 5-FU is obtained by the addition of folic acid in order to increase the intracellular level of 5,10-MTHF used to stabilize the inhibitory ternary complex and, therefore, obtain a highly efficient inhibition [4]. The long-term inhibition of TS gives rise to an accumulation of dUMP and consequently of deoxyuridine triphosphate, which can be misincorporated into DNA resulting in the formation of single- and double-strand DNA breaks that induce chromosome damage, fragile site formation and micronucleus formation [17,22]. As functional TS is required for effective cell proliferation, it is thought that functional genomic alterations in folate metabolism associated genes, especially in *TYMS*, or lower dietary consumption of folates, might

have an important role in the development of diseases [2]. Moreover, some studies have described *TYMS* polymorphisms as associated with clinical response to 5-FU-based therapy and/or colorectal cancer (CRC) and gastric cancer (GC) patients' survival [23–25].

Antifolates

Since folates are essential for cell proliferation, antifolates have been used in several diseases where cell proliferation is increased. These drugs are prescribed as anticancer, antibiotics or antiprotozoal agents and also as modifiers of

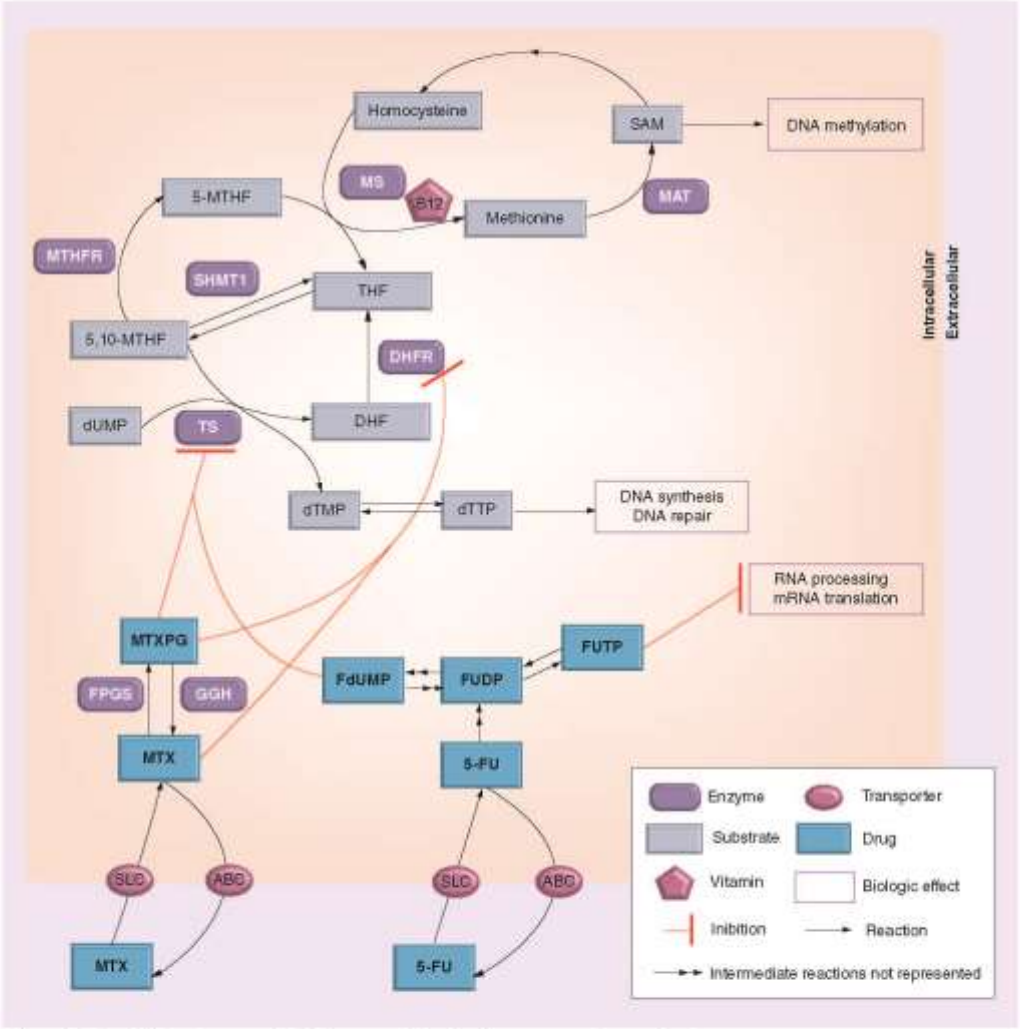

Figure 2. Metabolic pathways of TS, 5-fluorouracil and methotrexate action mechanisms.
5-FU: 5-fluorouracil; 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5-methylenetetrahydrofolate; ABC: ATP-binding cassette transporter; B12: Vitamin B12; DHF: Dihydrofolate; dTMP: Deoxythymidine monophosphate; dTTP: Deoxythymidine triphosphate; dUMP: Deoxyuridine monophosphate; FdUMP: 5-fluorodeoxyuridylate monophosphate; FUDP: Fluorouridine diphosphate; FUTP: 5-fluorouridine-5'-triphosphate; MTX: Methotrexate; MTXPG: Methotrexate polyglutamates; SAM: S-adenoryl methionine; SLC: Solute carriers; THF: Tetrahydrofolate.

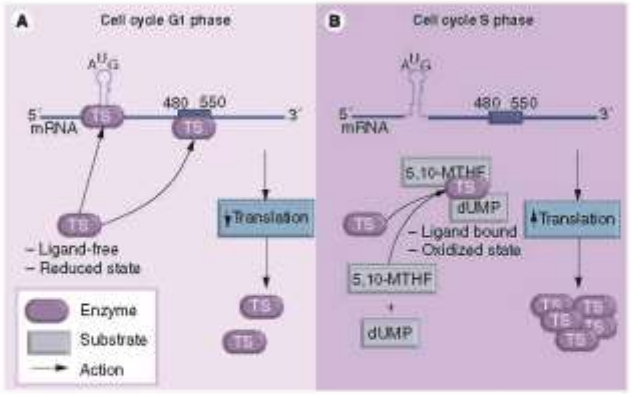

Figure 3. TS translational autoregulation mechanism during cell cycle stages. (A) G1 phase and **(B)** S phase.
5,10-MTHF: 5-methylenetetrahydrofolate; dUMP: Deoxyuridine monophosphate.

inflammatory disease courses such as rheumatoid arthritis (RA), psoriasis and inflammatory bowel disease [4,6,26].

Antifolates include a large group of drugs such as methotrexate (MTX), trimethoprim, pyrimethamine, pemetrexed and raltitrexed, which inhibit TS, DHFR and/or the purine *de novo* pathway [9,27]. MTX is one of the most well-known antifolate drugs and it is used frequently as the standard for several treatments [4,6,26]. Hence, in this review, it will be used to describe the TS inhibition as an example of a folate analogue.

MTX is transported across the cell membrane mainly via SLC19A1, a transmembrane protein that mediates the folates and antifolates entrance in the cell [28]. Inside cells, it is converted into polyglutamate derivatives by FPGS [27,28]. In addition, glutamate groups can be removed by GGH [29]. The higher glutamation will confer a negative charge to the compound, increasing its solubility [27,28], which will lead to:

- Reduced efflux from cells [4];
- Prolonged retention in tissues [4];
- Increased affinity for folate-dependent enzymes thus leading to an increase in the inhibition of TS [4].

Additionally, TS is also inhibited indirectly by 5,10-MTHF depletion induced by MTX (Figure 2) [30].

Several studies have shown that *TYMS* genotypes are associated with clinical response

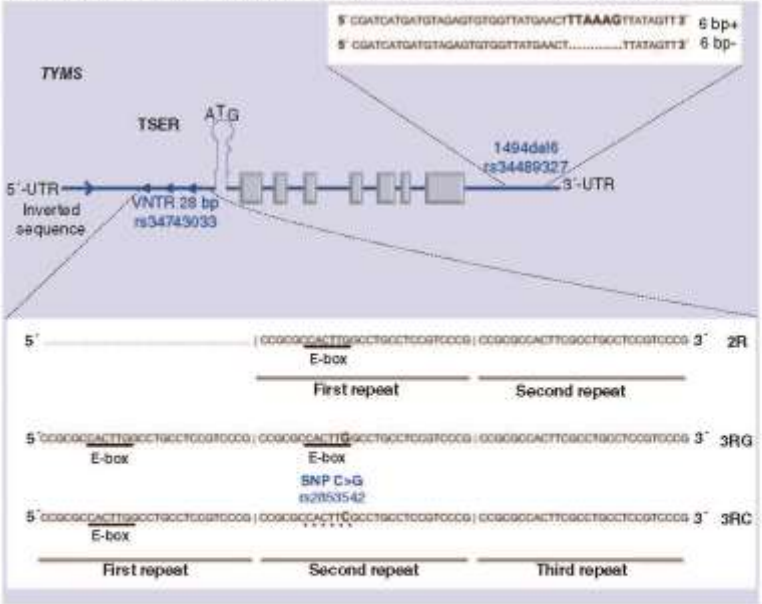

Figure 4. *TYMS* genetic polymorphisms.
del: Deletion; E-box: Enhancer box; R: Repeat; TSER: *TYMS* enhancer region; VNTR: Variable number tandem repeat.

Table 1. *TYMS* polymorphisms characteristics.

rs ID	Polymorphism	Alleles	Type	Region	Putative biochemical effects	Ref.
rs34743033	*TYMS* VNTR 28 bp	(CCGCGCCACTTCGCCTGCCTCCGTCCCG)2/3/4/5/9*	VNTR	5'-UTR	mRNA translational efficiency and TS expression	[5,6,22,83]
rs2853542	*TYMS* SNP C>G	C/G	SNP	5'-UTR	mRNA translational efficiency and TS expression	[32]
rs34489327	*TYMS* 1494del6	–/TTAAAG	Del/ins	3'-UTR	mRNA stability and TS expression	[47,77]

*2/3/4/5/9 represents the possible number of repeats
Del: Deletion; Ins: Insertion; VNTR: Variable number tandem repeat

to MTX in RA and/or acute lymphoblastic leukemia (ALL) patients' survival [31,32].

Resistance to inhibitors

Efforts to produce more effective TS inhibitors have been hindered due to the accumulation/overexpression of TS in cells that leads to loss of function [33]. Additionally, *in vitro* and *in vivo* studies have shown that the exposure of cells to TS inhibitors can lead to *TYMS* amplification and increased TS levels [34,35]. This is presumably explained by the mechanisms of autoregulation in which TS binds to the inhibitor and, consequently, is not available to attach to mRNA avoiding translation and, therefore, protein levels are expected to increase [13]. Since TS inhibitor bioavailability is dose dependent and correlated with TS expression, the overexpression of TS may have important consequences in clinical response, leading to less efficacy and reduced toxicity [21,33,36].

Beyond TS overexpression as a mechanism of resistance to inhibitors, there are several others such as:

- Diminished drug influx or increased drug efflux from cells, caused by a mutation of drug transporters;
- Decreased polyglutamation in case of antifolates;
- Genetic polymorphisms in *TYMS* that affect TS structure and function by changing the binding site of inhibitors [12,13].

TYMS genetic polymorphisms: from molecular epidemiology to pharmacogenetics

Genetic polymorphisms have been proven to have an increasing importance in the definition of risk for the occurrence/recurrence of various diseases and also for the prediction of patients' clinical outcome [37]. With the development of molecular epidemiology and pharmacogenetic studies, these recent advances offer a powerful tool for the early identification of individuals at risk of disease development and also for the improvement of the prediction of patients' outcome by increasing the individualization of treatments [37].

Since TS is involved in folate modulation and cancers have frequent genomic alterations in folate metabolism-associated genes, several authors have described the association between *TYMS* genetic polymorphisms and the development of several diseases or clinical outcome [1,3,23–25,31,32]. The most studied *TYMS* polymorphisms (rs34743033, rs2853542 and rs34489327) are located on UTRs and seem to influence TS expression (Figure 4 & Table 1). Despite not being translated into proteins, the 5'-UTR and 3'-UTR are transcribed into mRNA along with exons and are thought to be important in mRNA stability, localization and collaborate in translational efficiency [38].

rs34743033 (*TYMS* 28-bp VNTR)

The rs34743033 polymorphism consists of a 28 bp variable number tandem repeat (VNTR) polymorphism located on the 5'-UTR enhancer region of the *TYMS* promoter (thymidylate synthase enhancer region [TSER]) [39]. Molecular epidemiologic studies have described that the majority of populations harbor either a double repeat (2R) or a triple repeat (3R), although, there have been reports of four, five or nine repeats in some African and Asian populations [40,41].

Despite the complete mechanism still being unexplained, the presence of at least one unit of the repeat is necessary for transcription since the sequence is required for the stem-loop formation around the start codon site of *TYMS* [39].

Table 2. Potential molecular epidemiology and pharmacogenetic implications of rs34743033.

Study (year)	Cases/ controls	Population/ ethnicity	Model	Outcome	Ref.
Molecular epidemiology					
Skibola *et al.* (2002)	71/114	British/Caucasian	ALL	Increased risk for 2R2R	[46]
Chen *et al.* (2003)	270/454	American/Caucasian	CRC	No significant association	[49]
Graziano *et al.* (2004)	134/139	Italian/Caucasian	GC	No significant association	[48]
Zhang *et al.* (2004)	704/1085	American/Caucasian	HNSCC	Increased risk for 2R3R	[47]
Shi *et al.* (2005)	1055/1140	American/Caucasian	LC	No significant association	[15]
Tan *et al.* (2005)	324/492	Chinese/Asian	ESCC	No significant association	[14]
Tan *et al.* (2005)	231/492	Chinese/Asian	GCA	No significant association	[14]
Zhai *et al.* (2006)	432/473	Chinese/Asian	BC	No significant association	[54]
Hubner *et al.* (2007)	673/301	British/Caucasian	CRC	No significant association	[52]
Xu *et al.* (2007)	1102/1141	American/Caucasian	BC	No significant association	[55]
Carmona *et al.* (2008)	196/200	Portuguese/Caucasian	CRC	No significant association	[51]
Guimaraes *et al.* (2011)	113/188	Brazilians/Caucasian	CRC	No significant association	[53]
Gao *et al.* (2012)	315/439	Chinese/Asian	CRC	Increased risk for 2R allele	[45]
Nazki *et al.* (2012)	72/144	Kashmiri/Indian	ALL	No significant association	[2]
Yoshimitsu *et al.* (2012)	455/1052	Japanese/Asian	CRC	No significant association	[50]
Pharmacogenetics					
Villafranca *et al.* (2001)	65/–	American/Caucasian	CRC	Reduced downstaging for 3R3R No association with DFS	[56]
Chen *et al.* (2003)	270/–	American/Caucasian	CRC	No significant association with OS	[49]
Relling *et al.* (2004)	64/–	American/Caucasian	ALL	Increased toxicity for 2R2R	[60]
Lecomte *et al.* (2004)	90/–	French/Caucasian	CRC	Increased toxicity for 2R2R No association with efficacy or OS	[25]
Krajinovic *et al.* (2005)	259/–	Canadian/Caucasian	ALL	Reduced EFS for 3R3R	[6]
Rocha *et al.* (2005)	246/–	American/variable	ALL	Reduced EFS for 3R3R	[57]
Dotor *et al.* (2006)	129/–	Spanish/Caucasian	CRC	Increased OS for 3R3R	[63]
Gosens *et al.* (2008)	38/–	Dutch/Caucasian	CRC	Reduced OS for 3R3R	[59]
Graziano *et al.* (2008)	80/–	Italian/Caucasian	CRC	No significant association with clinical response	[67]
Lima *et al.* (2008)	152/–	Portuguese/Caucasian	NSCLC	No significant association with survival	[64]
Giusella *et al.* (2009)	130/–	Italian/Caucasian	CRC	No significant association with toxicity, DFS or OS	[65]
Etienne-Grimaldi *et al.* (2010)	117/–	French/Caucasian	CRC	No significant association with clinical response or DFS	[66]
Martinez-Balibrea *et al.* (2010)	149/–	Spanish/Caucasian	CRC	Better clinical response for 2R allele No significant association with OS	[60]
Erculj *et al.* (2012)	198/–	Slovenian/Caucasian	ALL	Increased toxicity for 2R2R	[62]
Sepe *et al.* (2012)	557/–	American/variable	ALL	Reduced EFS for 3R4R	[58]
Jekic *et al.* (2013)	184/–	Serbian/Caucasian	RA	No significant association with clinical response	[68]
Radtke *et al.* (2013)	499/–	Dutch/Caucasian	ALL	Increased toxicity for 2R2R	[36]

ALL: Acute lymphoblastic leukemia; BC: Breast cancer; CRC: Colorectal cancer; DFS: Disease-free survival; EFS: Event-free survival; ESCC: Esophageal squamous cell carcinoma; GC: Gastric cancer; GCA: Gastric cardia adenocarcinoma; HNSCC: Head and neck squamous cell carcinoma; LC: Lung cancer; NSCLC: Non-small-cell lung cancer; OS: Overall survival; R: Repeat; RA: Rheumatoid arthritis.

Additionally, it was shown that the first repeat of the 2R allele and the two first repeats of the 3R allele exhibit a putative enhancer box (E-box) sequence [72]. This short sequence (CANNTG) is recognized by several transcription factors, mainly the upstream stimulating factors (USF)

that stimulate gene transcription [42]. Therefore, it has been theorized that a higher number of repeats would increase the amount of USF recognition sites and, consequently, lead to an increased transcription of *TYMS* [39].

Kawakami *et al.* suggested that this region may also have a role in the translation regulation of TS mRNA and, therefore, 3R carriers are thought to have greater translation efficiency than 2R [43]. The mechanism is still unknown; however, there is an inverted repeat sequence upstream of the VNTR sequence that may have some implication in the regulation of translation [43]. Even though there is one study that reveals lower or equal levels of TS in 3R3R individuals when compared with 2R carriers [44], the majority of *in vitro* studies with HeLa and HEK immortalized cell lines confirmed that homozygous 3R are associated with higher TS expression compared to the other genotypes (2R3R and 2R2R) [1,22].

Several reports were performed to evaluate the impact of this polymorphism on the risk for development of several diseases. Taking into account that low-expression genotypes are related to decreased TS levels, which could be responsible for reduced DNA repair and lower folate levels, an increased cancer susceptibility is expected for 2R carriers. In fact, Gao *et al.* described an increased risk for CRC in 2R carriers compared with 3R homozygous carriers [45], and Skibola *et al.* reported the protective role of the 3R allele in adult ALL [46]. Zhang *et al.* have shown that individuals with the 2R3R genotype were associated

Table 3. Potential molecular epidemiology and pharmacogenetic implications of rs2853542.

Study (year)	Cases/ controls	Population/ ethnicity	Model	Outcome	Ref.
Molecular epidemiology					
Graziano *et al.* (2004)	134/139	Italian/Caucasian	GC	Increased risk for high-/median-expression genotypes	[48]
Tan *et al.* (2005)	324/492	Chinese/Asian	ESCC	Increased risk for low-expression genotypes	[14]
Tan *et al.* (2005)	231/492	Chinese/Asian	GCA	No significant association	[14]
Hubner *et al.* (2007)	673/301	British/Caucasian	CRC	No significant association	[52]
Pharmacogenetics					
Lecomte *et al.* (2004)	90/–	French/Caucasian	CRC	No association with clinical response, toxicity or OS	[24]
Krajinovic *et al.* (2005)	259/–	Canadian/Caucasian	Childhood ALL	No association with survival	[6]
Dotor *et al.* (2006)	129/–	Spanish/Caucasian	CRC	No association with survival	[63]
Gosens *et al.* (2008)	38/–	Dutch/Caucasian	CRC	No association with survival	[39]
Graziano *et al.* (2008)	80/–	Italian/Caucasian	CRC	Reduced clinical response for high-expression genotypes	[67]
Lima *et al.* (2008)	152/–	Portuguese/Caucasian	NSCLC	Increased survival for high-/median-expression genotypes	[64]
Gusella *et al.* (2009)	130/–	Italian/Caucasian	CRC	No significant association with toxicity, DFS or OS	[65]
Etienne-Grimaldi *et al.* (2010)	117/–	French/Caucasian	CRC	No significant association with clinical response or DFS	[66]
Farina-Sarasqueta *et al.* (2010)	251/–	Dutch/Caucasian	CRC	No association with DFS or OS	[74]
Erculj *et al.* (2012)	198/–	Slovenian/Caucasian	Childhood ALL	Reduced toxicity for high-expression genotypes	[62]
Jekic *et al.* (2013)	184/–	Serbian/Caucasian	RA	Reduced clinical response for high-expression genotype No association with toxicity	[68]

High-expression genotype: 3RG3RG; Median-expression genotypes: 3RG3RC and 2R3RG; Low-expression genotypes: 2R2R, 2R3RC and 3RC3RC.
ALL: Acute lymphoblastic leukemia; CRC: Colorectal cancer; DFS: Disease-free survival; ESCC: Esophageal squamous cell carcinoma; GC: Gastric cancer; GCA: Gastric cardia adenocarcinoma; NSCLC: Non-small-cell lung cancer; OS: Overall survival; RA: Rheumatoid arthritis.

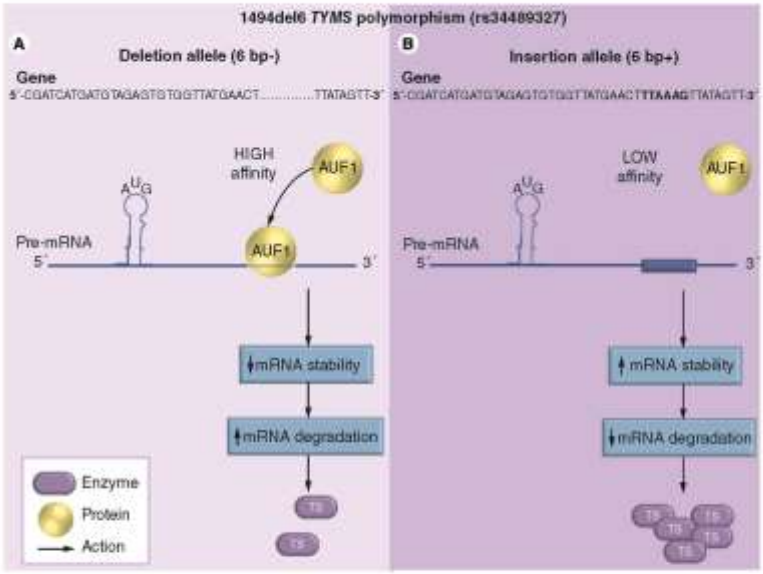

Figure 5. Mechanism of mRNA stability alteration and, indirectly, TS expression due to 1494del6 polymorphism in the 3'-UTR. (A) AUF1 has high affinity to TS mRNA when the 6 bp- allele is present. Its ligation diminishes TS mRNA stability leading to mRNA degradation and less protein. **(B)** AUF1 doesn't bind to TS mRNA when the 6 bp+ allele is present, leading to more mRNA stability, less mRNA degradation and more TS protein.
Del: Deletion.

with a borderline increased risk for head and neck squamous cell carcinoma (HNSCC) compared to 3R3R patients [47]. On the other hand, several studies found no statistically significant associations with risk for childhood ALL [2], esophageal squamous cell carcinoma (ESCC), gastric cardia adenocarcinoma [14], GC [48], CRC [49–53], breast cancer (BC) [54,55] and lung cancer [15] development.

Studies have been performed attempting to associate the *TYMS* 28-bp VNTR polymorphism with treatment and prognostic outcome. As previously described, regarding the functional role of *TYMS* polymorphisms on TS levels, the 3R allele causes higher TS expression, requiring higher concentrations of drugs for inhibition and cytotoxicity [56] and, consequently, it is expected to be associated with worse prognosis, poor drug efficacy and less toxicity. Reported studies in ALL patients treated with MTX, demonstrated a reduced event-free survival (EFS) in 3R homozygotes [6,57]. Sepe *et al.* demonstrated that 3R4R genotype had a poor prognosis in ALL patients treated with MTX and mercaptopurine [58]. Regarding CRC patients treated with 5-FU-based chemotherapy, it was shown that 3R homozygotes presented a poor prognosis [56,59,60]. Furthermore, other reports in ALL patients treated with MTX demonstrated a relationship between 2R carriers and increased toxicity [56,61,62]. Moreover, Lecomte *et al.* demonstrated in CRC patients treated with 5-FU-based chemotherapy, that 2R homozygotes presented a major incidence of adverse drug reactions, but no associations were found relating to therapeutic efficacy or overall survival (OS) [25]. Furthermore, another report in CRC patients treated with 5-FU showed an increased OS in 3R homozygotes and no association was found for disease-free survival [63]. Nevertheless, other studies showed no associations with patients' survival [60,64–66] and clinical response (efficacy or toxicity) to the TS inhibitor [65–68]. Such contrasting findings may be due to differences in MTX and 5-FU doses used in the studies and/or to the frequent loss of heterozygosity (LOH) at the *TYMS* locus in some tumors [69]. The LOH leads to *TYMS* genotype modification in tumor tissue when it is heterozygous in normal tissue, since one parental copy of this region is lost, possibly affecting drug response and survival [70]. Therefore, results for some cancer types where LOH is more frequent (e.g., CRC) should imply

careful analysis of tumor biopsies mainly when genotyping is performed [69]. Besides that, some authors have emphasized *TYMS* 28-bp VNTR as a relevant contributor to clinical response and/or survival variations and, so, propose the personalization of cancer therapy by using pharmacogenetic data. Accordingly, a study by Tan *et al.* was the first to prospectively use this type of genotyping to direct neoadjuvant fluoropyrimidine-based chemotherapy in 135 patients with rectal cancer [71]. The authors considered that the 2R allele will confer greater response to therapy than the 3R3R and 3R4R genotypes and the main conclusions were that both groups achieved high rates of downstaging and complete tumor response when treatment was personalized [71].

Table 2 summarizes relevant published results regarding the molecular epidemiology and pharmacogenetic implications of rs34743033.

■ rs2853542 (*TYMS* SNP C>G)

Studies have reported a SNP occurring on the 3R allele, located at the twelfth nucleotide of the second repeat consisting of a C>G substitution [44]. This C>G substitution has a large distribution among all major ethnic groups [22] and occurs in a critical nucleotide of the E-box sequence [23]. Consequently, in the presence of the cytosine (3RC) the E-box is disrupted and consequently USF transcription factors will no longer recognize the sequence, rendering transcription less stimulated than in the presence of the guanine (3RG) [23]. In fact, presence of the 3RG showed higher translational activity than the other genotypes in colorectal tissue samples [71] and in colon cancer cell lines [73].

Since this polymorphism occurs within the VNTR polymorphism, studies have been combining the information from both TSER polymorphisms (rs34743033 and rs2853542). Interestingly, some authors have discussed the potential impact of this event when assessing the abolishment of one E-box on the 3R allele, comparatively with the 2R allele, which has two functional E-box sequences [6,44]. Nevertheless, some studies have suggested the classification of patients according to their TS expression levels when TSER polymorphisms were considered: higher TS expression profile (3RG3RG), median TS expression profile (3RG3RC and 2R3RG) and low TS expression profile (2R2R, 2R3RC and 3RC3RC) [6,14,52,62,67,73–76].

Despite the small number of studies, TSER polymorphisms have been studied as risk factors for cancer development. Relating to the putative relationship between low TS levels and decreased DNA repair capability and folate levels, low TS expression genotypes should lead to an increased risk for cancer development in comparison to high TS expression genotypes. Tan *et al.* found when combining the TSER polymorphisms, that 2R homozygous had a threefold increased risk for ESCC than 3RG3RG genotype but no significant associations were found with cardia adenocarcinoma risk [14]. Furthermore, when the authors classified according to TS expression profiles, the low-expression patients had an increased risk for ESCC compared to the high/median TS expression profile patients [14]. In line with previous studies, Graziano *et al.* have not found association between the *TYMS* 28-bp VNTR polymorphism and GC risk but a significant higher risk was found in patients with a high-/median-expression profile (3RG3RG, 3RG3RC and 2R3RG genotypes) [48]. Additionally, Hubner *et al.* has shown no associations with the risk for CRC [52].

Although previous studies reported an association of TSER polymorphisms with risk for disease, other approaches were taken to explore its possible influence on clinical outcome. It is expected that low TS expression genotypes should present a better clinical response and/or survival but an increased toxicity. In fact, some studies have demonstrated that CRC [67] or RA [68] patients with the highest TS expression genotypes have worst therapeutic outcome when treated with 5-FU-based chemotherapy or MTX, respectively. Moreover, Krajinovic *et al.* showed that ALL individuals with 3R3R genotype subgroups (3RC3RC, 3RC3RG and 3RG3RG) were associated with low EFS [6]. Furthermore, and regarding toxicity, Erculj *et al.* demonstrated a protective role in high-/median-expression genotypes compared with low-expression genotypes in ALL patients treated with MTX for the development of leukocytopenia, thrombocytopenia and mucositis [62]. On the other hand, we have previously reported that high-/median-expression genotypes in non-small-cell lung cancer patients treated with platinum-based chemotherapy had a higher OS time [64]. Despite these results, other studies failed to show any correlation with survival [59,63,65,66,76] and/or with clinical response [25,65,66]. In addition, as previously suggested, patients with 3RC3RC are expected to have the same TS expression rate as 2R2R [73] and thus similar response, nevertheless there are studies in disagreement with this theory [25].

Table 3 summarizes relevant published results regarding the molecular epidemiology and pharmacogenetic implications of rs2853542.

rs34489327 (*TYMS* 1494del6)

Literature revealed that the presence of an insertion/deletion (ins/del) of a 6 bp sequence (TTAAAG) at the 3′-UTR of *TYMS* (1494del6) may be correlated with translation regulation by affecting mRNA stability and consequently TS expression [47,77].

The 3′-UTR of TS pre-mRNA contains *cis* adenylate-uridylate-rich elements and it was discovered that a *trans* RNA-binding protein (AUF1) binds to adenylate-uridylate-rich elements and diminishes the target mRNA stability. This AUF1 seems to preferentially attach to the deletion allele (6 bp-) TS pre-mRNA, thus leading to a less stable mRNA that will be more susceptible to degradation (Figure 5) [77]. This theory is supported by the findings of many *in vitro* studies that have demonstrated that the 6 bp- allele has decreased mRNA stability and was associated with lowest TS expression [47,77].

Several authors tried to find association of *TYMS* 1494del6 polymorphism with disease development. It is expected that the low TS expression genotypes (6 bp- carriers) should

Table 4. Potential molecular epidemiology and pharmacogenetic implications of rs34489327.

Study (year)	Cases/ controls	Population/ ethnicity	Model	Outcome	Ref.
Molecular epidemiology					
Chen *et al.* (2003)	270/454	American/Caucasian	CRC	No significant association	[49]
Graziano *et al.* (2004)	134/139	Italian/Caucasian	GC	Increased risk for 6 bp-/6 bp-	[48]
Zhang *et al.* (2004)	704/1085	American/Caucasian	HNSCC	Reduced risk for 6 bp-/6 bp-	[47]
Justenhoven *et al.* (2005)	688/724	German/Caucasian	BC	No significant association	[78]
Shi *et al.* (2005)	1055/1140	American/Caucasian	LC	Increased risk for 6 bp+ allele	[15]
Zhai *et al.* (2006)	432/473	Chinese/Asian	BC	Reduced risk for 6 bp+/6 bp+	[54]
Hubner *et al.* (2007)	673/301	British/Caucasian	CRC	Reduced risk for 6 bp-/6 bp-	[32]
Carmona *et al.* (2008)	196/200	Portuguese/Caucasian	CRC	Reduced risk for 6 bp- allele	[51]
Gao *et al.* (2012)	315/439	Chinese/Asian	CRC	No significant association	[45]
Yoshimitsu *et al.* (2012)	455/1052	Japanese/Asian	CRC	No significant association	[90]
Pharmacogenetics					
Lecomte *et al.* (2004)	90/–	French/Caucasian	CRC	Increased OS for 6 bp+/6 bp+ No association with clinical response or toxicity	[25]
Dotor *et al.* (2006)	129/–	Spanish/Caucasian	CRC	Increased OS for 6 bp- allele	[63]
Lu *et al.* (2006)	106/–	Chinese/Asian	GC	Increased clinical response for 6 bp- allele	[79]
Gosens *et al.* (2008)	38/–	Dutch/Caucasian	CRC	No association with survival	[59]
Graziano *et al.* (2008)	80/–	Italian/Caucasian	CRC	No significant association with clinical response	[67]
Lima *et al.* (2008)	152/–	Portuguese/Caucasian	NSCLC	Increased survival for 6 bp- allele	[64]
Gusella *et al.* (2009)	130/–	Italian/Caucasian	CRC	No significant association with toxicity, DFS or OS	[65]
Etienne-Grimaldi *et al.* (2010)	117/–	French/Caucasian	CRC	No significant association with clinical response or DFS	[66]
Martinez-Balibrea *et al.* (2010)	149/–	Spanish/Caucasian	CRC	No significant association with clinical response or OS	[60]
Gao *et al.* (2013)	125/–	Chinese/Asian	GC	Decreased OS for 6 bp+/6 bp+	[80]

BC: Breast cancer; CRC: Colorectal cancer; DFS: Disease-free survival; GC: Gastric cancer; HNSCC: Head and neck squamous cell carcinoma; LC: Lung cancer; NSCLC: Non-small-cell lung cancer; OS: Overall survival.

Table 5. Potential molecular epidemiology and pharmacogenetic implications of haplotypes.

Study (year)	Cases/ controls	Population/ ethnicity	Model	Outcome	Ref.
Molecular epidemiology					
Graziano *et al.* (2004)	134/139	Italian/Caucasian	GC	Increased risk for 3RG 6 bp- haplotype	[48]
Zhang *et al.* (2004)	704/1085	American/Caucasian	HNSCC	Reduced risk for 3R3R plus 6 bp-6 bp-	[47]
Carmona *et al.* (2008)	196/200	Portuguese/Caucasian	CRC	Reduced risk for 2R2R plus 6 bp- allele	[51]
Gao *et al.* (2012)	315/439	Chinese/Asian	CRC	Increased risk for 2R allele plus 6 bp-6 bp-	[45]
Pharmacogenetics					
Lecomte *et al.* (2004)	90/–	French/Caucasian	CRC	Increased toxicity for 2R 6 bp+ haplotype No association with clinical response, OS or progression time	[25]
Krajinovic *et al.* (2005)	259/–	Canadian/Caucasian	Childhood ALL	Increased EFS for 2R 6 bp- haplotype	[6]
Kawakami *et al.* (2005)	187/–	Italian/Caucasian	GC	Increased clinical response, DFS and OS for low expression genotypes and 6 bp- allele	[75]
Dotor *et al.* (2006)	129/–	Spanish/Caucasian	CRC	Increased OS for 3R 6 bp- haplotype	[63]
Gusella *et al.* (2009)	130/–	Italian/Caucasian	CRC	No significant association with toxicity, DFS or OS	[69]
Afzal *et al.* (2011)	501/–	Italian/Caucasian	CRC	No significant association with toxicity	[80]
Afzal *et al.* (2011)	592/–	Various/Caucasian	CRC	Reduced DFS and OS for low expression combined genotypes	[81]

ALL: Acute lymphoblastic leukemia; CRC: Colorectal cancer; DFS: Disease-free survival; EFS: Event-free survival; GC: Gastric cancer; HNSCC: Head and neck squamous cell carcinoma; OS: Overall survival; R: Repeat.

provide a greater risk for cancer when compared to the high-expression genotype. In accordance, Graziano *et al.* demonstrated an association between 6 bp- homozygous with an increased risk for GC [48] and Zhai *et al.* found the same relationship for BC [54]. However, a decreased risk associated with the 6 bp- allele for CRC [51,52], HNSCC [47] and lung cancer [15] was reported. Besides that, others studies have not found associations between the *TYMS* 1494del6 polymorphism and risk for CRC [45,49,50] or BC [78]. These controversial facts reveal that there is still much to clarify considering the risk association of this polymorphism with disease development.

When the implication of *TYMS* 1494del6 polymorphism in patients' outcome was addressed, it was suggested that homozygous patients for the deletion (6 bp-6 bp-) would have lower TS levels and, consequently, a better clinical outcome but increased toxicity. In fact, Lu *et al.* demonstrated that 6 bp- homozygotes had better response to 5-FU in GC [79]. For patients treated with 5-FU, at more advanced CRC stages, Dotor *et al.* found an association of the 6 bp- allele with better OS [63]. Moreover, another study revealed that 6 bp+ homozygotes presented a decreased OS compared to the remaining genotypes in GC patients receiving first-line capecitabine plus paclitaxel [80]. Lima *et al.* indicate a better survival for non-small-cell lung cancer 6 bp- carriers [64]. Nevertheless, one study in CRC demonstrated an association between 6 bp+ homozygotes and increased OS [75], while other studies have not found any correlation with prognosis [59,60,65,66], and neither with therapeutic outcome [25,60,65–67].

Table 4 summarizes the referred published results regarding molecular epidemiology and pharmacogenetic implications of rs34489327.

Haplotype analysis

Considering the existing discrepancies among several studies, the majority of the authors tried to explain it by providing evidence that the three polymorphisms of *TYMS* are in linkage disequilibrium [6,25,47,63,67,75]. Therefore, it appears to be important to evaluate the impact of these genetic variables as haplotypes and not separately.

Considering the haplotypes or the 5′-UTR and 3′-UTR polymorphisms combined for risk analysis, it was expected that low TS expression haplotypes (2R6 bp- and 3RC6 bp-) were related to decreased DNA repair and folate levels, which consequently, leads to higher cancer susceptibility. However, there is no consensus between the published reports. Gao *et al.*, demonstrated a higher risk for CRC in 6 bp-6 bp- patients presenting the 2R allele [45]. Graziano *et al.* described that the 3RG6 bp- haplotype had an

increased risk for GC [48]. Carmona *et al.* found a decreased risk for CRC in 2R2R and 6 bp- allele combined genotype patients [51]. Furthermore, in HNSCC patients, Zhang *et al.* concluded that 3R3R and 6 bp-6 bp- combined genotypes presented a decreased risk when compared with 2R2R combined with 6 bp+6 bp+ [47]. The controversial results can be due to different tumor models and/or LOH.

Moreover, other studies attempted to correlate the haplotypes with the clinical outcome. As described before, it is expected that patients presenting low TS expression haplotypes should present better clinical response and survival, but increased toxicity. Kawakami *et al.* demonstrated that patients with low TS expression genotypes (2R2R, 2R3RC and 3RC3RC) combined with 6 bp- carriers had the best outcome and improved disease-free survival and OS for GC treated with 5-FU-based chemotherapy [75]. Another study found an increased EFS for the 2R6 bp- haplotype in ALL treated with MTX [61]. Nevertheless, and regarding CRC patients treated with 5-FU-based chemotherapy, Dotor *et al.* demonstrated that 3R6 bp- haplotype had an increased OS when compared to 2R6 bp+ [63]. Leconte *et al.* showed that 2R6 bp+ haplotype was more prone to present severe adverse drug reactions but no associations were described for clinical response, OS or time of progression [35]. Afzal *et al.* demonstrated that low-expression genotypes (2R2R and 6 bp- carriers) presented a worse prognosis when compared to other genotypes [81]. Furthermore, other studies did not find an association between *TYMS* haplotypes and toxicity [83] or survival [65].

Table 5 summarizes the discussed published results with regards to molecular epidemiology and pharmacogenetic implications of haplotypes.

Conclusion & future perspective

Until now there have been many inconsistent results regarding the associations of *TYMS* polymorphisms with both disease risk and/or clinical outcome. These discrepancies could be explained by interstudy variability: small samples sizes with statistical underpowerment and a greater likelihood of false-positive associations; differences in disease models and disease stages; a variety of methods used to measure the risk, clinical response and/or survival; ethnicity variability; different genotyping protocols that limited the quality of results; and decreased effectiveness of different treatment regimens. Moreover, some results should be carefully analyzed when tumor biopsies are used for genotyping due to the frequency of LOH at the *TYMS* locus. Furthermore, owing to the complexity of cellular mechanisms involved, other factors could influence data analysis within conducted studies, such as, protein–protein interactions and regulatory mechanisms, and environmental factors such as folate status with the potential for gene–nutrient interactions.

In conclusion, the study of *TYMS* polymorphisms must be continued, aiming to find useful biomarkers for predicting disease risk and clinical efficacy. Therefore, it is essential that larger prospective studies are conducted, with the measure of TS levels *in vivo* and the study of its relationship with the presence of the three polymorphisms addressed in this review or even with novel *TYMS* polymorphisms, and with the correlation of haplotypes and with clinical pathological characteristics.

The progress of molecular epidemiology and pharmacogenetic studies is the key aim, to provide the needed translational element to clinical practice. Despite recent advances, a fairly long path has to be covered and unraveled until investigators will be able to accurately predict the risk for diseases and to reach therapy individualization.

Executive summary

- TS is a key factor for cell DNA repair and synthesis.
- TS is an important target for several drugs, such as 5-fluorouracil and methotrexate, in order to achieve therapeutic effects in various diseases.
- Efforts to produce more effective inhibitors have been limited owing to resistance to TS inhibitors, which is possibly due to *TYMS* overexpression.
- The three most studied *TYMS* polymorphisms are 28-bp VNTR (rs34743033); SNP C>G on 3R allele (rs2853542); and 1494del6 polymorphism (rs34489327). These polymorphisms putatively alter gene expression, TS mRNA stability and/or TS levels.
- Molecular epidemiology and pharmacogenetic studies of *TYMS* polymorphisms have produced inconsistent results.
- *TYMS* 1494del6 polymorphism (rs34489327) and haplotype analyses have shown encouraging results for elucidating if they influence risk for disease and clinical outcome.
- Many factors are behind these inconsistencies such as small sample sizes, ethnicity differences, variation in analysis methods and disparities in disease model and stage.

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CHAPTER IX

ROLE OF KEY TYMS POLYMORPHISMS ON METHOTREXATE THERAPEUTIC OUTCOME IN RHEUMATOID ARTHRITIS PORTUGUESE PATIENTS

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER IX. ROLE OF KEY *TYMS* POLYMORPHISMS ON METHOTREXATE THERAPEUTIC OUTCOME IN RHEUMATOID ARTHRITIS PORTUGUESE PATIENTS

This chapter elucidates the clinical relevance of the most studied thymidylate synthase polymorphisms (rs34743033, rs2853542 and rs34489327), using genotype and haplotype-based approaches, in Methotrexate therapeutic outcome (clinical response and toxicity occurrence) of Portuguese Rheumatoid Arthritis patients. The focus on thymidylate synthase stems from conclusions obtained in the review article that it is worth analyzing thymidylate synthase genetic polymorphisms association with Methotrexate therapeutic outcome.

Role of key *TYMS* polymorphisms on Methotrexate therapeutic outcome in Rheumatoid Arthritis Portuguese patients

SHORT TITLE

***TYMS* polymorphisms & MTX therapeutic outcome in RA**

ABSTRACT

Background: Therapeutic outcome of rheumatoid arthritis (RA) patients treated with methotrexate (MTX) can be modulated by thymidylate synthase (TS) levels, which may be altered by genetic polymorphisms in TS gene (*TYMS*). This study aims to elucidate the influence of *TYMS* polymorphisms in MTX therapeutic outcome (regarding both clinical response and toxicity) in Portuguese RA patients. Methods: Clinicopathological data from 233 Caucasian RA patients treated with MTX were collected, outcomes were defined and patients were genotyped for the following *TYMS* polymorphisms: 1) 28 base pairs (bp) variable number tandem repeat (rs34743033); 2) single nucleotide polymorphism C>G (rs2853542); and 3) 6bp sequence deletion (1494del6, rs34489327). Chi-square and binary logistic regression analyses were performed, using genotype and haplotype-based approaches. Results: Considering *TYMS* genotypes, 3R3R (p=0.005, OR=2.34), 3RC3RG (p=0.016, OR=3.52) and 6bp- carriers (p=0.011, OR=1.96) were associated with non-response to MTX. Multivariate analysis confirmed the increased risk for non-response to MTX in 6bp- carriers (p=0.016, OR=2.74). Data demonstrated that *TYMS* polymorphisms were in linkage disequilibrium (p<0.00001). Haplotype multivariate analysis revealed that haplotypes harboring both 3R and 6bp- alleles were associated with non-response to MTX. Regarding MTX-related toxicity, no statistically significant differences were observed in relation to *TYMS* genotypes and haplotypes. Conclusion: Our study reveals that *TYMS* polymorphisms could be important to help predicting clinical response to MTX in RA patients. Despite the potential of these findings, translation into clinical practice needs larger studies to confirm these evidences.

Key Words: Genotypes; Haplotypes; Methotrexate; Polymorphisms; Rheumatoid Arthritis; Therapeutic Outcome; Thymidylate Synthase; TS; TYMS.

INTRODUCTION

Methotrexate (MTX) is the cornerstone for rheumatoid arthritis (RA) treatment and is the most widely used disease-modifying antirheumatic drug (DMARD) in newly diagnosed patients [1,2]. Despite its cost-effectiveness, therapeutic outcome is variable mainly concerning to MTX clinical response and/or development of MTX-related toxicity [3-7]. MTX is an antifolate drug with important anti-inflammatory and antiproliferative effects, partly achieved by the intracellular inhibition of thymidylate synthase (TS) [8-10]. TS is a key protein for the *de novo* pyrimidine synthesis and is responsible for the simultaneous conversion of deoxyuridine monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (5,10-MTHF) to deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF). Subsequently, the dTMP is phosphorylated to deoxythymidine triphosphate (dTTP) and used for the deoxyribonucleic acid (DNA) synthesis and repair [6,11,12] (Figure 1A). Since TS levels were found to be predictive of MTX therapeutic outcome [13,14] and genetic polymorphisms in TS gene (*TYMS*) have been associated with TS levels [15,16], pharmacogenomics has raised great interest and, in fact, some studies have attempted to clarify the influence of genetic variations on clinical response to MTX in RA [17]. The most studied polymorphisms (rs34743033, rs2853542 and rs34489327) are represented on Figure 1B. Polymorphism rs34743033 is a 28 base pairs (bp) variable number tandem repeat (VNTR), located on 5' untranslated region (UTR) [18]. Is characterized by exhibiting a putative Enhancer box (E-box) sequence on the first 28bp repeat of 2R allele and on the two first repeats of 3R allele [15,19]. Therefore, a higher number of repeats should increase the amount of E-box binding sites for the upstream stimulating factors (USF), leading to an increased transcription of *TYMS* and, consequently, to higher TS levels [20]. In addition, a single nucleotide polymorphism (SNP) characterized by a cytosine to guanine (C>G) transition on the twelfth nucleotide of the second repeat of

VNTR 3R allele (rs2853542) has been described [15]. In the presence of cytosine (3RC) the E-box seems to be disrupted, reducing the stimulation of transcription in comparison to 3RG, thereby decreasing TS levels [15]. Since this SNP occurs within the *TYMS* 28bp VNTR polymorphism, several studies have been performed combining the information from both *TYMS* enhancer region (TSER) polymorphisms [6,21]. Another important polymorphism is a 6bp sequence (TTAAAG) deletion (1494del6, rs34489327) at 3´UTR, which seems to affect a region of TS pre-messenger ribonucleic acid (mRNA) that contains *cis* adenylate-uridylate-rich elements (AREs) [22,23]. These elements bind to a *trans* AU-rich factor 1 (AUF1), preferentially in the presence of deletion allele (6bp-), diminishing mRNA stability and, consequently, decreasing TS levels [16,22,23]. Therefore, the aim of this study was to elucidate the clinical relevance of these *TYMS* polymorphisms, by genotype and haplotype-based approaches, in MTX therapeutic outcome of Portuguese RA patients.

METHODS

Patients and study design

A retrospective study was performed between January 2009 and December 2012 at São João Hospital Center (Porto, Portugal) in a cohort of consecutive Caucasian patients (≥ 18 years) with RA treated with MTX. Patients were excluded from the study if there was history of drug abuse, recent pregnancy or desire to become pregnant. The study was approved by the Ethical Committee of São João Hospital Center (reference 33/2009), procedures were considered to be according to the standards of the Helsinki Declaration and all patients provided an informed written consent.

After diagnosis, patients were classified according the 1987 criteria of the American College of Rheumatology (ACR) and reclassified according the 2010 criteria of the ACR and the European League Against Rheumatism (EULAR) [24]. All patients were initially treated with 10mg *per os* (PO)/week of MTX in monotherapy. This dose was increased 5mg at each three weeks if the patients did not meet the EULAR criteria for response, i.e., if presented a

Disease Activity Score in 28 joints (DAS28) >3.2. Every 3 months treatment response was evaluated and, on the: 1) first evaluation, if patients have no response or show gastrointestinal toxicity, administration route was changed to subcutaneous (SC); 2) second evaluation, if maximum tolerable dose was used without response, MTX therapy was discontinued or associated with other synthetic DMARD; and 3) third evaluation, in patients without response and other contraindication, therapy was changed by associating a biological DMARD. The occurrence of MTX-related toxicity was registered at each visit and, according to severity, MTX dose was adjusted or discontinued. Folic acid supplementation was prescribed to all patients for the prevention of toxicity occurrence and their regular compliance was registered [7,25,26]. Other concomitant drugs, such as corticosteroids and non-steroidal anti-inflammatories (NSAIDs) were allowed during the study.

Outcome definition

Non-response. MTX clinical response was recorded at time of each visit. Non-response was defined when patients presented a DAS28 >3.2, calculated and defined as described by Prevoo *et al.* [27], in two consecutive evaluations. Therefore, non-response to MTX had a minimum period of MTX therapy, at least, of six months.

Toxicity. The occurrence of MTX-related toxicity, defined when patients presented any adverse drug reaction (ADR) related to MTX, was recorded upon each visit. The type of ADR was classified in System Organ Class (SOC) disorders, in accordance with Common Terminology Criteria for Adverse Events (CTCAE) [28].

Samples handling and *TYMS* genotyping

Whole blood samples from each patient were obtained with standard venipuncture technique in ethylenediaminetetraacetic acid (EDTA) containing tubes. Genomic DNA was extracted with QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer instructions and total

genomic DNA was quantified, and its purity analyzed, using the NanoDrop® 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA).

TSER polymorphisms. 28bp VNTR polymorphism (rs34743033) and SNP C>G (rs2853542) at the twelfth nucleotide of the second repeat of 3R allele were genotyped as described by Lima *et al.* [29]. For quality control, 10% of the samples were randomly selected for a second analysis and 10% percent of cases were confirmed by automated sequencing in a 3130xl Genetic Analyzer using the Kit BigDye Terminator v3.1 (Life Technologies, Foster City, CA, USA). Results were 100% concordant.

TYMS 1494del6 polymorphism. 1494del6 polymorphism (rs34489327) was genotyped as described by Lima *et al.* [29] with slight modifications. PCR products were purified with USB® ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) before cycle sequencing. Sequence reactions were carried out using the sequencing Kit BigDye Terminator v.3.1 (Life Technologies, Foster City, CA, USA) according to manufacturer's specifications. The sequencing profile was 30 cycles at 96°C for 10 seconds, 55°C for 10 seconds and 60° for 60 seconds, followed by an extension cycle at 60°C for 10 minutes. The sequence products were purified with illustra Sephadex G-50 Fine DNA Grade (GE Healthcare, Fairfield, CT, EUA) columns, denatured with Hi-Di™ Formamide and run in an 3130xl Genetic Analyzer (Life Technologies, Foster City, CA, USA). For quality control, 10% of the samples were randomly selected for a second analysis and results were 100% concordant.

Polymorphisms classification and linkage disequilibrium measure

TSER polymorphisms were classified according to their theoretical TS functional *status* as previously described [6] and grouped by predicted expression levels, as follow: low expression genotypes (2R2R, 2R3RC and 3RC3RC), median expression genotypes (2R3RG and 3RC3RG) and high expression genotype (3RG3RG). Haplotype analysis was performed using a two-stage iterative method named expectation maximization algorithm

(SNPStats software) [30]. In order to estimate LD between pairs of alleles at TSER and *TYMS* 1494del6 *loci*, *D'* coefficients were calculated in Arlequin for Windows, Version 3.11 (University of Berne, Bern, Switzerland) [31] with 100,000 number of steps in Markov chain. The measure was interpretable as the proportion of maximum possible level of association between two *loci*, given the allele frequencies, ranging from 0 (linkage equilibrium) to 1 (complete LD) [32]. Possible haplotypes were tested for association with risk for non-response to MTX and for MTX-related toxicity by taking the most frequent haplotype as reference.

Statistical analysis

Statistical analyses were performed with either IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA), OpenEpi for Windows, Version 2.3.1 [33] and SNPStats software [30]. Genotype and allele frequencies were assessed and tested for Hardy-Weinberg equilibrium (HWE). All statistical tests were two-sided and a probability (*p*) value of 5% or less was considered as statistically significant. The Pearson Chi-square test or Fisher's exact test were used to compare the outcome variables and *TYMS* polymorphisms. The odds ratio (OR) and the correspondent 95% confidence intervals (CI) were calculated as a measure of the association between the categorical variables. To correct for multiple comparisons, Bonferroni's method was applied in order to control the false positive rate, and a significance level of α=0.05/(n comparisons) was used [34]. Forest plot was performed using MedCalc® software for Windows, Version 13.1.2 [35]. Multivariate analysis with binary logistic regression was used to identify which *TYMS* genotypes or haplotypes could predict the occurrence of non-response to MTX and MTX-related toxicity. This analysis was performed adjusting to potential confounding clinicopathological variables in three steps. In the first step patient-related variables (age, gender and smoking) were considered; in a second step, beyond patient-related variables, disease-related variables (diagnosis age and disease duration) were added; and in a third step, beyond patient and disease-related variables, treatment-related

variables (folic acid supplementation, corticosteroids therapy, use of NSAIDs, other concomitant DMARDs used and MTX administration characteristics - dose, treatment duration and administration route) were also considered.

RESULTS

Population description

This study included follow-up data of 233 patients, 196 (84.1%) females and 37 (15.9%) males, with a mean age of 51±11.6 years old, of which 32 (13.7%) were smokers. Considering the disease-related variables, the mean age at diagnosis was 40.3 ± 13.2 years old and the median disease duration was 7.0 years (0.3-51.0). All 233 (100.0%) patients were treated with MTX with a median dose of 15.0 mg/week (2.5-25.0), 118 (50.6%) complied regularly to folic acid supplementation, 188 (80.7%) were under corticosteroid therapy and 170 (73.0%) used NSAIDs.

Non-response to MTX (DAS28 >3.2 in two consecutive evaluations) was observed in 128 (54.9%) patients. Regarding disease activity, the mean for DAS28 was 4.2 ± 1.3. MTX-related toxicity was registered in 77 (33.0%) patients. The observed ADRs were classified in SOCs disorders as follow: 58 (75.3%) gastrointestinal disorders (abdominal distension, diarrhea, dyspepsia, nauseas, stomach pain and/or vomiting); 9 (11.7%) skin and subcutaneous tissue disorders (alopecia, rash maculo-papular and rheumatoid nodulosis exacerbation); 5 (6.5%) hepatobiliary disorders (determined by transaminases serum elevation); and 5 (6.5%) respiratory, thoracic and mediastinal disorders (hypersensitivity pneumonitis). Since the number of cases in each SOCs disorders were small, the evaluation of *TYMS* polymorphisms with clinical relevance as possible biomarkers of MTX-related toxicity was performed for MTX-related overall toxicity.

TYMS genotype and haplotype analyses

Genotypes distribution of *TYMS* polymorphisms was in HWE (p>0.050) in the studied population. Frequencies of 28bp VNTR alleles and genotypes were: 2R allele 41.8%; 3R allele 57.3%; 4R allele 0.9%; 2R2R 15.0% (n=35); 2R3R

53.7% (n=125); 3R3R 29.6% (n=69); and 3R4R 1.7% (n=4). Due to the low frequency of 3R4R genotype, it was excluded from the analyses. Considering TSER polymorphisms, genotypes distribution was: 2R allele 42.6%; 3RC allele 33.0%; 3RG allele 24.4%; 2R2R 15.3% (n=35); 2R3RC 29.7% (n=68); 2R3RG 24.9% (n=57); 3RC3RC 12.6% (n=29); 3RC3RG 10.9% (n=25); and 3RG3RG 6.6% (n=15). According to TS theoretical functional *status*, genotypes frequencies were: low expression 57.6% (n=132); median expression 35.8% (n=82); and high expression 6.6% (n=15). Frequencies of 1494del6 alleles and genotypes were: 6pb+ allele 70.0%; 6bp- allele 30.0%; 6bp+6bp+ 48.9% (n=114); 6bp+6bp- 41.6% (n=97); and 6bp-6bp- 9.5% (n=22).

Haplotype analysis revealed that 28bp VNTR and 1494del6 polymorphisms were in LD (p<0.00001). Alleles 2R and 6bp+, and alleles 3R and 6bp- were the most linked ones (D'=0.67 for both). The analysis demonstrated four haplotypes: 2R6bp+ 38.4%; 2R6bp- 4.1%; 3R6bp+ 31.7% and 3R6bp- 25.8%. TSER and 1494del6 polymorphisms were also in LD (p<0.00001). Alleles 2R and 6bp+ (D'=0.67) and 3RG and 6bp- (D'=0.48) demonstrated to be the most linked ones. This analysis showed six haplotypes: 2R6bp+ 38.4%; 2R6bp- 4.1%; 3RC6bp+ 22.7%; 3RG6bp+ 9.0%; 3RC6bp- 10.3%; and 3RG6bp- 15.5%.

TYMS genotypes and MTX therapeutic outcome

Table 1 reports the relation between *TYMS* polymorphisms and MTX therapeutic outcome both regarding MTX non-response and toxicity.

Non-response. In relation to 28bp VNTR polymorphism, 3R allele was significantly associated with non-response to MTX when compared to 2R allele (p=0.012, OR=1.61). In addition, 3R homozygotes were associated with more than 2-fold increased risk for non-response to MTX when compared to 2R homozygotes (p=0.018, OR=2.71) and 2R carriers (p=0.005, OR=2.34) and remained significant after corrected for multiple comparisons. For TSER polymorphisms, 3RC allele shown to be associated with non-response to MTX when compared to 2R allele (p=0.008, OR=1.79). Furthermore, and attending to functional 3R, 3RC3RG was related with more than 3-fold increased risk

for non-response to MTX when compared to 2R3RG (*p*=0.016, OR=3.52), which remained significant after multiple comparisons correction. Considering the 1494del6 polymorphism, 6bp- allele was significantly associated with non-response to MTX when compared to 6bp+ allele (*p*=0.006, OR=1.76). Moreover, and compared to 6bp+ homozygotes, 6bp+6bp- (*p*=0.038, OR=1.79), 6bp-6bp- (*p*=0.024, OR=3.07) and 6bp- carriers (*p*=0.011, OR=1.96) presented a statistically significant increased risk for non-response to MTX and, excepting for 6bp+6bp-, continued significant after correcting for multiple comparisons.

Toxicity. No statistically significant differences were observed in relation to *TYMS* genotypes and MTX-related overall toxicity.

TYMS haplotypes and MTX therapeutic outcome

Table 2 represents the relationship between *TYMS* haplotypes and MTX therapeutic outcome both regarding MTX non-response and toxicity.

Non-response. 3R6bp- haplotype was found significantly associated with non-response to MTX when compared to 2R6bp+ haplotype (*p*=0.001, OR=2.54). Moreover, 3RC6bp+, 3RC6bp- and 3RG6bp- haplotypes were statistically significant associated with non-response to MTX when compared to 2R6bp+ haplotype (*p*=0.041, OR=1.79; *p*=0.013, OR=2.80; and *p*=0.009, OR=2.39, respectively).

Toxicity. No statistically significant differences were observed in relation to *TYMS* haplotypes and MTX-related overall toxicity.

Multivariate analysis

Multivariate analysis was performed in three steps adjusting to potential confounding variables. Table 3 shows multivariate analysis results of *TYMS* genotypes and haplotypes and clinical response to MTX. Figure 2 resumes the impact of all potential confounding variables in the association of *TYMS*

genotypes and haplotypes with clinical response to MTX. Regarding *TYMS* genotypes, results demonstrated that 6bp- carriers were statistically significant associated with more than 2-fold increased risk for non-response to MTX when compared to 6bp+ homozygotes (p=0.016, OR=2.74). According to *TYMS* haplotypes, our results shown that haplotypes harboring simultaneously 3R and 6bp- alleles were statistically significant associated with almost 3-fold increased risk for non-response to MTX when compared to 2R6bp+ haplotype.

DISCUSSION

Thymidylate synthase is a key enzyme for DNA synthesis and repair [11,12] inhibited by MTXPGs and, therefore, contributes for MTX antiproliferative and anti-inflammatory effects [10]. In fact, TS levels were found to be predictive of MTX therapeutic outcome [13,14]. Since genetic polymorphisms in *TYMS* have been associated with TS levels [6], in this study we aimed to elucidate the influence of *TYMS* polymorphisms (28bp VNTR, SNP C>G and 1494del6) in MTX therapeutic outcome of Portuguese RA patients.

All patients enrolled in this study were recruited within a well-defined geographical area and were of Caucasian ethnicity, with gender and age at time of diagnosis distributions similar to other reported populations [36,37]. Genotypes distribution of *TYMS* polymorphisms was in HWE and was similar to those found for other Caucasian populations [21,38,39]. Nevertheless, and despite the potential of our results, possible study limitations include: 1) relatively reduced population size; 2) presence of other *TYMS* polymorphisms that possibly could alter TS expression or functionality; 3) limited screen of some important genes that codify other enzymes involved in MTX action mechanism.

TYMS genotypes and MTX therapeutic outcome

Non-response. Among our population and regarding 28bp VNTR polymorphism, 3R allele was associated with risk for non-response to MTX,

which increases in the presence of both 3R alleles, in accordance to previous studies [13,40]. Literature describes 3R allele as associated with higher TS levels [19,20] and TS levels as predictive of clinical response to MTX [13,40]. Moreover, 3R allele has been associated with higher MTX doses required [13] and higher RA disease activity [40]. Despite the significant univariate analysis results, multivariate analysis did not confirm them. Additionally, other studies demonstrated associations between 3R homozygotes and response to MTX [39] or showed no association [21,41-43]. It has been suggested by some authors that it is of greater importance to consider the SNP C>G on 3R allele and analyze the TSER polymorphisms instead of studying 28bp VNTR polymorphism alone. 3RG allele was associated with higher transcriptional activity and translation efficiency due to its increased ability to complex with the USF protein [15,44]. Accordingly, the number of functional E-box in both 2R and 3RC alleles should be the same [6,15], which should reveal that patients with these genotypes would have similar TS expression and, consequently, a resembling clinical response. However, our results seem to demonstrate that 2R and 3RC alleles are different since 3RC3RG genotype was associated with over 3-fold increased risk for non-response to MTX when compared to 2R3RG. In addition, our results showed that 3RC allele was associated with non-response to MTX, when compared to 2R allele, and 3RC3RC genotype has a non-significant trend for non-response to MTX when compared to 2R2R genotype. Nevertheless, no statistically significant differences were observed attending to TSER polymorphisms grouped according to theoretically TS expression levels and to multivariate analysis. Moreover, a previous study demonstrated that non-response to MTX was associated to 3RG3RG patients [21]. Therefore, the putative relationship between TSER polymorphisms and clinical response to MTX outcome needs further clarification.

In relation to 1494del6 polymorphism, our results demonstrated that 6bp-allele was associated with non-response to MTX. Additionally, multivariate analysis showed that 6bp- carriers were associated with about 3-fold increased risk for non-response to MTX. *In vitro* studies have demonstrated

that 6bp- allele has decreased mRNA stability and, thereby reduced TS expression [22,23], however, in other previously reported studies in RA Caucasian patients no association was observed [41]. Moreover, one study in Psoriasis, a disease where MTX is used in similar doses than RA, 6bp- allele demonstrated a trend for non-response, however, this study included Caucasian and non-Caucasian patients [45]. Studies in Asiatic patients have reported different results, some of them reported an association between 6bp- allele and response [13,43], while others reported no associations [42,46]. From all of these results it seems that ethnicity could be an important factor to predict the clinical response to MTX.

Toxicity. Regarding the occurrence of MTX-related overall toxicity, our results did not reach significance pertaining to TSER and 1494del6 polymorphisms, in accordance with previously reported studies [21,41,42,46,47]. Nevertheless, other studies reported significant associations of 28bp VNTR polymorphism with MTX-related toxicity [38,48]. To the best of our knowledge this is the first report evaluating the influence of TSER polymorphisms in MTX-related toxicity in RA.

TYMS haplotypes and MTX therapeutic outcome

Non-response. Haplotypes may have a particular significance in regard to functionality or as genetic markers for unknown functional variants. Therefore, haplotype analysis was performed, to assess of possible consequences on the phenotype in the copresence of several variants of the same gene. As reported by others [6,39,49,50], *TYMS* polymorphisms were in LD, especially 2R6bp+ and 3RG6bp- haplotypes. Univariate haplotype analysis demonstrated that 3R6bp-, 3RC6bp+, 3RC6bp- and 3RG6bp- haplotypes (haplotypes harboring 3R allele for 28bp VNTR, 3RC allele for TSER and 6bp- allele for 1494del6) were associated with almost 3-fold increased risk for non-response to MTX. Nevertheless, multivariate analysis showed that haplotypes harboring simultaneously 3R and 6bp- alleles (3R6bp-, 3RC6bp-

and 3RG6bp-) were associated with non-response to MTX. This suggests a prominent role of the 3'-UTR polymorphism in predicting the clinical response to MTX and it seems that 6bp- allele can interact differently with 2R and 3R alleles, in agreement with Lurje *et al*. [51]. Additionally, our results suggested that the haplotype revealing more risk for non-response to MTX was 3RC6bp-, which combines the major risk alleles from the 5´UTR (3RC) and from the 3´UTR (6bp-). Only one study in RA has performed haplotype analysis, where an association between 3R6bp- haplotype and response to MTX was demonstrated [39]. Nevertheless, there are some important differences: no reference to SNP C>G; studied population included patients with early RA; and the study evaluated the impact in clinical response to MTX combined therapy with sulfasalazine. Thus, we propose that *TYMS* haplotype analysis should be used in future studies to elucidate the influence of *TYMS* in MTX therapeutic outcome, which could help to interpret these preliminary conflicting data.

Toxicity. Regarding MTX-related toxicity, no differences were observed attending to *TYMS* haplotypes. Despite it was expected that *TYMS* haplotypes follow the same tendency as *TYMS* genotypes, to the best of our knowledge no studies analyzed the *TYMS* haplotypes and the development of toxicity arising from MTX in RA.

The observed discrepancies among different studies could be explained by inter-study variability, ethnicity variability, samples sizes, variety of methods used to measure the MTX therapeutic outcome, different treatment regimens, and different genotyping protocols with limited quality of results. Therefore, functional TS studies in RA should be conducted to better understanding TS expression regulation mechanism and its putative importance in establishing more effective clinical therapeutic strategies when MTX is used in RA patients. To the best of our knowledge, this is the first report regarding the study of the association of *TYMS* polymorphisms with MTX therapeutic outcome in Portuguese RA patients. This study concluded that *TYMS*

polymorphisms seem to be important to predict clinical response to MTX in RA patients; *TYMS* genotypes and haplotypes harboring 6bp- allele were associated with non-response to MTX; *TYMS* haplotypes harboring simultaneously 3R and 6bp- alleles seem to be predictors of non-response to MTX; and, to elucidate the role of *TYMS* on MTX therapeutic outcome full haplotypic information should be exploited. Despite the potential of our findings, translation into clinical practice requires larger and multicentric studies in order to clearly endorse the utility of these polymorphisms.

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FIGURES

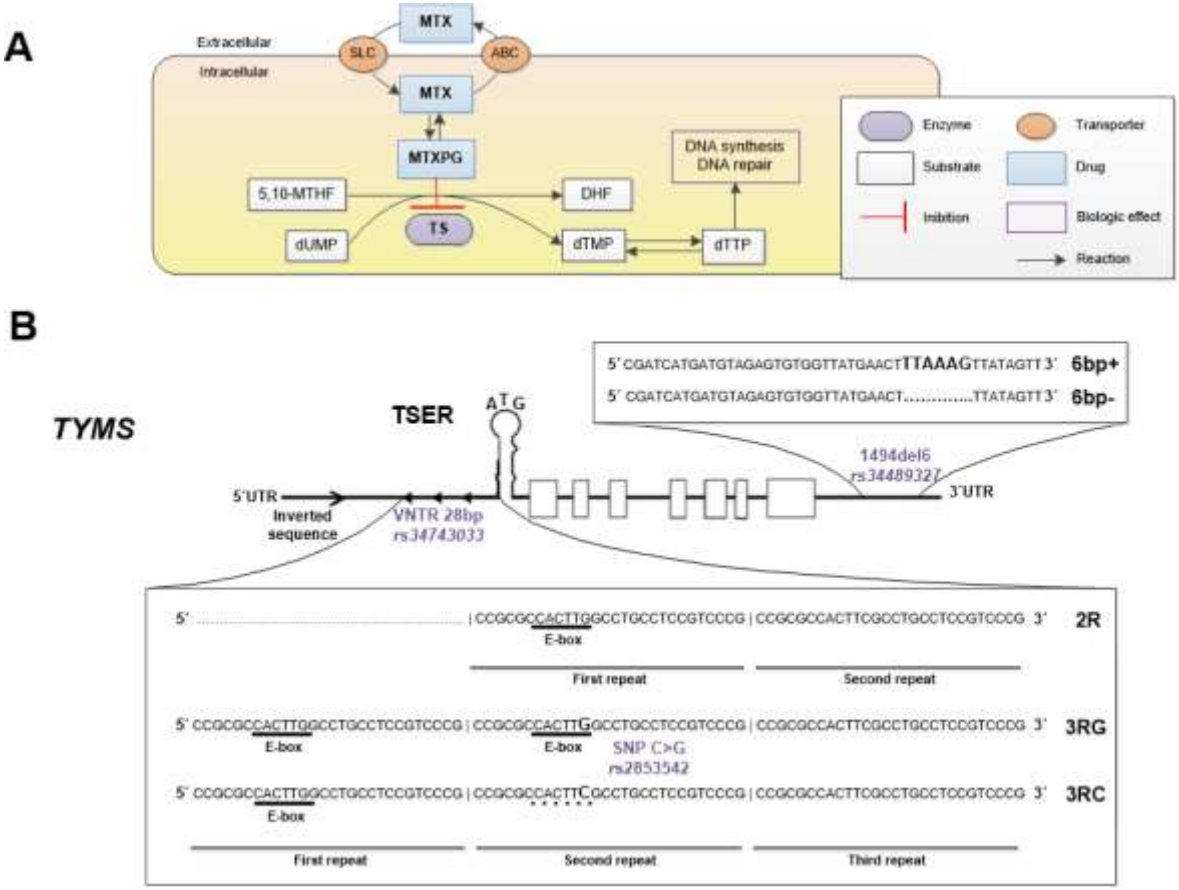


Figure 1. A. Part of MTX action mechanism in which thymidylate synthase (TS) is involved. MTX enters the cell after binding to folate transporters, mainly by solute carriers (SLC), and can be exported by members of the ATP-binding cassette (ABC) transporters family. To prevent MTX rapid efflux from cells and enhance its intracellular retention, MTX is polyglutamated by the enzyme folylpolyglutamyl synthase into MTX polyglutamates (MTXPGs) which inhibit TS activity. TS is a key protein for the *de novo* pyrimidine synthesis and is responsible for the simultaneous conversion of deoxyuridine monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (5,10-MTHF) to deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF). Subsequently, the dTMP is phosphorylated to deoxythymidine triphosphate (dTTP) and used for the DNA synthesis and repair. **B. *TYMS* structure and location of VNTR 28bp (rs34743033), SNP C>G (rs2853542) and 1494del6 (rs34489327) polymorphisms.** 5,10-MTHF: 5,10-methylenetetrahydrofolate; A: adenine; ABC: ATP-binding cassette; bp: base pairs; C: cytosine; del: deletion; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dTTP: deoxythymidine triphosphate; dUMP: deoxyuridine monophosphate; E-box: enhancer box; G: guanine; MTXPG: methotrexate polyglutamates; R: repeat; SLC: solute carrier; SNP: single nucleotide polymorphism; TS: thymidylate synthase (protein); TSER: thymidylate synthase enhancer region; T: thymine; *TYMS*: thymidylate synthase (gene); UTR: untranslated region; VNTR: variable number tandem repeat.

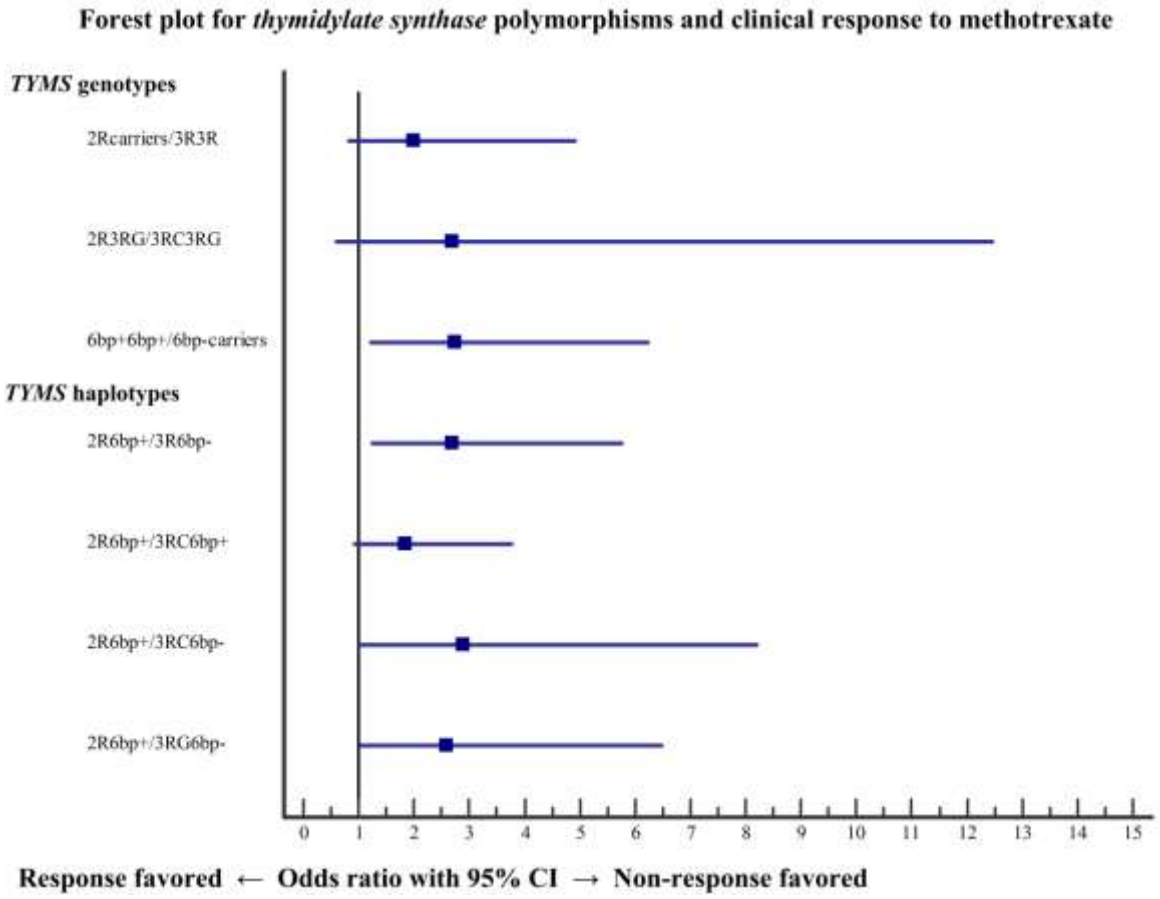


Figure 2. Forest plot of multivariate analysis in the association of *thymidylate synthase* genotypes and haplotypes with clinical response to methotrexate. Odds ratio and 95% confidence intervals are reported for clinical response to methotrexate. bp: base pairs; C: cytosine; CI: confidence interval; del: deletion; G: guanine; R: repeat; TYMS: thymidylate synthase (gene).

TABLES

Table 1. *Thymidylate synthase* polymorphisms and methotrexate therapeutic outcome

	MTX Response				MTX Toxicity			
	Response	Non-Response	*p*	OR (95% CI)	Non-Toxicity	Toxicity	*p*	OR (95% CI)
***TYMS* 28bp VNTR (rs34743033) #**								
2R2R	19 (54.3)	16 (45.7)		Reference	25 (71.4)	10 (28.6)		Reference
2R3R	62 (49.6)	63 (50.4)	0.624	1.21 (0.57-2.56)	78 (62.4)	47 (37.6)	0.324	1.51 (0.67-3.41)
3R3R	21 (30.4)	48 (69.6)	0.018 ˅	2.71 (1.17-6.29)	50 (72.5)	19 (27.5)	0.911	0.95 (0.39-2.35)
2R carriers	81 (50.6)	79 (49.4)		Reference	103 (64.4)	57 (35.6)		Reference
3R3R	21 (30.4)	48 (69.6)	0.005 ˅	2.34 (1.29-4.27)	50 (72.5)	19 (27.5)	0.233	0.69 (0.37-1.28)
2R2R	19 (54.3)	16 (45.7)		Reference	25 (71.4)	10 (28.6)		Reference
3R carriers	83 (42.8)	111 (57.2)	0.208	1.59 (0.77-3.27)	128 (66.0)	66 (34.0)	0.529	1.29 (0.58-2.84)
2R allele	100 (51.3)	95 (48.7)		Reference	128 (65.6)	67 (34.4)		Reference
3R allele	104 (39.5)	159 (60.5)	0.012 ˅	1.61 (1.09-2.38)	178 (67.7)	85 (32.3)	0.647	0.91 (0.60-1.38)
TSER polymorphisms (rs2853542* and rs34743033)								
Functional 2R								
2R2R	19 (54.3)	16 (45.7)		Reference	25 (71.4)	10 (28.6)		Reference
2R3RC	32 (47.1)	36 (52.9)	0.487	1.34 (0.59-3.03)	44 (64.7)	24 (35.3)	0.492	1.36 (0.56-3.31)
3RC3RC	9 (31.0)	20 (69.0)	0.062	2.64 (0.94-7.39)	23 (79.3)	6 (20.7)	0.469	0.65 (0.20-2.08)
Functional 3R								
2R3RG	30 (52.6)	27 (47.4)		Reference	34 (59.6)	23 (40.4)		Reference
3RC3RG	6 (24.0)	19 (76.0)	0.016 ˅	3.52 (1.23-10.10)	16 (64.0)	9 (36.0)	0.710	0.83 (0.31-2.20)
3RG3RG	6 (40.0)	9 (60.0)	0.384	1.67 (0.52-5.30)	11 (73.3)	4 (26.7)	0.384§	0.54 (0.15-1.90)
2R allele	100 (51.3)	95 (48.7)		Reference	128 (65.6)	67 (34.4)		Reference
3RC allele	56 (37.1)	95 (62.9)	0.008 ˅	1.79 (1.13-2.82)	106 (70.2)	45 (29.8)	0.369	0.81 (0.50-1.31)
3RG allele	48 (42.9)	64 (57.1)	0.155	1.40 (0.86-2.30)	72 (64.3)	40 (35.7)	0.810	1.06 (0.63-1.78)
TSER polymorphisms grouped according to theoretically TS expression levels**								
Low expression	60 (45.5)	72 (54.5)		Reference	92 (69.7)	40 (30.3)		Reference
Median expression	36 (43.9)	46 (56.1)	0.824	1.07 (0.61-1.85)	50 (61.0)	32 (39.0)	0.189	1.47 (0.83-2.63)
High expression	6 (40.0)	9 (60.0)	0.687	1.25 (0.42-3.71)	11 (73.3)	4 (26.7)	1.000§	0.84 (0.25-2.79)
Low + Median expression	96 (44.9)	118 (55.1)		Reference	142 (66.4)	72 (33.6)		Reference
High expression	6 (40.0)	9 (60.0)	0.714	1.22 (0.42-3.55)	11 (73.3)	4 (26.7)	0.778§	0.72 (0.22-2.33)
Low expression	60 (45.5.)	72 (54.5)		Reference	92 (69.7)	40 (30.3)		Reference
Median + High expression	42 (43.4)	55 (56.7)	0.746	1.09 (0.64-1.85)	61 (62.9)	36 (37.1)	0.279	1.36 (0.78-2.36)
***TYMS* 1494del6 (rs34489327)**								
6bp+6bp+	61 (53.5)	53 (46.5)		Reference	78 (68.4)	36 (31.6)		Reference
6bp+6bp-	38 (39.2)	59 (60.8)	0.038	1.79 (1.03-3.10)	59 (60.8)	38 (39.2)	0.249	1.40 (0.79-2.46)
6bp-6bp-	6 (27.3)	16 (72.7)	0.024 ˅	3.07 (1.12-8.41)	19 (86.4)	3 (13.6)	0.122§	0.34 (0.10-1.23)
6bp+6bp+	61 (53.5)	53 (46.5)		Reference	78 (68.4)	36 (31.6)		Reference
6bp- carriers	44 (37.0)	75 (63.0)	0.011 ˅	1.96 (1.16-3.31)	78 (65.5)	41 (34.5)	0.641	1.14 (0.66-1.97)
6bp+ carriers	99 (46.9)	112 (53.1)		Reference	137 (64.9)	74 (35.1)		Reference
6bp-6bp-	6 (27.3)	16 (72.7)	0.078	2.36 (0.89-6.26)	19 (86.4)	3 (13.6)	0.055§	0.29 (0.08-1.02)
6bp+ allele	160 (49.2)	165 (50.8)		Reference	215 (66.2)	110 (33.8)		Reference
6bp- allele	50 (35.5)	91 (64.5)	0.006 ˅	1.76 (1.15-2.71)	97 (68.8)	44 (31.2)	0.578	0.89 (0.57-1.38)

Results are expressed in n (%). *p* value <0.05 was considered to be of statistical significance (highlighted in bold). §Fisher's exact test used when number of cases of one cell was less than 5.
˅Statistically significant when *p* values were adjusted for multiple comparisons correction using Bonferroni's method (α=0.05/n comparisons).
#3R4R genotype (n=4) was excluded from analyses due to the low frequency. *rs2853542 - *TYMS* SNP C>G on 3R allele. **Genotypes theoretically associated with TS expression: a) high: 3RG3RG; b) median: 2R3RG and 3RC3RG; c) low: 2R2R, 2R3RC and 3RC3RC.
bp: base pairs; C: cytosine; del: deletion; G: guanine; OR: odds ratio; R: repeat; SNP: single nucleotide polymorphism; TS: thymidylate synthase (protein); TSER: *TYMS* enhancer region; *TYMS*: thymidylate synthase (gene); VNTR: variable number tandem repeat.

Table 2. *Thymidylate synthase* haplotypes and methotrexate therapeutic outcome

	MTX Response				MTX Toxicity			
TYMS Haplotypes	Response	Non-Response	*p*	OR (95% CI)	Non-Toxicity	Toxicity	*p*	OR (95% CI)
Based on *TYMS* 28bp VNTR and *TYMS* 1494del6 polymorphisms								
2R6bp+	43.0	30.0		Reference	36.2	33.9		Reference
2R6bp-	6.0	7.4	0.360	1.70 (0.54-5.32)	5.7	10.2	0.190	2.20 (0.69-7.03)
3R6bp+	33.9	34.5	0.100	1.55 (0.92-2.60)	33.1	37.8	0.490	1.23 (0.69-2.20)
3R6bp-	17.1	28.1	0.001	2.54 (1.46-4.43)	25.0	18.1	0.320	0.74 (0.41-1.34)
Based on TSER and *TYMS* 1494del6 polymorphisms								
2R6bp+	43.2	30.2		Reference	36.2	34.8		Reference
2R6bp-	5.8	7.2	0.360	1.70 (0.55-5.24)	5.6	9.3	0.220	2.02 (0.66-6.20)
3RC6bp+	21.2	25.6	0.041	1.79 (1.03-3.12)	23.8	23.6	0.820	1.07 (0.59-1.95)
3RC6bp-	6.2	11.8	0.013	2.80 (1.25-6.25)	10.9	6.0	0.240	0.55(0.21-1.47)
3RG6bp+	12.5	8.7	0.880	1.06 (0.50-2.24)	9.3	13.3	0.300	1.53 (0.69-3.38)
3RG6bp-	11.1	16.5	0.009	2.39 (1.24-4.59)	14.2	13.0	0.810	0.92 (0.46-1.82)

Results are expressed in estimated frequencies (%) under linkage disequilibrium. *p* value <0.05 was considered to be of statistical significance (highlighted in bold).
bp: base pairs; C: cytosine; del: deletion; G: guanine; OR: odds ratio; R: repeat; TSER: *TYMS* enhancer region; *TYMS*: thymidylate synthase (gene); VNTR: variable number tandem repeat.

Table 3. Multivariate analysis of *thymidylate synthase* polymorphisms and clinical response to methotrexate

Genetic variables \ Adjusted variables	Patient-related		Patient-related + Disease-related		Patient-related + Disease-related + Treatment-related	
	P	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)
***TYMS* genotypes**						
***TYMS* 28bp VNTR (rs34743033)**						
2R carriers		Reference		Reference		Reference
3R3R	0.013	2.23 (1.19-4.17)	0.013	2.24 (1.19-4.21)	0.135	1.99 (0.81-4.91)
TSER polymorphisms						
Functional 3R						
2R3RG		Reference		Reference		Reference
3RC3RG	0.069	2.90 (0.92-9.13)	0.071	2.91 (0.91-9.25)	0.203	2.70 (0.59-12.47)
***TYMS* 1494del6 (rs34489327)**						
6bp+6bp+		Reference		Reference		Reference
6bp- carriers	0.003	2.33 (1.32-4.10)	0.003	2.38 (1.34-4.23)	0.016	2.74 (1.21-6.23)
***TYMS* haplotypes**						
Based on *TYMS* 28bp VNTR and *TYMS* 1494del6 polymorphisms						
2R6bp+		Reference		Reference		Reference
3R6bp-	<0.001	2.87 (1.59-5.19)	<0.001	2.92 (1.60-5.32)	0.012	2.68 (1.25-5.75)
Based on TSER and *TYMS* 1494del6 polymorphisms						
2R6bp+		Reference		Reference		Reference
3RC6bp+	0.041	1.81 (1.03-3.20)	0.035	1.86 (1.05-3.31)	0.090	1.85 (0.91-3.76)
3RC6bp-	0.012	2.97 (1.28-6.93)	0.018	2.75 (1.19-6.32)	0.048	2.89 (1.01-8.21)
3RG6bp-	0.004	2.78 (1.39-5.56)	0.003	3.06 (1.49-6.31)	0.043	2.60 (1.04-6.49)

***P* value <0.05 is considered to be of statistical significance (highlighted in bold).**
Adjusted variables include: 1) patient-related variables (age, gender and smoking); 2) disease-related variables (diagnosis age and disease duration); and 3) treatment-related variables (folic acid supplementation, corticosteroids, non-steroidal anti-inflammatories, other concomitant disease-modifying antirheumatic drugs and methotrexate administration characteristics - dose, treatment duration and administration route). Genetic variables include: *TYMS* genotypes and *TYMS* haplotypes.
bp: base pairs; C: cytosine; del: deletion; G: guanine; OR: odds ratio; R: repeat; TSER: *TYMS* enhancer region; *TYMS*: thymidylate synthase (gene); VNTR: variable number tandem repeat.

CHAPTER X

GENERAL DISCUSSION

Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER X - GENERAL DISCUSSION

This chapter discusses the overall study's results regarding the association of clinicopathological and genetic variables with Methotrexate therapeutic outcome, as also explores its limitations.

Despite MTX is extensively used in RA treatment, there is a great interpatient variability to MTX therapeutic outcome, which can be partly determined by clinicopathological and genetic factors. Therefore, this work evaluated the influence of clinicopathological variables and several genetic polymorphisms in genes encoding for MTX membrane transport proteins as also for proteins involved in MTX action mechanism pathways, as predictors of clinical response to MTX and/or MTX-related adverse drug reactions occurrence in Portuguese RA patients.

1. POPULATION

The patients enrolled in this work were recruited based on inclusion criteria applied in the study (adults, Caucasian, without drug abuse history, recent pregnancy or desire to become pregnant during the study, diagnosed with RA, treated with MTX, with well characterized clinicopathological variables). The size of population was in accordance to the prevalence of the disease in the Portuguese population with reported disease gender epidemiology (22), suggesting an occurrence of two to four times higher in females, and age of diagnosis range, occurring between the third and the fifth decades of life, similar to other reported populations (24, 321). In addition, the studied population was a representative clinical practice cohort of established and well-defined RA patients (224, 320).

Additionally, the evaluated outcomes (non-response to MTX and MTX-related ADRs occurrence) were in accordance with literature. Non-response to MTX was registered for 128 (54.9%) patients and, literature reports that 40%

to 60% of patients fail to achieve a good response profile (322). Non-response was measured only after six months of treatment with MTX because the maximum improvement tends to occur only after approximately six months of starting with MTX (166), even it is dependent of other factors such, for example, comorbidities and patients' age. Considering MTX-related toxicity, 77 (33.0%) patients presented ADRs, which is in accordance with literature that reports about 15% to 30% of patients experience ADRs (322). Similarly to literature descriptions, the most frequent observed ADRs were minor GI disorders (75.3%), including abdominal distension, diarrhea, dyspepsia, nauseas, stomach pain and/or vomiting, and none has led to MTX discontinuation (191, 200, 206, 225). Other observed ADRs included skin and subcutaneous tissue disorders (alopecia, rash maculo-papular and rheumatoid nodulosis exacerbation); hepatobiliary disorders (determined by transaminases serum elevation); and, respiratory, thoracic and mediastinal disorders (hypersensitivity pneumonitis), which are in agreement with the descriptions in literature (323).

2. CLINICOPATHOLOGICAL VARIABLES & MTX THERAPEUTIC OUTCOME

Several clinicopathological variables were reported as associated with MTX therapeutic outcome variability among RA patients.

2.1. Clinical Response to MTX

In accordance to MTX clinical response, results demonstrated that non-current smokers were associated with non-response to MTX. Although some studies have pointed out that smokers had a worst response to MTX, presenting a higher disease activity and severity (329, 330), others were able to demonstrate that tobacco exposure reduced radiographic progression and favored a better functional score (331, 332). In fact, literature describes the association between smoking and decreased folate levels which enhance the

antifolate effect of MTX and, therefore, improve the response to MTX (324-326). Furthermore, cigarette nicotine seems to potentiate the immunosuppressive and anti-inflammatory effects by acting on the immunological system (327, 328).

Results also demonstrated that anti-CCP and ANAs positivity were associated with more than 2-fold increased risk of non-response to MTX. These autoantibodies are presented in RA patients and are strongly correlated with erosive disease, worse functional status, higher disease activity and with non-response to MTX (2, 333-337). Nevertheless, other studies have shown a correlation between anti-CCP positivity and a better response to MTX or absence of associations in early RA populations (338, 339). The discrepancy with prior studies may be explained by the fact that our population had established disease. To the best of my knowledge there are no studies in RA associating ANAs and MTX response.

Additionally, this study revealed that an increased score in HAQ was associated with an increased risk for non-response to MTX. As reported by others, the higher HAQ score corresponds to an increased disease activity, thus it was expected that these patients would have a worst response to MTX (338, 340).

The concomitant use of NSAIDs was correlated with non-response to MTX. These results could be explained by the existence of drug-drug interactions, since NSAIDS are known to alter MTX and 7-OH-MTX binding to plasmatic proteins and to impair MTX hepatic metabolism (161). This translates into low amount of free MTX and reduced formation of active MTX metabolites in hepatocytes. Due to the importance of NSAIDs as symptomatic therapy in RA, and to contradictory results, further studies are required to clarify this association (316, 338).

In addition, SC administration route was significantly associated with better response to MTX. This result can be explained by the higher MTX bioavailability associated with this route of administration (341), which consequently leads tissues to a greater exposure to MTX, higher cellular polyglutamation and retention and, therefore, to better response to MTX.

2.2. Toxicity to MTX

According to MTX-related toxicity, results demonstrated statistically significant associations between lower MTX treatment duration and MTX-related toxicity occurrence (overall and GI) in univariate analysis and with MTX-related GI toxicity in multivariate analysis. These results are in accordance with published literature, where the median time for MTX-related ADRs occurrence was 36.5 months (323). Moreover, results confirmed the protective effect afforded by folic acid supplementation. Since it is expected that folic acid supplements reduce the toxicity of low-dose MTX therapy (143, 342-345), the concomitant use of folic acid with MTX is considered the standard treatment (142, 143). Even though folic acid supplementation was prescribed to all patients, only 118 (50.6%) complied with the treatment. Becker *et al.* reported an association between MTX-related toxicity occurrence and lower intracellular folates (346). Furthermore, two meta-analyses support the obtained findings by concluding that the low-doses of folate supplementation have protective effects for MTX-related GI toxicity (142, 143). Also, one study demonstrated that GI toxicity was the main reason for MTX withdrawal when related with the absence of folate supplementation (316). In fact, studies reported that MTX treatment leads to a folate deficiency status, given that mammalian cells cannot synthesize folates *de novo* (347). Moreover, RA itself also conditioned a decrease in folate levels (348) and, consequently, without the appropriate reposition of folates, several metabolic pathways, such as the biosynthesis of purines, pyrimidines and some amino acids (such serine and methionine), processes which maintain normal cell growth and replication, will be compromised (347). For this reason, rapidly dividing tissues, such as of the GI tract, are more susceptible to folate depletion and, therefore, the supplementation with folic acid will help to protect from GI toxicity (347). Other reason contributing for the protective mechanism resulting from folic acid supplementation may be the interaction of folic acid with MTX (161). This interaction causes the block of MTX reabsorption in the renal distal tubule

leading to a decrease of MTX circulating levels, and consequently to lower toxicity (161).

Additionally, results demonstrated an association between increased levels of ESR and MTX-related overall toxicity but not with GI toxicity. The DAS28 is widely used for the assessment of RA patients in the clinic to monitor disease activity (349-353) and is calculated from four components: TJC, SJC, VAS and ESR or CRP. Reasons for using CRP include not only its potential to be a more direct measure of inflammation, but also the increasing usage of B-cell directed therapies that may have differential effects on these inflammatory markers (354). However, recent data from two large observational studies suggested that DAS28(CRP) scores tended to be lower than DAS28(ESR) scores and Inoue *et al.* suggested potential new thresholds for disease activity categories for DAS28(CRP) (116, 117). Since no threshold has been defined, we have used the ESR for DAS28 calculation. The ESR measure is a simple and inexpensive laboratory test for assessing the inflammatory or acute response (353) with reduced sensitivity and specificity. Consequently, the obtained results can be explained by the greater impact of the other observed ADRs in the inflammatory process, such skin and subcutaneous tissue disorders; hepatobiliary disorders; and, respiratory, thoracic and mediastinal disorders, when compared to GI toxicity. In fact, other studies have reported an increase of acute phase reactants in rheumatoid nodulosis (355, 356), hepatotoxicity (357), and hypersensitivity pneumonitis (358), that were also observed as ADRs in studied population, which can explain the reason why higher ESR levels were associated with MTX-related overall toxicity and not with MTX-related GI toxicity.

3. GENETIC VARIABLES & MTX THERAPEUTIC OUTCOME

The genotypes of studied polymorphisms were in HWE and the distribution was similar to the published literature for other Caucasian

populations (183, 223, 224, 227, 232, 259, 264, 273, 286, 320, 322, 359-361) and with the National Center for Biotechnology Information (NCBI) database, except for *SLC19A1* G80A polymorphism (rs1051266). Nevertheless, it is well known that NCBI database does not contemplate all the populations and, there are no studies available for this polymorphism regarding the Portuguese population to allow comparisons of the obtained genotypes distribution.

3.1. MTX Carrier-Mediated Transport Pathway & MTX-Related Toxicity

The SLC19A1 is a bidirectional transporter, described as being expressed in the majority of tissues, with relevance in enterocytes and hepatocytes (177, 215). Genotype analysis, pertaining the influence of *SLC19A1* G80A polymorphism (rs1051266) on overall toxicity, demonstrated no statistically significant differences but, G carriers were at a significant increased risk of MTX-related GI toxicity when compared to A homozygotes. Bohanec Grabar *et al.* have previously reported that G homozygotes had an increased risk for MTX-related overall toxicity (224, 225) but did not clearly described the impact on GI disorders, and, the majority of other studies did not shown associations with MTX-related toxicity (228, 232, 233). Considering the obtained data and literature, it can be hypothesized that GG genotype could provide an increased influx capacity. Here, we can hypothesize that GG genotype could provide an increased influx capacity leading to higher bioavailability, increased MTX tissues exposure, mainly in tissues where SLC19A1 is highly expressed and, consequently, resulting in GI toxicity. Moreover, results demonstrated that *SLC19A1* rs7499 G carriers were associated with an increased risk for MTX-related toxicity (overall and GI). This SNP occurs in a portion of 3'-UTR believed to be important for mRNA stability, localization and translational efficiency (216) and, thus, important to membrane transporter expression. Although, its effect in this bidirectional transporter function is unknown, it can be proposed that GG genotype could provide an increased influx capacity leading to higher bioavailability,

increased MTX tissues exposure and, consequently, to toxicity. In the literature, only one study in Caucasian patients addressed the influence of this SNP in MTX-related toxicity, but no associations were observed (232). In addition, A carriers for *SLC19A1* rs2838956, presented a borderline trend towards significance for MTX-related toxicity (overall and GI). This is possibly due to an increased influx capability of A carriers, which consequently, leads to greater MTX tissues exposure and toxicity. Results are in agreement with a previously report by Bohanec Grabar *et al.* that demonstrated a borderline significant trend towards MTX-related overall toxicity for A carriers, particularly for skin and subcutaneous tissue disorders (225), yet another study reported no statistically significant association (232). The impact of this variant in SLC19A1 is currently unknown and functional studies are essential since intronic SNPs can potentially influence RNA splicing, which may affect transporter structure and function (234).

As for *SLC46A1* rs2239907, G homozygotes revealed association with MTX-related overall toxicity. This SNP is also located in a 3´UTR region, which is considered as important in mRNA stability, localization and translational efficiency (216). Despite the effect of this SNP in transporter function is currently unknown, it is regarded as a potential functional SNP. The SLC46A1 is mostly expressed in apical membrane of enterocytes but also can be found in other cells (177). Thus, it can be proposed that GG genotype could provide an increased influx capacity leading to higher bioavailability and, consequently, to higher MTX tissues exposure and toxicity. To the best of my knowledge this is the first report to analyze the effect of this SNP with MTX-related toxicity in RA.

Regarding *SLCO1B1* rs4149056, results demonstrated that T carriers were associated with MTX-related toxicity (overall and GI). Literature reports that T carriers are associated with an increased membrane expression of SLCO1B1 and higher MTX influx and clearance (362). Despite the SLCO1B1 transporter is mainly expressed on basolateral membrane of hepatocytes (236), its mRNA also has been detected in other tissues, including enterocytes (238), which can explain the MTX intracellular retention (GI and

hepatic) leading to cytotoxicity. The association of T allele with MTX-related toxicity has been previously described for high-dose MTX (362) but this is the first report to analyze the influence of this SNP with MTX-related toxicity in RA.

In *ABCG2*, rs13120400 C carriers showed a trend towards for MTX-related toxicity. The ABCG2 transporter, located in apical membranes of enterocytes, hepatocytes and kidney tubular cells, is responsible for MTX efflux from the enterocytes to intestinal tract lumen and MTX excretion into bile and urine (173). Then, it is plausible that C carriers would cause a reduced efflux capability, which is translated in less MTX elimination and higher MTX bioavailability, leading to toxicity. This is the first report to associate this SNP with MTX-related toxicity in RA patients.

From haplotype analyses, this work showed that GGAG haplotype for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200 was associated with MTX-related GI toxicity when compared to AAGA haplotype. The association of GGAG haplotype with GI toxicity was expected from the genotype analyses obtained results for those SNPs in *SLC19A1*. Hence, it can be hypothesize that GGAG haplotype could provide an increased MTX influx capability, leading to higher MTX intracellular levels, mainly in tissues where SLC19A1 is highly expressed (177) and, thus, have an increased risk for GI toxicity development. In fact, it appears more evident that, to better characterize the role of a candidate gene, the full haplotypic information should be exploited in order to assess the possible consequences on the phenotype in the copresence of several variants of the same gene (317, 318).

Accordingly to TRI for MTX-related overall toxicity, an increased TRI value was associated with an increased incidence of ADRs. From this work, results demonstrated that patients with Index 3 were 18-fold more likely to present an ADR when compared to those with Index 1. Regarding to the occurrence of MTX-related GI disorders, the TRI demonstrated that patients with Index 4, when compared to those with Index 3 and 1, were 5-fold and 9-fold, respectively, more likely to present GI disorders. This highlights the importance of genotyping patients and the urgency of developing the field of

therapy personalization for the prediction of MTX-related toxicity development.

3.2. MTX Cellular Pathway & MTX-Related Toxicity

To evaluate the influence of pyrimidine pathway in the MTX-related toxicity, the influence of *TYMS* polymorphisms was studied. Similarly to other reported studies, presented data showed no association of MTX-related toxicity with *TYMS* TSER and 1494del6 polymorphisms (226, 232, 246, 264, 363), while other studies reported significant associations of 28bp VNTR polymorphism with MTX-related toxicity occurrence (224, 269). Regarding *TYMS* haplotypes, no differences were observed too. Despite it was expected that *TYMS* haplotypes follow the same tendency as *TYMS* genotypes, to the best of my knowledge this is the first report evaluating the influence of TSER polymorphisms in MTX-related toxicity and no previously studies analyzed *TYMS* haplotypes and the development of toxicity arising from MTX in RA patients.

3.3. MTX Cellular Pathway & Clinical Response to MTX

To evaluate the influence in MTX clinical response of genetic polymorphisms in genes encoding to proteins involved in MTX cellular pathways, four genetic polymorphisms were analyzed, which are related with folate and *de novo* nucleotides synthesis pathways.

3.3.1. Folate pathway

Methylenetetrahydrofolate reductase is not directly inhibited by MTX or MTXPG but its expression levels seemed to influence MTX action by modifying the folate status (221). The obtained results demonstrated a significant association between *MTHFR* C677T polymorphism (rs1801133) T homozygotes and non-response to MTX. Despite the lack of consistency of some studies (259, 273), the results presented in this work are in accordance

with others previously reported (183, 227). Literature describes that TT genotype is responsible for a reduction of MTHFR activity (267), leading to reduced 5-MTHF and another folate cofactors levels, affecting both folate and methionine pathways (190, 191). This will lead to a decreased release of the anti-inflammatory adenosine (192) and, consequently, to MTX non-response.

3.3.2. *De novo* pyrimidine synthesis pathway

Regarding the *de novo* pyrimidine synthesis pathway, 3R allele for *TYMS* 28bp VNTR polymorphism (rs34743033) was associated with increased risk for non-response to MTX, which increased in the presence of both 3R alleles, as suggested by other authors (221, 291). In fact, literature describes 3R allele as associated with higher TS levels (288, 290) and TS levels as predictive of clinical response to MTX (221, 291). Moreover, 3R allele has been associated with higher MTX doses required (291) and of higher RA disease activity (221). Despite the statistically significance for the univariate analysis results, multivariate analysis did not confirm them. Additionally, other studies demonstrated associations between 3R homozygotes and better response to MTX (223) or showed no association (232, 264, 292, 363). It has been suggested that it is of great importance to consider the C>G modification on 3R allele of *TYMS* (rs2853542) and analyze the TSER polymorphisms instead of studying the 28bp VNTR polymorphism isolated (286). The 3RG allele was associated with higher transcriptional activity and translation efficiency due to its increased ability to complex with the USF protein (289, 364). Nevertheless, the number of functional E-box in both 2R and 3RC alleles should be the same (289), which should reveal that patients with these genotypes would have similar TS expression and, consequently, a resembling clinical response. Results seemed to point out that 2R and 3RC alleles are different, as previously suggested by Lima *et al.* (286), since 3RC3RG genotype was associated with over 3-fold increased risk for non-response to MTX when compared to 2R3RG. In addition, the obtained results showed that 3RC allele was associated with non-response to MTX, when compared to 2R allele, and 3RC3RC genotype has a non-significant trend for

non-response to MTX when compared to 2R2R genotype. Nevertheless, no statistically significant differences were observed regarding to TSER polymorphisms grouped according to theoretically TS expression levels, as suggested by Lima *et al.* (286). A previously study demonstrated that non-response to MTX was associated to 3RG3RG patients (264). Therefore, the putative relationship between TSER polymorphisms and clinical response to MTX outcome needs further clarification.

For *TYMS* 1494del6 polymorphism (rs34489327), the obtained results demonstrated that 6bp- allele was associated with non-response to MTX. Additionally, multivariate analysis showed that 6bp- carriers were associated with about 3-fold increased risk for non-response to MTX. *In vitro* studies have demonstrated that 6bp- allele has decreased mRNA stability and, thereby reduced TS expression (293, 294); however, in other previously reported study in RA Caucasian patients no associations were observed (232). Studies including Asiatic patients have reported different results, some of them reported an association between 6bp- allele and better response to MTX (291, 292), while others reported no associations (226, 363). From all of these results it seems that ethnicity could be an important factor to predict the clinical response to MTX attending to *TYMS* 1494del6 polymorphism.

As reported by other authors, *TYMS* polymorphisms were in LD, especially 2R6bp+ and 3RG6bp- haplotypes (223, 286, 366). Univariate haplotype analysis demonstrated that 3R6bp-, 3RC6bp+, 3RC6bp- and 3RG6bp- haplotypes (haplotypes harboring 3R allele for 28bp VNTR, 3RC allele for TSER and 6bp- allele for 1494del6) were associated with almost 3-fold increased risk for non-response to MTX. Nevertheless, multivariate analysis showed that haplotypes harboring simultaneously 3R and 6bp- alleles (3R6bp-, 3RC6bp- and 3RG6bp-) were associated with non-response to MTX. This suggests a prominent role of the 3'-UTR polymorphism in predicting the clinical response to MTX and it seems that 6bp- allele can interact differently with 2R and 3R alleles, in agreement with Lima *et al.* (286) and with Lurje *et al.* (367). Additionally, results suggested that the haplotype revealing increased risk for non-response to MTX was 3RC6bp-,

which combines the major risk alleles from the 5´UTR (3RC) and from the 3´UTR (6bp-). The only study in RA were haplotype analysis was performed demonstrated an association between 3R6bp- haplotype and better response to MTX (223); nevertheless, there are some important differences in relation to the obtained results from this thesis work: no reference to SNP C>G; studied population included patients with early RA; and the study evaluated the impact in clinical response to MTX combined therapy with SSZ.

3.3.3. *De novo* purine synthesis pathway

Regarding the *de novo* purine synthesis pathway, results indicated that T carriers for *ATIC* T675C polymorphism (rs4673993) are at increased risk for non-response to MTX, as previously suggested (273, 322). Since ATIC is directly inhibited by MTXPG, the intracellular accumulation of AICAR and its metabolites causes the inhibition of ADA and AMPD1, thus leading to increased intracellular concentrations of adenosine and its consequent release for the extracellular space, contributing for the anti-inflammatory effects of MTX (184). To the best of my knowledge, there are no functional studies reporting the effect of this polymorphism in ATIC activity. Nevertheless, it can be hypothesize that in the presence of T allele it might lead to MTX non-response due to increased conversion of AICAR to FAICAR, causing the degradation of adenosine and its non-liberation, thus leading to less MTX anti-inflammatory effects. In addition, T allele seems to contribute for the increase of the *de novo* purine synthesis and for the decrease of MTX anti-proliferative effect (161), leading to non-response to MTX. Therefore, more studies are required to elucidate the impact of this polymorphism in the ATIC activity and clinical response to MTX. Moreover, this polymorphism seems to be in LD with *ATIC* C347G (rs2372536), a SNP of which the G allele carriers (minor allele) have been reported as related with better response (221, 273, 368, 369). Hence, results are consistent with the obtained, reporting an association between *ATIC* 675C homozygotes (minor allele) and clinical response to MTX.

4. STUDY LIMITATIONS

Albeit the potential importance of the results presented in this thesis, the limitations of this study should be considered. The sample size of studied population could be one of the limitations, although it is in accordance to the epidemiology data of the disease in the Portuguese population (22). Moreover, and due to the low frequency of SOC disorders, other than GI disorders, the influence of the studied polymorphisms in the occurrence of these other SOC disorders were not possible to test.

Despite the pharmacogenetic potential of the obtained data, it is important to consider that MTX transporters are expressed in different tissues and, therefore, this line of investigation could be proved of remarkable relevance since it would enable the prediction of toxicity for each tissue and help to guide therapeutic choices. Moreover, it is important to analyze the influence of polymorphisms in folate and MTX intracellular and circulating levels in order to better elucidate the impact on MTX PK and PD.

Furthermore, and due to complexity of MTX action mechanism, as previously reported by other studies (163, 189, 232, 259, 320, 370), multiple factors, such as the clinicopathological and other genetic variables, can influence and/or be associated with the MTX non-response and/or related toxicity occurrence. Although in this work a relative great portion of potentially implicated polymorphisms were studied, many of which had never been studied before in both RA and Caucasian populations, the presence of other genetic polymorphisms in genes encoding for MTX transporters and for proteins involved in folate, *de novo* purine and pyrimidine synthesis, adenosine and methionine pathways, deserves further evaluation. Thus, combined genetic and functional studies are needed to address the impact of polymorphisms in protein levels and function.

As referred previously, controversial results regarding clinicopathological variables and the studied genetic polymorphisms association with clinical response to MTX and MTX-related toxicity development have been published, motivating the need for more and larger

studies, necessary to clarify raised questions and support the obtained results.

The reported variations on different studies should be discussed regarding the possible misleading drawn conclusion for each study, either bias related to study design and settings, sample size/power, ethnicity, the population disease duration (early or established RA), changes in folate status and gene-gene interactions. Additionally, one of the major issues found was the different definition of concepts such as toxicity and clinical response to MTX, thus influencing conclusions drawn and rendering comparisons difficult. In this work, non-response to MTX was defined when patients presented a DAS28 >3.2 in two consecutive evaluations after at least six months of MTX therapy (114) and ADR was defined according to the International Committee on Harmonization as "*a response - mild, moderate or severe - to a drug that is noxious and unintended and occurs at doses normally used in humans for the prophylaxis, diagnosis and treatment of disease or for modification of physiological function*" (371), with ADRs classified in SOC disorders according to CTCAE (301).

Furthermore, another factor that must be highlighted is that, reported studies relating MTX-related overall toxicity association for the studied polymorphisms are not entirely clear regarding the incidence of other ADRs results. Since membrane transport proteins expression level is different depending the tissue (215), different impacts on tissue toxicity are expected and so, considering only the concept of overall toxicity will increase the difficulty for establish comparisons. Moreover, membrane transport proteins mechanisms have been referred as different depending on MTX dose (low-dose implicates the transporters and high-dose implicates both transporters and passive diffusion (372)). Consequently, this point is of great importance when previous studies are scrutinized to evaluate the influence of polymorphisms on MTX PK and PD. In fact, in this field, when studying MTX action in individuals, it is crucial to focus on these two major determinants: (1) PK, how much of a drug is needed to reach its target in the body, and (2)

PD, how well the target cells respond to the drug, which are both critical for consideration in the field of Pharmacogenomics (179).

Therefore, when analyzing the Pharmacogenomics of a specific disease it is important to investigate and discuss all the possible interactions and the integration of the different mechanisms of disease development and treatment response (Figure 27).

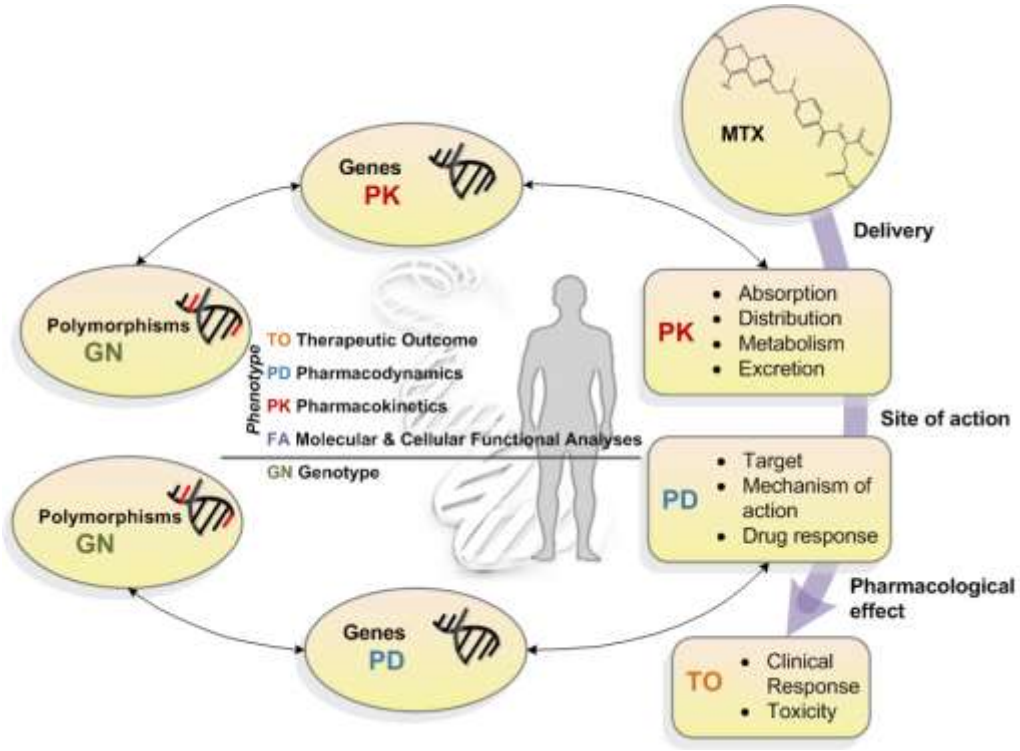
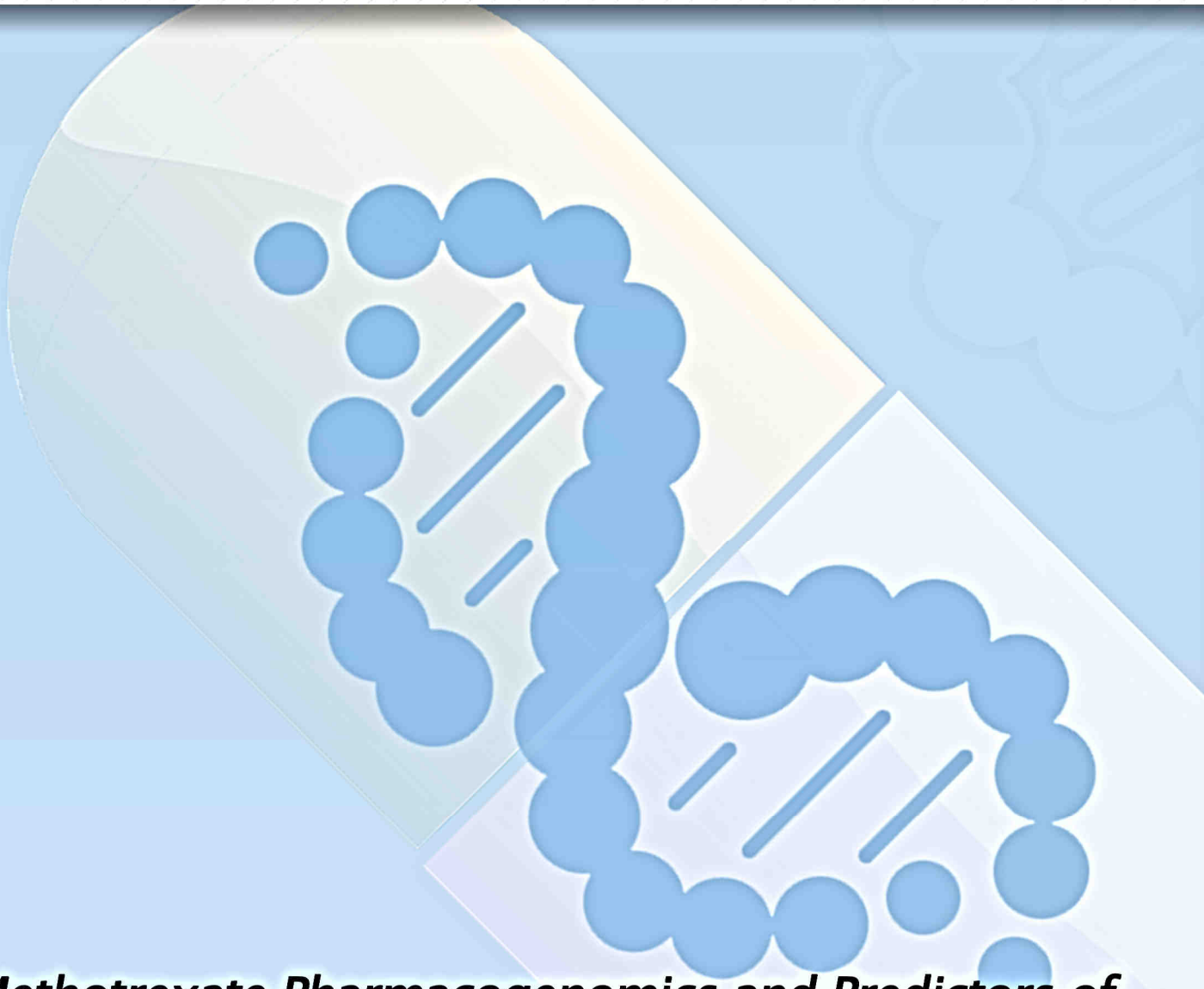


Figure 27. The interplay of disease, treatment and patient for therapeutic outcome prediction.

CHAPTER XI

CONCLUDING REMARKS AND FUTURE PERSPECTIVES



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER XI – CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This chapter presents the main conclusions and highlights the future work that is required and its importance.

Methotrexate therapeutic outcome is variable among RA patients both regarding clinical response and toxicity occurrence. This interpatient variability can be caused by different clinicopathological variables, patient-, disease- and treatment-related variables, as well as genetic polymorphism in genes encoding for proteins involved in MTX action mechanism.

Due to the inconsistent results reported in the literature about the putative predictors of MTX therapeutic outcome in RA patients, in order to reduce these discrepancies and provide more robust evidences, ideal future studies should consider as crucial several factors as follow: 1) the existence of distinct transporters, granting MTX different flux directions, expressed simultaneously, in the same cells and/or in different tissues; 2) the presence of endogenous substances (e.g. folates) or other drugs that could interact with MTX and/or with MTX transporters; 3) pH differences that could alter MTX solubility and, thus, dissolution (crucial for absorption when MTX is administered by PO route); 4) alterations in polyglutamation turnover, and thus, in MTX cellular retention, possibly by changing the functionality of FPGS and GGH; 5) the existence of other polymorphisms that could alter transporter levels, function or expression; and, 6) the presence of genetic polymorphisms in genes encoding for proteins involved in MTX action pathways than that under study in this work. The analysis of polymorphisms in genes encoding for MTX-carrier mediated transport pathway and its association with clinical response, and in *MTHFR* and *ATIC* with MTX-related toxicity should be analysed in future studies. Moreover, in order to improve the detection of further genetic factors responsible for variability in low-dose MTX therapeutic outcome, future studies should follow validated guidelines defining therapeutic outcomes, in order to harmonize definitions;

study design should be prospective, due to the fewer potential sources of bias and confounding factors when compared to retrospective studies; studies should be larger and multicentric (a large sample size can greatly improve statistical power and multicentre national and/or international collaborations can rapidly generate large samples); and, studies should undertake genotype and haplotype based approaches, in order to clarify the influence in PK parameters and in predicting MTX therapeutic outcome.

From the obtained results, it is possible to conclude that several genetic polymorphisms (rs7499, rs1051266, rs1801133, rs2239907, rs2838956, rs2853542, rs34489327, rs34743033, rs3788200, rs4149056 and rs4673993) encoding for MTX membrane transporters, as also for proteins involved in MTX intracellular pathways (specifically for SLCs, ABCs, TS, MTHFR and ATIC proteins) are useful predictors for MTX clinical response and/or of MTX-related toxicity development. Methotrexate is the cornerstone disease-modifying antirheumatic drug for RA treatment and, with the ageing of population and longer working life span, RA represents an expanding social and economic problem. Rheumatoid arthritis appears now to be a "syndrome", with different mechanisms underlying different clinical subsets, and is a major cause of morbidity and reduction in life expectancy, due to its systemic involvement, affecting patients, in particular, and the society, in general.

These results are clearly important for RA patients, since they can be helpful as biomarkers of MTX therapeutic outcome to elucidate which patients will not benefit from MTX treatment, both because of non-response and/or because the ADRs occurrence. Hence, it is of great importance to improve the knowledge of personalized treatment with MTX in RA patients.

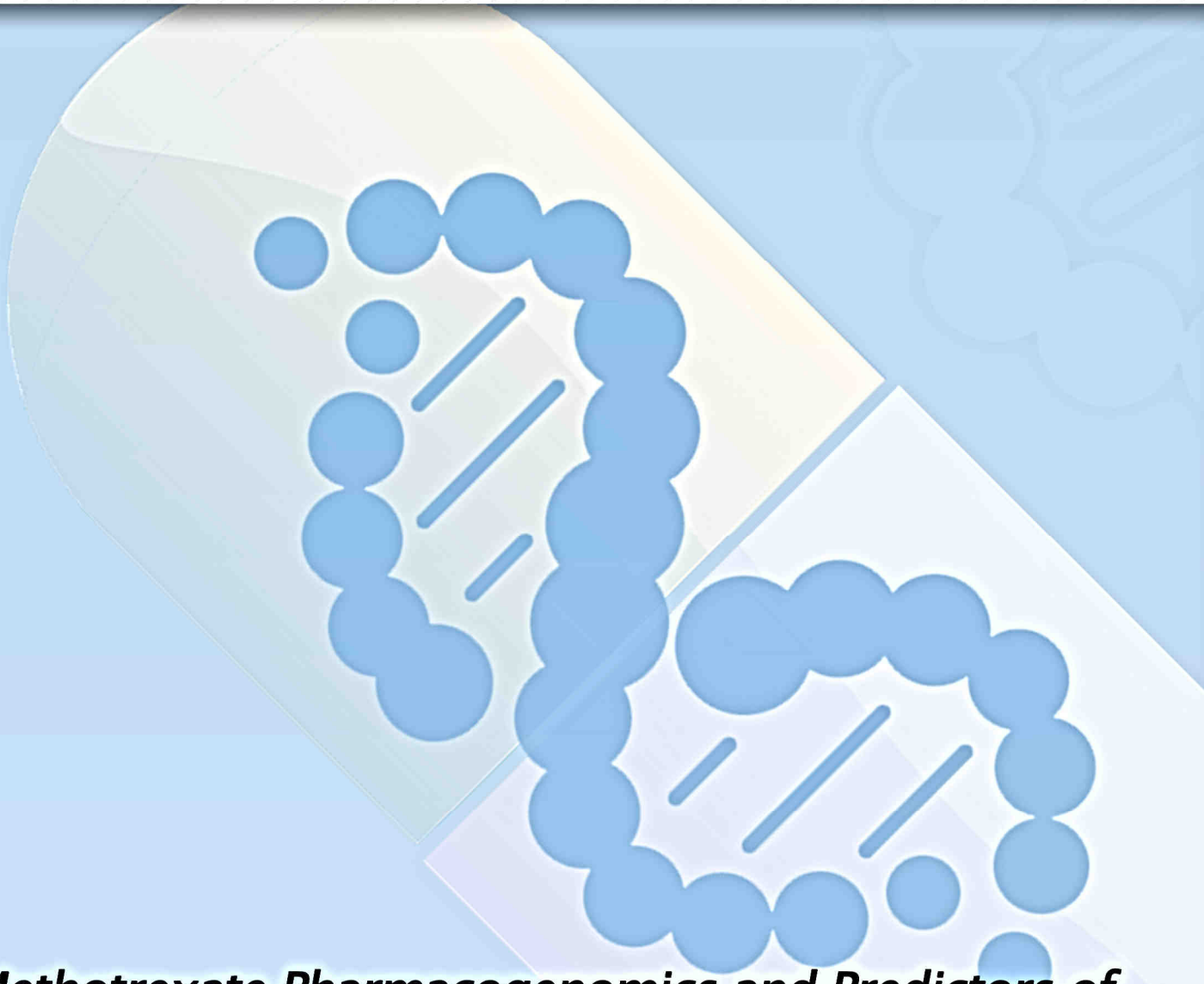
The obtained results indicate that genotyping of patients with RA clearly will contribute to the development of a personalized medicine approach for MTX therapeutic strategies. Nevertheless, it is imperative to validate these results in prospective studies, that will reduce potential sources of bias and confounding factors when compared to retrospective studies; with a large number of cases that can greatly improve statistical

power; with a multicentre design that could rapidly generate large samples and better comparison of results); with better definition of outcome variables; preferably using both genotype and haplotype based approaches, in order to clarify the influence in PK parameters and in predicting MTX therapeutic outcome. Hence, these polymorphisms could be validated as biomarkers of MTX therapeutic outcome, helping clinicians to select patients that will not benefit from MTX treatment contributing for the personalization of treatment for each patient.

In conclusion, several polymorphisms were demonstrated to be associated with MTX therapeutic outcome. After further validation, the studied polymorphisms can be useful as biomarkers of MTX therapeutic outcome, helping to elucidate which patients will not benefit from MTX treatment. All together, these results indicate that genotyping of patients with RA clearly will contribute to the development of a personalized medicine approach for MTX therapeutic strategies.

However, scientists also recognize that even as the knowledge continues to expand, its translation into clinics still requires evidences, needed to be generated for a particular disease and drug combination, before treatment can be customized to a patient's genotype. Thus, much work remains to be done before personalized medicine can reach its fullest potential.

EXECUTIVE SUMMARY



Methotrexate Pharmacogenomics and Predictors of Therapeutic Outcome in Rheumatoid Arthritis

CHAPTER XII – EXECUTIVE SUMMARY

INTRODUCTION

Rheumatoid Arthritis

- *Rheumatoid Arthritis is a complex, systemic autoimmune disease, characterized by a chronic inflammation of multiple peripheral joints.*
- *The incidence and prevalence of RA, a "New World disease", vary across populations and it's higher in North America and Northern Europe and in certain native American-Indian populations.*
- *Rheumatoid Arthritis is a multifactorial disease, involving both genetic and environmental factors, which pathophysiology mechanisms are currently not fully established.*
- *The 2010 ACR/EULAR classification criteria provide diagnostic support for recognition of early RA.*
- *Rheumatoid Arthritis monitoring is performed taking into account the quantification of joint damage, disease activity and health-related quality of life impact.*
- *Earlier detection of RA and a rapid, effective and aggressive therapeutic institution are the key factors to achieve the disease remission and improve survival rates.*

Methotrexate

- *Methotrexate is the most used and cost-effective disease-modifying antirheumatic drug for RA treatment.*
- *Low-dose MTX passes through several biological membranes by mediation of membrane transporters, which may be classified as influx (SLCs) and efflux (ABCs).*

- *Once inside the cells, MTX and/or MTXPGs inhibit multiple enzymes involved in folate pathway and de novo nucleotides synthesis. This inhibition capacity is then reflected in other pathways, such as methionine and adenosine pathways, which will along contribute to MTX Pharmacodynamics (antiproliferative and anti-inflammatory effects).*

Pharmacogenomics and Predictors of Therapeutic Outcome

- *Patients' clinicopathological variables, as also as genetic polymorphisms in genes encoding for proteins involved in MTX carrier-mediated transport systems and/or in intracellular pathways, have been described as contributors to the observed therapeutic outcome variability among RA patients.*

OBJECTIVES

- *The main aim of this study was to evaluate the influence of clinicopathological variables and genetic polymorphisms in genes encoding for proteins involved in MTX action mechanism as predictors of clinical response to MTX and/or of MTX-related toxicity occurrence in Portuguese RA patients.*

MATERIALS AND METHODS

- *A retrospective study, in a cohort of 233 consecutive Caucasian patients with RA treated with MTX was conducted.*
- *Clinicopathological data were collected from individual clinical records by physicians and include variables possibly influencing disease state and clinical response to MTX, selected based either in literature review and/or clinical significance. Whole blood samples for genotyping*

techniques were obtained in the time of the patient inclusion in the study.

- *Therapeutic outcome endpoints, non-response to MTX and MTX-related toxicity occurrence, were defined, as a DAS28 >3.2 in two consecutive evaluations after at least six months of MTX therapy and as the presence of any ADR related to MTX, respectively.*
- *Statistical analyses were performed whenever relevant and included: univariate and multivariate analyses by genotypes and haplotypes and toxicogenetic risk indexes, Forest plot and correction for multiple comparisons.*

RESULTS

Clinicopathological variables & clinical response to MTX

- *Non-current smokers, positivity to anti-CCP and to ANA antibodies, higher HAQ score and NSAIDs users were associated with non-response to MTX.*
- *Subcutaneous administration route was associated with better response to MTX.*

Clinicopathological variables & MTX-related toxicity occurrence

- *Lower MTX treatment duration and non-folic acid supplementation were associated with MTX-related GI toxicity occurrence.*
- *Higher ESR levels were associated with MTX-related overall toxicity.*

MTX-carrier mediated transport pathway & MTX-related toxicity occurrence

- *Increased risk for MTX-related overall toxicity was associated with SLC19A1 rs7499 G carriers, SLC46A1 rs2239907 G homozygotes and SLCO1B1 rs4149056 T carriers and T homozygotes.*

- *Toxicogenetic risk index analysis revealed that patients with Index 3 were 18-fold more likely to present an ADR when compared to those with Index 1.*
- *An increased risk for MTX-related GI toxicity was associated with SLC19A1 rs7499 G carriers and G homozygotes, SLC19A1 rs1051266 G carriers, SLC19A1 rs2838956 A carriers and, SLCO1B1 rs4149056 T carriers and T homozygotes.*
- *A higher risk of earlier MTX-related GI toxicity was registered for SLC19A1 rs1051266 G carriers.*
- *Haplotype GGAG for SLC19A1 rs7499, rs1051266, rs2838956 and rs3788200 was associated with increased risk for MTX-related GI toxicity.*
- *Toxicogenetic risk index analysis revealed that patients with Index 4 were 9-fold more likely to present a GI disorder when compared to those with Index 1.*

Folate pathway & clinical response to MTX

- *The T homozygotes for MTHFR rs1801133 were associated with over 4-fold increased risk for non-response to MTX.*

***De novo* pyrimidine synthesis pathway & clinical response to MTX**

- *Genotypes 3R3R, 3RC3RG and 6bp- carriers for TYMS rs34743033, rs34743033+rs2853542 and rs34489327, respectively, were associated with non-response to MTX.*
- *Haplotypes harboring both 3R and 6bp- alleles were associated with increased risk for non-response to MTX.*

De novo pyrimidine synthesis pathway & MTX-related toxicity occurrence

- *No statistically significant differences were observed regarding MTX-related toxicity and TYMS polymorphisms.*

De novo purine synthesis pathway & clinical response to MTX

- *The T carriers for ATIC rs4673993 were associated with over 4-fold increased risk for non-response to MTX.*

CONCLUDING REMARKS & FUTURE PERSPECTIVES

- *This work suggests that genetic polymorphisms combined with clinicopathological data may help to identify patients whom will not benefit from MTX treatment but validation from other studies is essential.*
- *Consideration of clinicopathological data and genotyping of patients with RA clearly will contribute to the development of a personalized medicine approach for MTX therapeutic strategies but much work remains to be done before personalized medicine can reach its fullest potential.*

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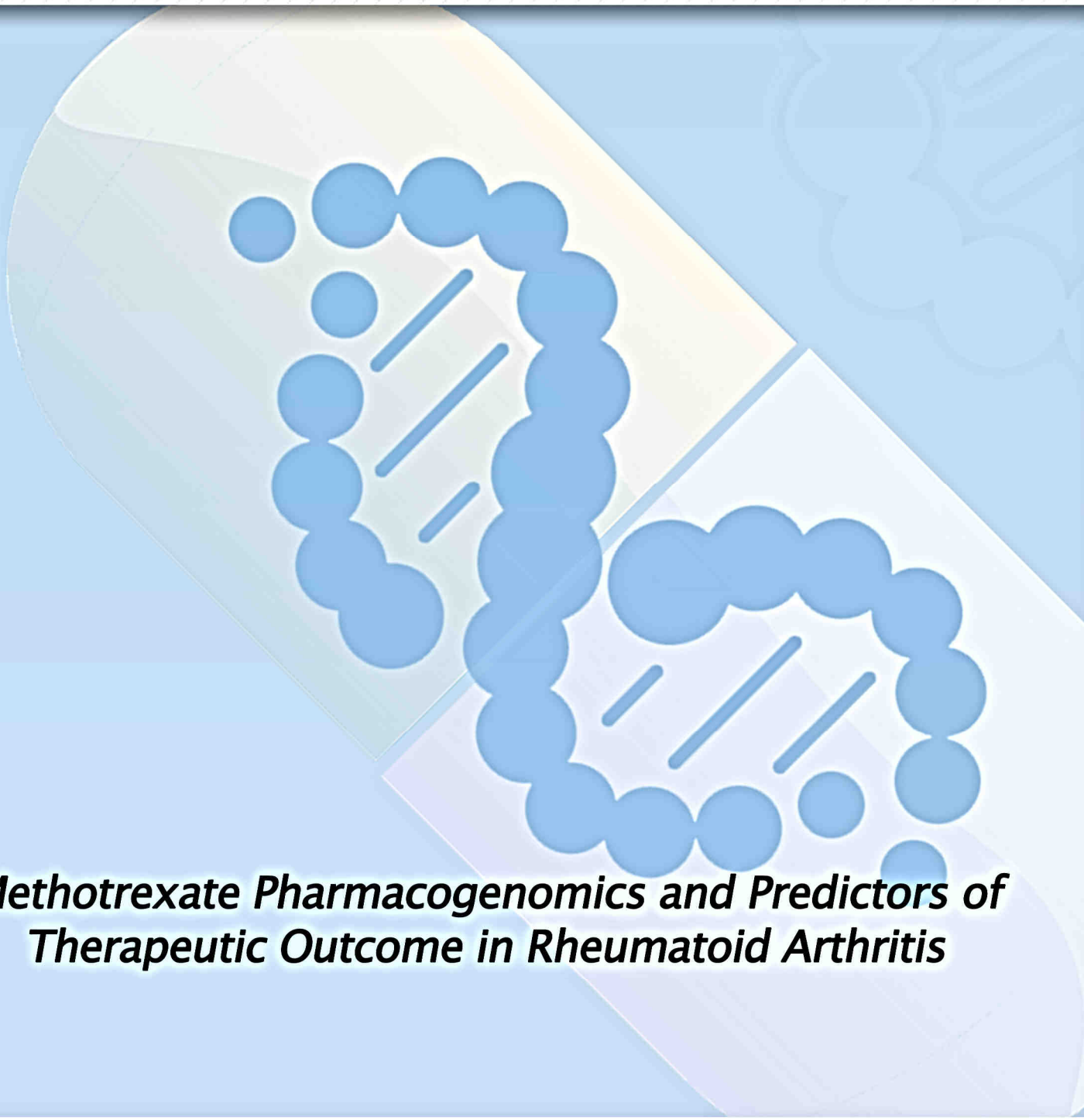

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