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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

DISSERTAÇÃO

MESTRADO INTEGRADO EM MEDICINA

Artigo de Investigação Médica

Moving Towards Personalized Medicine in Rheumatoid Arthritis

Methotrexate Intracellular Pathways and Phase II Reactions as Pharmacogenetic Predictors of Methotrexate Therapeutic Outcome

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Porto 2016

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Dissertação de Mestrado Integrado em Medicina submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador: Rui Manuel de Medeiros Melo Silva, PhD
Categoria: Professor Associado
Afiliação: Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto

“A força do querer não tem limites conhecidos.

Não é previsível o que se consegue quando se quer atingir as coisas.

O querer leva-nos até onde ninguém sabe, nem o próprio.”

Nuno Grande

NOTA PRELIMINAR

Declara-se, como comunicação do papel do aluno na execução desta dissertação, que o autor participou da conceção e execução do trabalho experimental que deu origem aos resultados, bem como realizou a análise, a interpretação e a elaboração do documento integrante da presente dissertação. O autor escreveu ainda a introdução, a discussão e as conclusões, descritas no presente documento, salvaguardando todas as correções e recomendações providenciadas pelo seu orientador.

O trabalho de investigação foi realizado no Grupo de Oncologia Molecular e Patologia Viral do Centro de Investigação do Instituto Português de Oncologia do Porto, em colaboração com o Instituto Universitário de Ciências da Saúde e o Centro Hospitalar de São João.

Porto, 19 de maio de 2016

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Áurea Rosa Nunes Pereira Lima

PREFÁCIO DO AUTOR

No âmbito da Unidade Curricular “Dissertação/Projeto/Relatório de Estágio” do Mestrado Integrado em Medicina do Instituto de Ciências Biomédica Abel Salazar – Universidade do Porto, foi proposta a elaboração de uma Dissertação, Projeto ou Relatório de Estágio. De entre as três modalidades possíveis, o autor optou pela elaboração de uma Dissertação, mais precisamente na forma de artigo de investigação médica.

Pelo exposto, o presente documento apresenta o artigo de investigação médica, intitulado “Moving Towards Personalized Medicine in Rheumatoid Arthritis: Methotrexate Intracellular Pathways and Phase II Reactions as Pharmacogenetic Predictors of Methotrexate Therapeutic Outcome”, cumprindo as regras da revista de referência “Pharmacogenomics” da Future Medicine, à qual o artigo foi submetido a 13 de abril de 2016, encontrando-se neste momento “sob revisão”.

Mais ainda, e porque o presente trabalho de investigação surge na sequência de outros já publicados pelo autor, nos quais a população alvo de estudo se encontra devidamente descrita, o artigo de investigação médica aqui apresentado remete para outras publicações do autor quando este entende ser necessário. Todavia, e para facilidade de leitura desta Dissertação, a descrição da população alvo de estudo encontra-se como Anexo no presente documento.

RESUMO CIRCUNSTANCIADO DA DISSERTAÇÃO

A artrite reumatoide é uma doença autoimune, caracterizada pela inflamação crónica de múltiplas articulações, condicionando considerável incapacidade. O metotrexato é o fármaco antirreumático modificador da doença de primeira linha no tratamento da artrite reumatoide, devido à boa relação custo-efetividade. Não obstante, a variabilidade observada no perfil de resposta terapêutica (Figura 1) é uma realidade, pelo que esforços têm sido realizados no sentido da identificação de preditores genéticos de resultados negativos da medicação ao metotrexato, mais precisamente preditores da ocorrência de não efetividade e/ou de reações adversas, a fim de personalizar e otimizar a terapêutica a instituir e, desde modo, contribuir para mais e melhor Saúde dos doentes com artrite reumatoide.

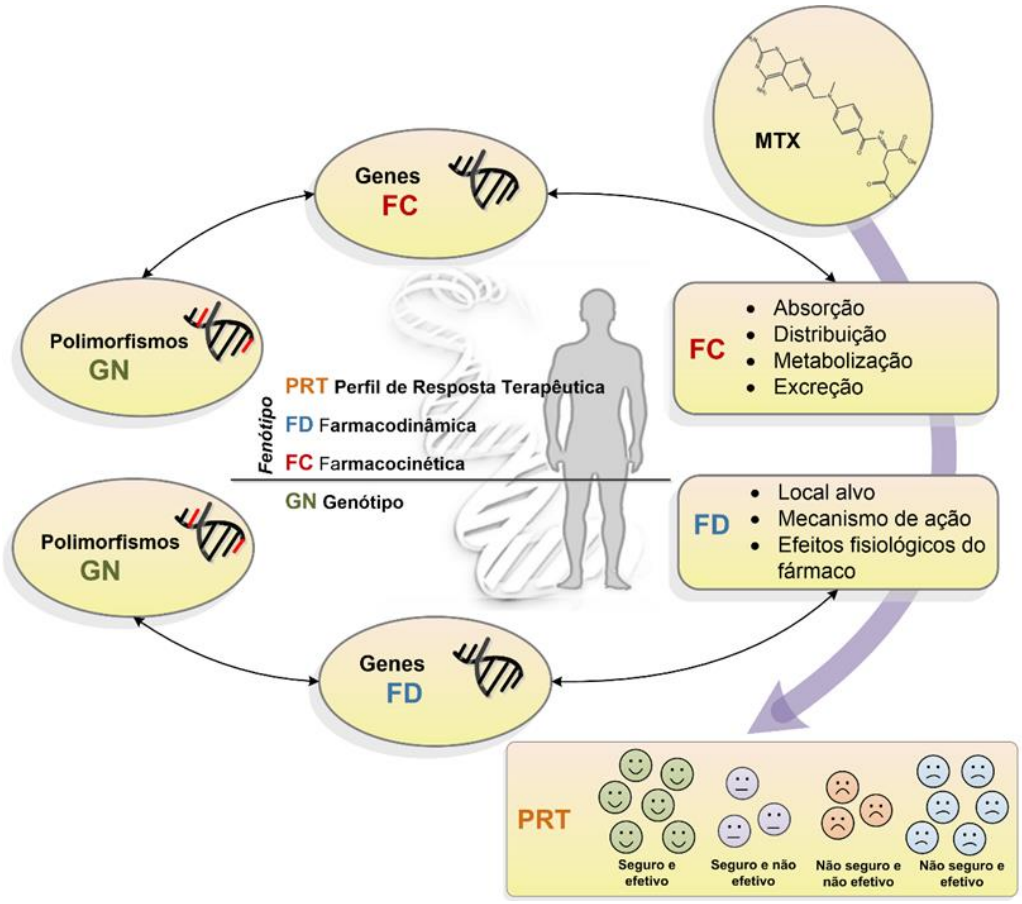

Figura 1. Fatores influenciadores do Perfil de Resposta Terapêutica.

Neste sentido, polimorfismos genéticos em genes codificantes de proteínas envolvidas no mecanismo de ação do metotrexato (Figura 2) têm sido estudados, não havendo, à data, dados relativos à população Portuguesa.

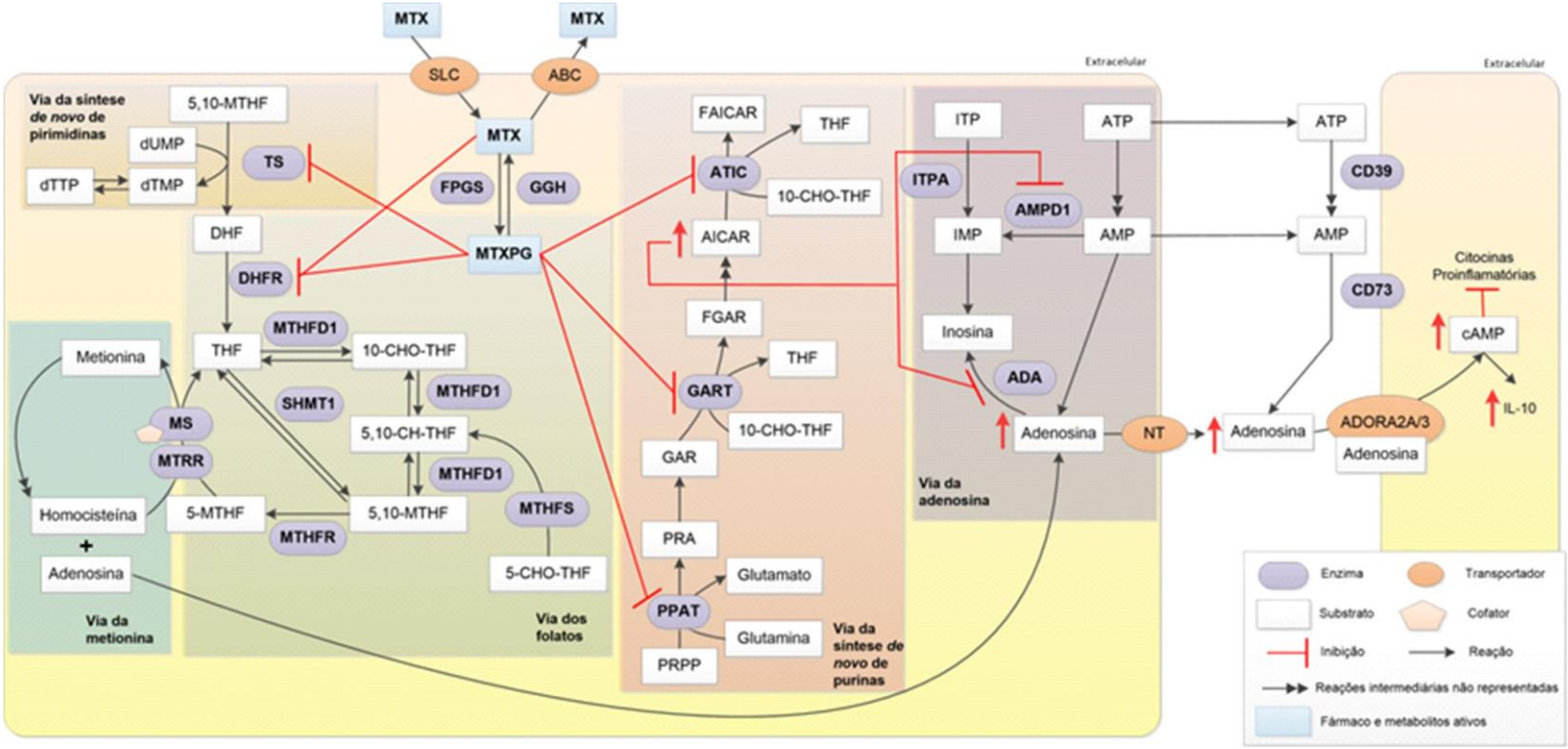

Figura 2. Mecanismo de ação do metotrexato.

Legenda: 5-MTHF: 5-metiltetrahidrofolato; 5,10-MTHF: 5,10-metilenotetrahidrofolato; 5,10-CH-THF: 5,10-meteniltetrahidrofolato; 10-CHO-THF: 10-formiltetrahidrofolato; ABC: adenosina trifosfato (ATP)-"binding cassette"; ADA: adenosina deaminase; ADORA2A/3: recetor de adenosina subtipo A2A ou subtipo 3; AICAR: aminoimidazol carboxamida adenosina ribonucleótido; AMP: adenosina monofosfato; AMPD1: adenosina monofosfato deaminase 1; ATIC: 5-aminoimidazol-4-carboxamida ribonucleótido transformilase; ATP: adenosina trifosfato; cAMP: monofosfato de adenosina cíclica; CD: cluster de diferenciação DHF: dihidrofolato; DHFR: dihidrofolato redutase; dTMP: deoxitimidina monofosfato; dTTP: deoxitimidina trifosfato; dUMP: deoxiuridina monofosfato; FAICAR: formil-aminoimidazol carboxamida adenosina ribonucleótido; FGAR: formil glicinamida ribonucleótido; FPGS: folilpoliglutamato sintetase; GAR: glicinamida ribonucleótido; GART: glicinamida ribonucleótido formil transferase; GGH: gama-glutamil hidrolase; IL: interleucina; IMP: inosina monofosfato; ITP: inosina trifosfato; ITPA: inosina trifosfato pirofosfatase; MS: metionina sintetase; MTHFD1: metilenotetrahidrofolato desidrogenase 1; MTHFR: metilenotetrahidrofolato redutase; MTHFS: meteniltetrahidrofolato sintetase; MTRR: metionina redutase; MTX: metotrexato; MTXPG: metotrexato poliglutamato; NT: transportador de nucleosídeo; PPAT: fosforribosil pirofosfato aminotransferase; PRA: 5-fosfosrribosil amina; PRPP: 5-fosforribosil-1-pirofosfato; SAH: S-adenosil homocisteína; SAM: S-adenosil metionina; SHMT1: serina hidroximetiltransferase 1; SLC: "solute carrier"; THF: tetrahidrofolato.

Pelo exposto, o presente trabalho visa analisar a influência de um conjunto de polimorfismos genéticos em genes codificantes de proteínas intervenientes nas vias intracelulares do metotrexato como potenciais preditores da ocorrência de resultados negativos da medicação em doentes Portugueses com artrite reumatoide tratados com metotrexato.

Pertinência do objeto da investigação

Com base no conhecimento atual na área da Farmacogenómica e da Medicina/Terapêutica Personalizada, o trabalho a é pioneiro no que respeita à avaliação da influência de um conjunto alargado de polimorfismos genéticos, em genes codificantes para proteínas envolvidas no mecanismo de ação do metotrexato, como potenciais preditores de resultados negativos da medicação.

O facto de poder vir a ser possível predizer, recorrendo ao património genético dos doentes com artrite reumatoide, quais aqueles que não beneficiarão do tratamento com metotrexato antes de iniciarem a terapêutica com o fármaco em causa, revela-se promissor e de extrema utilidade na prática clínica, permitindo que outras estratégias terapêuticas, mais efetivas e seguras, possam ser equacionadas mais precocemente.

Materiais e métodos utilizados

Foi realizado um estudo retrospetivo num coorte de 233 doentes com artrite reumatoide e sob terapêutica com metotrexato, recrutados consecutivamente na consulta de Reumatologia do Centro Hospitalar de São João, EPE, entre janeiro de 2009 e dezembro de 2012, tendo por base um conjunto de critérios de inclusão/exclusão. Vinte variáveis clínico-patológicas foram colhidas dos registos clínicos individuais dos doentes. Após esclarecimento do estudo em causa e assinatura do consentimento informado por parte dos doentes, foram colhidas amostras de sangue total para posterior genotipagem de 35 polimorfismos genéticos em 14 genes codificantes de enzimas envolvidas em reações de fase II e de proteínas envolvidas em vias intracelulares do MTX, mais precisamente das vias da síntese *de novo* de purinas, dos folatos, da metionina e da adenosina.

Para cada doente foi definido o perfil de resposta terapêutica ao metotrexato atendendo a critérios clínicos descritos na literatura. A análise estatística realizada incluiu análises uni e multivariada (regressão logística binária ajustada a variáveis

clínico-patológicas potencialmente influenciadoras do perfil de resposta terapêutico), atendendo a genótipos, haplótipos e índices de risco genético.

Resultados e Conclusões

A amostra estudada revelou-se homogénea relativamente à origem étnica (todos Caucasianos e do Norte de Portugal), à epidemiologia da doença face ao género (2-4 vezes mais prevalente nas mulheres), à idade de diagnóstico da doença (entre a 3ª e a 5ª década de vida), além de representativa da prática clínica de doentes com artrite reumatoide bem estabelecida.

Também no que se refere ao perfil de resposta terapêutica, a amostra revelou-se de acordo com a literatura. Verificou-se que o metotrexato foi não efetivo em 54,9% (n=128) e não seguro em 33,0% (n=77) dos doentes. Mais ainda, em 9,0% (n=20) foi simultaneamente não efetivo e não seguro; em 46,0% (n=108) foi não efetivo mas seguro; em 24,0% (n=57) foi efetivo mas não seguro; e, em apenas 21,0% (n=48) dos doentes foi simultaneamente efetivo e seguro.

No que respeita à não efetividade do metotrexato, o estudo farmacogenético demonstrou uma associação estatisticamente significativa entre a não efetividade e oito genótipos: *MTHFR* rs1801131 AA e rs1801133 TT; *MS* rs1805087 AA; *MTRR* rs1801394 portadores do alelo A; *ATIC* rs2372536 portadores do alelo C, rs4673993 portadores do alelo T, rs7563206 portadores do alelo T e rs12995526 portadores do alelo T; e, três haplótipos: CC para *GGH* rs3758149 e rs12681874; CGTTT para a combinação 1 de polimorfismos do *ATIC* e, CTTTC para a combinação 2 de polimorfismos do *ATIC*. (*ATIC* combinação 1: rs2372536, rs3821353, rs4673993, rs7563206 e rs12995526; *ATIC* combinação 2: rs2372536, rs4673993, rs7563206, rs12995526 e rs16853834. Mais ainda, a avaliação do índice de risco genético global para não efetividade demonstrou que os doentes com Índices 6-8 apresentavam um risco acrescido para não efetividade em cerca de 6 vezes superior, quando comparados com os doentes com Índices 0-5.

Relativamente à não segurança do metotrexato, o estudo farmacogenético demonstrou uma associação estatisticamente significativa entre a falta de segurança do metotrexato e cinco genótipos: *ATIC* rs2372536 portadores do alelo G, rs3821353 portadores do alelo T, rs7563206 CC e rs12995526 CC; e *ADORA2A* rs2267076 TT; e, dois haplótipos: CTTCC para a combinação 1 de polimorfismos do *ATIC* e, TC para *ADORA2A* rs2267076 e rs2298383. A avaliação do índice de risco genético global

para não segurança demonstrou que os doentes com Índices 3-4 apresentavam um risco acrescido para falta de segurança cerca de 7 vezes superior, quando comparados com os doentes com Índices 0-3.

Concluindo, e tendo por base os resultados obtidos e aqui apresentados, o património genético dos doentes poderá ser útil para predizer os doentes com perfil de resposta terapêutica desfavorável e, assim, identificar aqueles que não beneficiarão do metotrexato como fármaco antirreumático modificador da doença de primeira linha no tratamento da artrite reumatoide. Consequentemente, estes dados poderão ser de grande utilidade na prática clínica orientando para a Medicina/Terapêutica Personalizada, com vista à obtenção de um perfil de resposta terapêutica simultaneamente efetivo e seguro adequado a cada doente. No entanto, mais estudos são necessários para validar esses resultados.

Interesse, relevância e aplicabilidade dos resultados do trabalho

O presente trabalho de investigação sugere que o património genético dos doentes, mais precisamente que polimorfismos genéticos em genes codificantes de proteínas envolvidas no mecanismo de ação de determinado fármaco, poderá ser útil na orientação da prática clínica e instituição de terapêutica personalizada, de forma a obter-se o perfil de resposta terapêutica desejado, isto é, seguro e efetivo. No trabalho apresentado, o modelo de doença usado foi a Artrite Reumatoide, o fármaco estudado foi o Metotrexato e os polimorfismos genéticos selecionados de acordo com o mecanismo de ação do fármaco em causa, bem como os indicadores de efetividade e não segurança foram os adequados à doença em estudo. Não obstante, e porque muitas são as doenças crónicas em que os doentes apresentam resultados negativos à medicação instituída, o património genético poderá auxiliar a prática clínica, pela orientação da terapêutica personalizada, com o objetivo de alcançar o perfil de resposta terapêutica ideal para muitas outras doenças, além da artrite reumatoide, e para muitos outros fármacos, além do metotrexato. Assim, a translação dos polimorfismos genéticos para a prática clínica com vista à previsão do perfil de resposta terapêutica dos doentes no momento do diagnóstico, orientando a estratégia terapêutica a seguir, poderá tornar-se uma realidade. Em suma, o interesse, relevância e aplicabilidade dos resultados do presente trabalho demonstram-se pela contribuição para o progresso da Farmacogenómica, o que será essencial para sustentar um avanço na área da Medicina e da Terapêutica Personalizada.

ÍNDICE

TITLE PAGE

Moving towards Personalized Medicine in Rheumatoid Arthritis: Methotrexate Intracellular Pathways and Phase II Reactions as Pharmacogenetic Predictors of Methotrexate Therapeutic Outcome

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ABSTRACT

Aims: Evaluate the potential of selected Single Nucleotide Polymorphisms (SNPs) as predictors of methotrexate (MTX) therapeutic outcome in rheumatoid arthritis (RA). **Patients & Methods:** Thirty-five SNPs in 14 genes involved in MTX intracellular pathways and Phase II reactions were genotyped in 233 RA patients treated with MTX. Binary logistic regressions were performed by genotype/haplotype-based approaches. Non-Response- and Toxicity-Genetic Risk Indexes (Non-RespGRI and ToxGRI) were created. **Results:** MTX non-response was associated to 8 genotypes and 3 haplotypes: *MTHFR* rs1801131 AA and rs1801133 TT; *MS* rs1805087 AA; *MTRR* rs1801394 A carriers; *ATIC* rs2372536 C carriers, rs4673993 T carriers, rs7563206 T carriers and rs12995526 T carriers; CC for *GGH* rs3758149 and rs12681874; CGTTT for *ATIC* combination 1; and CTTTC for *ATIC* combination 2. From Overall Non-RespGRI patients with Indexes 6-8 had more than 6-fold increased risk for MTX non-response. MTX-related toxicity was associated to 5 genotypes and 2 haplotypes: *ATIC* rs2372536 G carriers, rs3821353 T carriers, rs7563206 CC and rs12995526 CC; *ADORA2A* rs2267076 T; CTTCC for *ATIC* combination 1; and TC for *ADORA2A* rs2267076 and rs2298383. From Overall ToxGRI patients with Indexes 3/4 had more than 7-fold increased risk for MTX-related toxicity. **Conclusions:** Genotyping may be helpful to identify which RA patients will not benefit from MTX treatment and, consequently, important to personalized medicine in RA. Nevertheless, further studies are required to validate these findings.

Keywords: Genetic risk index; Methotrexate; Pharmacogenomics; Personalized medicine; Polymorphisms; Response; Rheumatoid arthritis; Therapeutic outcome; Toxicity.

INTRODUCTION

During this century a new field has been discussed – the personalized medicine! The use of the right drug at the right dose and time, for the right patient, aimed to maximize the likelihood of therapeutic effectiveness and minimize the occurrence of adverse drug reactions (ADRs) [1]. Medicine was always personalized but only in this century the integration of patient genome was considered as important factor responsible for intra- and interpatient drug therapeutic outcome variability [2-4]. One of the major contributors of this development is Pharmacogenomics (PGx), which represents the use of individual genetic data to predict therapeutic outcome and tailor the best medical intervention to each patient [1]. During the past decade there has been considerable research into PGx but translating PGx knowledge into clinical practice is a major challenge, particularly in the case of highly complex diseases such as rheumatoid arthritis (RA) [5]. RA is a systemic autoimmune disease characterized by chronic inflammation of multiple peripheral joints for which the “gold standard” drug is methotrexate (MTX) [5-7]. Literature suggests that RA patients’ genetic profile may have a significant role in therapeutic outcome variability observed among patients [8-10], particularly genes encoding proteins involved in RA pathophysiology and MTX action mechanism [9, 11, 12]. MTX action mechanism include: 1) MTX membrane transport pathways, a network of transporters which allow MTX influx and efflux, belonging to two major superfamilies’ - solute carriers (SLCs) and adenosine triphosphate (ATP)-binding cassette (ABCs) transporters [11, 13]; and, 2) MTX intracellular pathways, which include polyglutamation, folate, methionine, adenosine and *de novo* synthesis of purines and pyrimidines pathways [11, 14-17]. Polyglutamation pathway is responsible for MTX intracellular retention by the sequential addition (by folylpolyglutamate synthetase - FPGS) or removal (by γ-glutamyl hydrolase - GGH) of glutamic acid residues [18]. The amount of intracellular MTX polyglutamates (MTXPGs) depends on polyglutamation net rate [14, 19, 20]. Folate pathway, essential for providing the required folate cofactors for several pathways such *de novo* purine and pyrimidine synthesis, includes several enzymes such dihydrofolate reductase (DHFR), methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), methenyltetrahydrofolate synthetase (MTHFS) and methylenetetrahydrofolate reductase (MTHFR). Methionine pathway, beyond other functions, is responsible for adenosine biosynthesis (via transmethylation), a potent anti-inflammatory agent with a key role on MTX anti-inflammatory effect [21, 22]. This effect can be modulated by the activity of methionine synthase (MS) and methionine synthase reductase (MTRR) in the presence of a methyl(III)cobalamin cofactor [21, 22]. *De novo* synthesis of purines pathway is one of the most prominent for MTX therapeutic effects [8, 23] because of the involvement of several key proteins such as glycinamide ribonucleotide formyl transferase (GART), phosphoribosyl pyrophosphate amidotransferase (PPAT) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) [23]. Literature hypothesizes that AICAR accumulation, as a consequence of ATIC inhibition by MTXPGs, is responsible for intracellular accumulation of adenosine and its release to extracellular space [8, 16]. Adenosine pathway is responsible for the other three ways of adenosine

biosynthesis (beyond transmethylation): 1) cyclic adenosine monophosphate (cAMP) conversion to adenosine [24]; 2) intracellular ATP dephosphorylation to adenosine, activated when energy demand exceeds energy supply [25, 26]; and 3) extracellular ATP conversion to adenosine by ectoenzymes cluster of differentiation (CD) 39 and CD73 [27, 28]. *In vitro*, *in vivo* and clinical data studies theorize that adenosine is released in high concentrations from cells by nucleoside transporters (NTs) after MTX treatment [16, 29], then adenosine exerts its anti-inflammatory effects on target cells via interaction with G-coupled adenosine receptors (ADOR): ADORA2A and ADORA3 **(Figure 1)** [30].

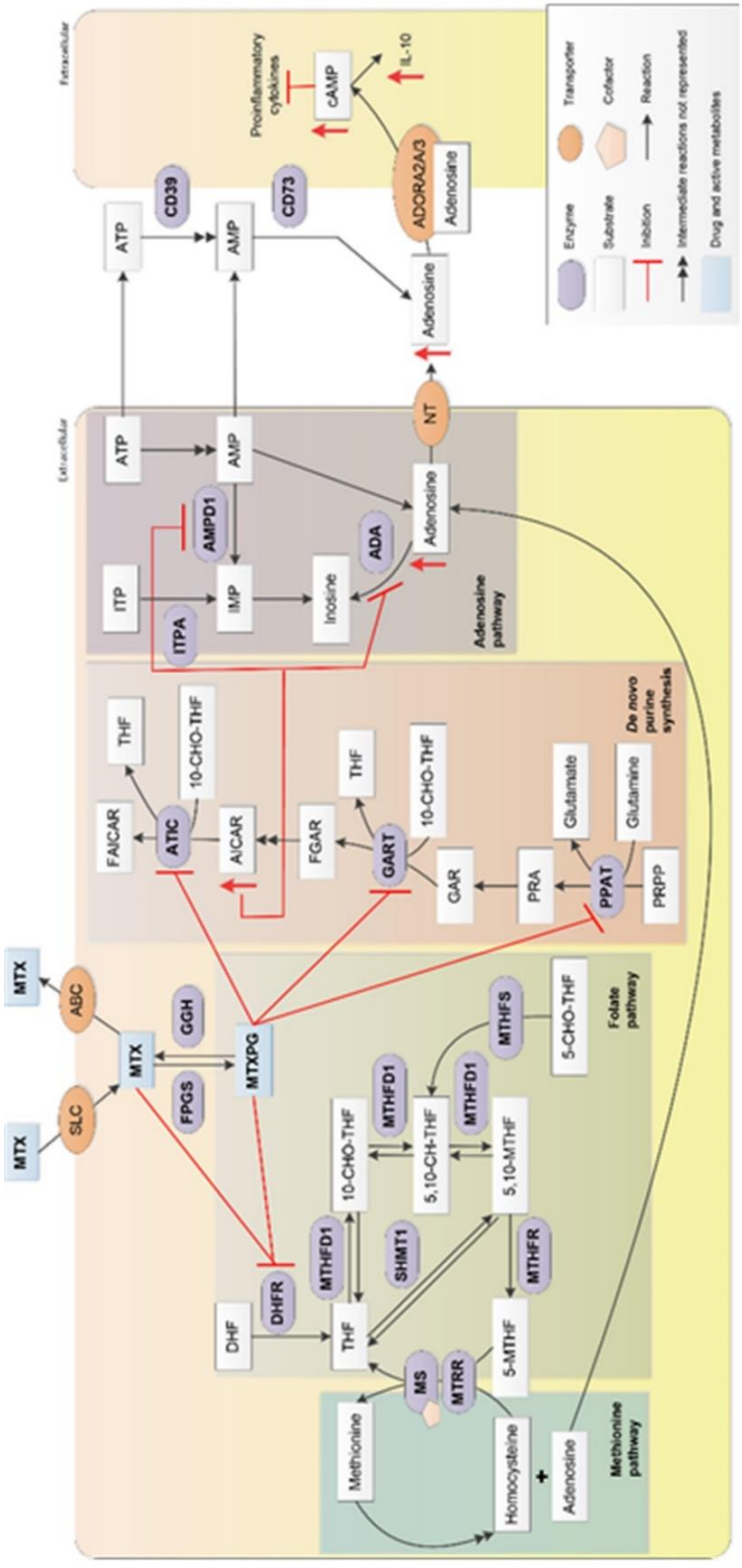


Figure 1. Role of intracellular pathways in the methotrexate action mechanism.
5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; 5,10-CH-THF: 5,10-methenyltetrahydrofolate; 10-CHO-THF: 10-formyltetrahydrofolate; ABC: adenosine triphosphate (ATP)-binding cassette; ADA: adenosine deaminase; ADORA2A/3: adenosine receptor subtype A2A or subtype 3; AICAR: aminoimidazole carboxamide adenosine ribonucleotide; AMP: adenosine monophosphate; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; AMP: adenosine monophosphate; ATP: adenosine triphosphate; cAMP: cyclic-adenosine monophosphate; CD: cluster of differentiation 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; GART: glycineamide ribonucleotide formyl transferase; GGH: gamma-glutamyl hydrolase; IL: interleukin; IMP: inosine monophosphate; ITP: inosine triphosphate; ITPA: inosine triphosphate pyrophosphatase; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methenyltetrahydrofolate synthetase; MTRR: methionine synthase reductase; MTX: methotrexate; MTXPG: methotrexate polyglutamate; NT: nucleoside transporter; 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.

In addition, proteins that inactivate MTX, such glutathione S-transferase P1 (GSTP1), a Phase II reaction enzyme, can be also important as predictor of MTX therapeutic outcome [31]. Therefore, and given the importance of all MTX intracellular pathways and Phase II reactions, genetic polymorphisms involved in these pathways seem to be crucial targets to evaluate their potential as predictors of MTX therapeutic outcome. Emphasizing that present work follows up another four, where relevant results were obtained for MTX membrane transporters and *de novo* pyrimidine synthesis pathways [13, 32-34], it is aimed to evaluate selected SNPs in genes encoding for other MTX pathways as potential predictors of MTX therapeutic outcome for Portuguese RA patients, hoping to bring awareness among scientific community about the importance of translation knowledge into clinical practice, towards personalized medicine.

PATIENTS & METHODS

Patients & Study Design

This study, conducted between January 2009 and December 2012 at São João Hospital Center (Porto, Portugal), was developed as a retrospective study in a cohort of consecutive Caucasian patients (≥ 18 years) with RA treated with MTX. All patients had to meet the 2010 revised classification criteria of American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) [35]. Patients were excluded if had drug abuse history, recent pregnancy or desire to become pregnant during the study. The study was approved by local research ethics committee (reference 33/2009) and informed written consent was obtained from all patients according to the standards of Helsinki Declaration.

Therapeutic strategy: All patients were initially treated with 10mg *per os* (PO)/week of MTX in monotherapy. This dose was increased 5mg at each 3 weeks if patients did not meet EULAR criteria for response, i.e. if presented a Disease Activity Score in 28 joints (DAS28) >3.2. Every 3 months MTX clinical response was evaluated and therapeutic strategies were defined as follow: 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 disease modifying anti-rheumatic drug (DMARD); and 3) third evaluation, in patients without response and other contraindication, therapy was changed by associating a biological DMARD. Folic acid supplementation was prescribed to all patients and its regular compliance was registered. Other concomitant drugs, such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and other DMARDs were allowed during the study.

Data collection and variable definition: Patient demographics, clinicopathological and treatment characteristics were collected from clinical records. MTX clinical response was recorded at each visit and assessed using the DAS28. Estimated glomerular filtration rate (eGFR) was calculated using the

follow Modification of Diet in Renal Disease (MDRD) equation: 186x(creatinine/88.4)-1.154x(Age)-0.203x(0.742 if female)x(1.210 if black).

Outcome definition: ***Clinical response to MTX.*** Non-response was defined when patients presented a DAS28 >3.2, calculated and defined as described by Prevoo *et al.* [36], in two consecutive evaluations. Therefore, non-response to MTX was defined only after a minimum period of at least 6 months of MTX therapy. Response to MTX was defined when patients presented a DAS28 ≤3.2. ***MTX-related toxicity.*** The occurrence of MTX-related toxicity, defined when patients presented any ADR related to MTX, was recorded upon each visit. ADR type was classified in System Organ Class (SOC) disorders, in accordance with Common Terminology Criteria for Adverse Events (CTCAE) [37].

SNPs Selection & Genotyping

A total of 35 SNPs in 14 genes encoding for proteins involved in MTX intracellular pathways and Phase II reactions were selected based on literature, attending to their putative effects on proteins activity and/or MTX therapeutic outcome [4, 9, 10, 19, 38-48]. Whole blood samples from each patient were obtained with standard venipuncture technique. 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 NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA). Sequenom® Assay Design 3.1 software was used to primers design and genotyping was performed according to standard Sequenom® iPLEX protocol [49]. For quality control, 10% of the samples were randomly selected for a second analysis and results were 100% concordant.

Statistical Analysis

Statistical analysis was performed with either IBM® SPSS® Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA) and SNPStats software [50]. 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%. 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 same gene was estimated and expressed as *D´* coefficients.

Multivariate analyses by binary logistic regression were used to compare genotypes and haplotypes with MTX therapeutic outcome. Binary logistic regression was adjusted to clinicopathological variables possibly influencing disease state and/or clinical response to MTX. Such variables were selected based either in literature review and/or clinical significance [38, 51-54] and included the following variables: 1) patient-related: age, gender, smoking *status* and renal function (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). Possible haplotypes were tested for association with MTX therapeutic outcome by taking the most frequent haplotype as reference. Rare haplotypes (estimated haplotype frequency <2.0%) were excluded. Non-Response- and Toxicity-Genetic Risk Indexes (Non-RespGRI and ToxGRI, respectively) were created for each pathway and for all pathways by the sum of risk genotypes that revealed to be statistically significant with MTX non-response and MTX-related toxicity. Index number was in accordance to the possible combinations between risk genotypes and tested for association with MTX therapeutic outcome in a binary logistic regression. Forest plots were constructed using MedCalc® software for Windows, Version 13.1.2 [55]. 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 mean age of 51±11.6 years old, of which 32 (13.7%) were smokers. Median of SCr was 8.20mg/L (4.00-19.80) and of eGFR was 82.0ml/min/1.73m^2 (29.00-186.00), and 30 patients (12.9%) presented renal insufficiency (eGFR<60ml/min/1.73m^2). Considering disease-related variables, the mean age at diagnosis was 40.3±13.2 years old, the median disease duration was 7.0 years (0.3-51.0) and the mean for DAS28 was 4.2±1.3. All patients (100.0%) were treated with MTX with a median dose of 15.0mg/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. 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 maculopapular 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 putative SNPs predictors of MTX-related toxicity occurrence was performed for overall toxicity.

Genotypes and Haplotypes Characteristics

Genotypes distribution of studied SNPs is represented in Table 1. SNPs were excluded from analysis when minor allele frequency was less than 10.0% and/or call rates were less than 95%: 1) *FPGS* rs10760502; 2) *GGH* rs1800909, rs11545076 and rs11545078; 3) *DHFR* rs1105525, rs1650697 and rs34764978; 4) *PPAT* rs3796548; and, 5) *ADA* rs73598374. Therefore, 26 SNPs were considered.

Genotypes distribution was in HWE (p >0.050) except for *GGH* rs3758149 and *ATIC* rs3821353. *FPGS*, *GGH*, *DHFR*, *MTHFR* and *ADORA2A* SNPs were in LD. For *ATIC*, SNPs were in LD except for rs3821353 and rs16853834. Hence analyses regarding *ATIC* were performed considering the following combinations: 1) *ATIC* combination 1: rs2372536, rs3821353, rs4673993, rs7563206 and rs12995526; and 2) *ATIC* combination 2: rs2372536, rs4673993, rs7563206, rs12995526 and rs16853834.

Table 1. Genotypes frequency of studied SNPs

rs ID	Alleles	Genotype frequency		
		Ancestral allele homozygotes	Heterozygotes	Minor allele homozygotes
Polyglutamation pathway				
FPGS				
rs10106	A>G	78 (33.5)	105 (45.1)	50 (21.5)
rs1054774	T>A	88 (37.8)	109 (46.8)	36 (15.5)
rs1544105	G>A	78 (33.5)	110 (47.2)	45 (19.3)
rs4451422 [a)]	A>C	79 (34.1)	105 (45.3)	48 (20.7)
rs10760502 [#]	G>A	63 (40.7)	50 (32.2)	42 (27.1)
GGH				
rs1800909 [#]	T>C	163 (85.3)	28 (14.7)	0 (0.0)
rs3758149 [a)]	C>T	125 (53.9)	80 (34.5)	27 (11.6)
rs11545076 [#]	G>T	0 (0.0)	233 (100.0)	0 (0.0)
rs11545078 [#]	C>T	203 (87.1)	29 (12.4)	1 (0.4)
rs12681874	C>T	183 (78.5)	46 (19.7)	4 (1.7)
Folate pathway				
DHFR				
rs7387	T>A	106 (45.7)	106 (45.5)	21 (9.1)
rs1105525 [#]	C/G>A	0 (0.0)	0 (0.0)	0 (0.0)
rs1232027	G>A	94 (40.3)	113 (48.5)	26 (11.2)
rs1643657	A>G	105 (45.1)	107 (45.9)	21 (9.0)
rs1650697 [#]	C>T	5 (2.1)	2 (0.9)	0 (0.0)
rs10072026	T>C	181 (77.7)	47 (20.2)	5 (2.1)
rs34764978 [#]	A>G	0 (0.0)	0 (0.0)	0 (0.0)
MTHFR				
rs1801131	A>C	126 (54.1)	85 (36.5)	22 (9.4)
rs1801133	C>T	105 (45.1)	99 (42.5)	29 (12.4)
MTHFS				
rs8923	A>G	184 (79.0)	46 (19.7)	3 (1.3)
Methionine pathway				
MS				
rs1805087	A>G	158 (67.8)	70 (30.0)	5 (2.1)
MTRR				
rs1801394	G>A	62 (26.6)	118 (50.6)	53 (22.7)

Results are expressed as n (%). [a)] Genotypes technique failed to 1 patient. [#] SNPs were excluded from analysis because minor allele frequency was <10.0% and/or call rates were <95%.
A: adenine; ADA: adenosine deaminase; ADORA2A: adenosine receptor subtype A2A; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; C: cytosine; DHFR: dihydrofolate reductase; FPGS: folylpolyglutamate synthetase; G: guanine; GART: glycinamide ribonucleotide formyl transferase; GGH: gamma-glutamyl hydrolase; GSTP1: human glutathione S-transferase P1; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methenyltetrahydrofolate synthetase; MTRR: methionine synthase reductase; PPAT: phosphoribosyl pyrophosphate amidotransferase; SHMT1: serine hydroxymethyltransferase 1; T: thymine.

Table 1. Genotypes frequency of studied SNPs (cont.)				
rs ID	Alleles	Genotype frequency		
		Ancestral allele homozygotes	Heterozygotes	Minor allele homozygotes
De novo purines synthesis pathway				
ATIC				
rs2372536	C>G	110 (47.2)	99 (42.5)	24 (10.3)
rs3821353	G>T	166 (71.2)	46 (19.7)	21 (9.0)
rs4673993	T>C	110 (47.2)	99 (42.5)	24 (10.3)
rs7563206	T>C	65 (27.9)	109 (46.8)	59 (25.3)
rs12995526	T>C	67 (28.8)	110 (47.2)	56 (24.0)
rs16853834	C>T	160 (68.7)	68 (29.2)	5 (2.1)
GART				
rs8971	A>G	128 (54.9)	86 (36.9)	19 (8.2)
PPAT				
rs3796548 [#]	C>T	191 (82.0)	41 (17.6)	1 (0.4)
Adenosine pathway				
ADA				
rs73598374 [#]	G>A	0 (0.0)	0 (0.0)	0 (0.0)
ADORA2A				
rs2267076	C>T	91 (39.1)	112 (48.1)	30 (12.9)
rs2298383	T>C	77 (33.0)	110 (47.2)	46 (19.7)
AMPD1				
rs17602729	C>T	180 (77.3)	48 (20.6)	5 (2.1)
Phase II reaction				
GSTP1				
rs1695	A>G	103 (44.2)	104 (44.6)	26 (11.2)

Results are expressed as n (%). [a)] Genotypes technique failed to 1 patient. [#] SNPs were excluded from analysis because minor allele frequency was <10.0% and/or call rates were <95%.
A: adenine; ADA: adenosine deaminase; ADORA2A: adenosine receptor subtype A2A; AMPD1: adenosine monophosphate deaminase 1; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; C: cytosine; DHFR: dihydrofolate reductase; FPGS: folylpolyglutamate synthetase; G: guanine; GART: glycinamide ribonucleotide formyl transferase; GGH: gamma-glutamyl hydrolase; GSTP1: human glutathione S-transferase P1; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methenyltetrahydrofolate synthetase; MTRR: methionine synthase reductase; PPAT: phosphoribosyl pyrophosphate amidotransferase; SHMT1: serine hydroxymethyltransferase 1; T: thymine.

Genotypes, Haplotypes, Genetic Risk Indexes & Methotrexate Therapeutic Outcome

Polyglutamation pathway: Table 2 represents the relation between MTX therapeutic outcome and SNPs in *FPGS* and *GGH* by genotype- and haplotype-based approaches. Considering genotype approach, our results demonstrated non-statistically significantly results for *FPGS* (rs10106, rs1054774, rs1544105 and rs4451422) and *GGH* (rs3758149 and rs12681874). Nevertheless, CC haplotype for *GGH* rs3758149 and rs12681874 was associated with an increased risk for MTX non-response, when compared to TC haplotype. No statistically significant associations were observed for haplotypes and MTX-related toxicity.

Table 2. Association of SNPs involved in Polyglutamation Pathway with MTX Therapeutic Outcome

	Clinical Response				Overall Toxicity			
	Response	Non-response	*p*	OR (95%CI)	Non-toxicity	Toxicity	*p*	OR (95%CI)
GENOTYPES								
***FPGS* A>G (rs10106)**								
AA+AG	83 (45.4)	100 (54.6)		1.00	123 (67.2)	60 (32.8)		1.00
GG	22 (44.0)	28 (56.0)	0.317	1.50 (0.68-3.29)	33 (66.0)	17 (34.0)	0.850	1.09 (0.46-2.59)
AA	28 (35.9)	50 (64.1)		1.00	57 (73.1)	21 (26.9)		1.00
AG+GG	77 (49.7)	78 (50.3)	0.070	0.51 (0.25-1.06)	99 (63.9)	56 (36.1)	0.267	1.57 (0.71-3.46)
***FPGS* T>A (rs1054774)**								
TT+TA	91 (46.2)	106 (53.8)		1.00	131 (66.5)	66 (33.5)		1.00
AA	14 (38.9)	22 (61.1)	0.172	1.87 (0.76-4.57)	25 (69.4)	11 (30.6)	0.960	0.97 (0.36-2.62)
TT	33 (37.5)	55 (62.5)		1.00	63 (71.6)	25 (28.4)		1.00
TA+AA	72 (49.7)	73 (50.3)	0.070	0.52 (0.26-1.06)	93 (64.1)	52 (35.9)	0.507	1.29 (0.61-2.73)
***FPGS* G>A (rs1544105)**								
GG+GA	85 (45.2)	103 (54.8)		1.00	127 (67.6)	61 (32.4)		1.00
AA	20 (44.4)	25 (55.6)	0.316	1.53 (0.67-3.50)	29 (64.4)	16 (35.6)	0.797	1.12 (0.46-2.76)
GG	29 (37.2)	49 (62.8)		1.00	56 (71.8)	22 (28.2)		1.00
GA+AA	76 (49.0)	79 (51.0)	0.115	0.56 (0.27-1.15)	100 (64.5)	55 (35.5)	0.557	1.27 (0.57-2.84)
***FPGS* A>C (rs4451422)**								
AA+AC	84 (45.7)	100 (54.3)		1.00	123 (66.8)	61 (33.2)		1.00
CC	21 (43.8)	27 (56.2)	0.270	1.57 (0.70-3.49)	32 (66.7)	16 (33.3)	0.950	0.97 (0.40-2.34)
AA	29 (36.7)	50 (63.3)		1.00	58 (73.4)	21 (26.6)		1.00
AC+CC	76 (49.7)	77 (50.3)	0.077	0.52 (0.25-1.07)	97 (63.4)	56 (36.6)	0.192	1.70 (0.77-3.76)
***GGH* C>T (rs3758149)**								
CC+CT	91 (44.4)	114 (55.6)		1.00	138 (67.3)	67 (32.7)		1.00
TT	14 (51.9)	13 (48.1)	0.169	0.47 (0.16-1.38)	17 (63.0)	10 (37.0)	0.368	1.66 (0.55-5.01)
CC	53 (42.4)	72 (57.6)		1.00	81 (64.8)	44 (35.2)		1.00
CT+TT	52 (48.6)	55 (51.4)	0.065	0.53 (0.27-1.04)	74 (69.2)	33 (30.8)	0.515	1.27 (0.62-2-60)
***GGH* C>T (rs12681874)**								
CC+CT	102 (44.5)	127 (55.5)		1.00	154 (67.2)	75 (32.8)		1.00
TT	3 (75.0)	1 (25.0)	0.460	0.38 (0.03-4.98)	2 (50.0)	2 (50.0)	0.636	1.70 (0.19-15.21)
CC	82 (44.8)	101 (55.2)		1.00	122 (66.7)	61 (33.3)		1.00
CT+TT	23 (46.0)	27 (54.0)	0.703	0.86 (0.40-1.85)	34 (68.0)	16 (32.0)	0.410	0.68 (0.27-1.69)

HAPLOTYPES								
***FPGS* A>G (rs10106)**	***FPGS* T>A (rs1054774)**	***FPGS* G>A (rs1544105)**	***FPGS* A>C (rs4451422)**	**% ***	**Clinical Response**		**Overall Toxicity**	
					p	**OR (95%CI)**	***p***	**OR (95%CI)**
A	T	G	A	54.7		1.00		1.00
G	A	A	C	38.6	0.590	0.88 (0.55-1.41)	0.610	1.15 (0.67-1.96)
G	T	A	C	2.75	0.600	1.50 (0.33-6.85)	0.500	1.70 (0.37-7.82)
***GGH* C>T (rs3758149)**			***GGH* C>T (rs12681874)**					
C			C	66.0		1.00		1.00
T			C	22.4	**0.037**	0.50 (0.26-0.95)	0.150	1.62 (0.85-3.09)
T			T	6.4	0.740	0.84 (0.30-2.34)	0.490	0.69 (0.24-1.97)
C			T	5.1	0.440	0.65 (0.22-1.92)	0.840	1.13 (0.34-3.72)

Results are expressed in n (%). *p* ≤0.05 was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). Non-RespGRI and ToxGRI were not performed because studied genotypes for polyglutamation pathway did not revealed to be statistically significant with MTX therapeutic outcome. *Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. A: adenine; C: cytosine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; FPGS: folylpolyglutamate synthetase; G: guanine; GGH: γ-glutamyl hydrolase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine; ToxGRI: genetic risk index for MTX-toxicity.

Folate pathway: Table 3 represents the relation between MTX therapeutic outcome and SNPs in *DHFR*, *MTFHR* and *MTHFS* by genotype-, haplotype- and genetic risk index-based approaches. Considering genotype approach, 2 SNPs were associated with about 2-fold increased risk for MTX non-response: *MTHFR* rs1801131 A homozygotes and *MTHFR* rs1801133 T homozygotes. No significant

associations with MTX non-response were observed for *DHFR* polymorphisms (rs7387, rs1232027, rs1643657 and rs10072026) and *MTHFS* rs8923. Moreover, no statistically significant associations were observed attending to studied SNPs of folate pathway and MTX-related toxicity occurrence. Moreover, no associations were obtained in haplotype approach. Non-RespGRI from folate pathway demonstrated that patients with Index 2 (*MTHFR* rs1801131 AA + *MTHFR* rs1801133 TT) presented an increased risk for MTX non-response of about 5-fold when compared to those with Index 0.

Table 3. Association of SNPs involved in Folate Pathway with MTX Therapeutic Outcome

	Clinical Response				Overall Toxicity			
	Response	Non-response	*p*	OR (95%CI)	Non-toxicity	Toxicity	*p*	OR (95%CI)
GENOTYPES								
***DHFR* T>A (rs7387)**								
TT+TA	93 (43.9)	119 (56.1)		1.00	145 (68.4)	67 (31.6)		1.00
AA	12 (57.1)	9 (42.9)	0.947	0.96 (0.31-2.99)	11 (52.4)	10 (47.6)	0.338	1.80 (0.54-5.98)
TT	49 (46.2)	57 (53.8)		1.00	74 (69.8)	32 (30.2)		1.00
TA+AA	56 (44.1)	71 (55.9)	0.078	1.84 (0.93-3.63)	82 (64.6)	45 (35.4)	0.498	1.28 (0.63-2.59)
***DHFR* G>A (rs1232027)**								
GG+GA	91 (44.0)	116 (56.0)		1.00	142 (68.6)	65 (31.4)		1.00
AA	14 (53.8)	12 (46.2)	0.395	1.55 (0.57-4.25)	14 (53.8)	12 (46.2)	0.587	1.35 (0.46-4.00)
GG	42 (44.7)	52 (55.3)		1.00	63 (67.0)	31 (33.0)		1.00
GA+AA	63 (45.3)	76 (54.7)	0.287	1.44 (0.73-2.83)	93 (66.9)	46 (33.1)	0.968	0.98 (0.48-2.03)
***DHFR* A>G (rs1643657)**								
AA+AG	93 (43.9)	119 (56.1)		1.00	145 (68.4)	67 (31.6)		1.00
GG	12 (57.1)	9 (42.9)	0.947	0.96 (0.31-2.99)	11 (52.4)	10 (47.6)	0.338	1.80 (0.54-5.98)
AA	49 (46.7)	56 (53.3)		1.00	73 (69.5)	32 (30.5)		1.00
AG+GG	56 (43.8)	72 (56.2)	0.066	1.89 (0.96-3.72)	83 (64.8)	45 (35.2)	0.514	1.27 (0.62-2.57)
***DHFR* T>C (rs10072026)**								
TT+TC	103 (45.2)	100 (54.3)		1.00	152 (66.7)	76 (33.3)		1.00
CC	2 (40.0)	27 (56.2)	0.864	1.24 (0.11-14.43)	4 (80.0)	1 (20.0)	0.392	0.31 (0.02-4.50)
TT	76 (42.0)	105 (58.0)		1.00	123 (68.0)	58 (32.0)		1.00
TC+CC	29 (55.8)	23 (44.2)	0.144	0.56 (0.25-1.22)	33 (63.5)	19 (36.5)	0.452	1.37 (0.60-3.13)
***MTHFR* A>C (rs1801131)**								
AA+AC	93 (44.1)	118 (55.9)		1.00	144 (68.2)	67 (31.8)		1.00
CC	12 (54.5)	10 (45.5)	0.910	1.07 (0.35-3.28)	12 (54.5)	10 (45.5)	0.761	1.22 (0.33-4.53)
AA	48 (38.1)	78 (61.9)		1.00	91 (72.2)	35 (27.8)		1.00
AC+CC	57 (53.3)	50 (46.7)	**0.045**	0.51 (0.26-0.98) [a)]	65 (60.7)	42 (39.3)	0.369	1.39 (0.68-2.85)
***MTHFR* C>T (rs1801133)**								
CC+CT	98 (48.0)	106 (52.0)		1.00	135 (66.2)	69 (33.8)		1.00
TT	7 (24.1)	22 (75.9)	**0.019**	3.86 (1.25-11.89)	21 (72.4)	8 (27.6)	0.302	1.80 (0.59-5.51)
CC	52 (49.5)	53 (50.5)		1.00	62 (59.0)	43 (41.0)		1.00
CT+TT	53 (41.4)	75 (58.6)	0.536	1.23 (0.63-2.39)	94 (73.4)	34 (26.6)	0.373	0.72 (0.35-1.48)
***MTHFS* A>G (rs8923)**								
AA+AG	105 (45.7)	125 (54.3)		1.00	153 (66.5)	77 (33.5)		1.00
GG	0 (0.0)	3 (100.0)	0.999	1.00 (1.00-1.00)	3 (100.0)	0 (0.0)	0.999	1.00 (1.00-1.00)
AA	81 (44.0)	103 (56.0)		1.00	125 (67.9)	59 (32.1)		1.00
AG+GG	24 (49.0)	25 (51.0)	0.657	1.19 (0.54-2.62)	31 (63.3)	18 (36.7)	0.718	1.17 (0.49-2.79)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). ToxGRI was not performed because genotypes from studied SNPs for folate pathway did not revealed to be statistically significant with MTX-related toxicity.*Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. [a)] When reference was C carriers: OR=1.96, 95%CI: 1.01-3.79. A: adenine; C: cytosine; CI: confidence interval; DHFR: dihydrofolate reductase; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methylenetetrahydrofolate synthetase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine; ToxGRI: genetic risk index for MTX-toxicity.

Table 3. Association of SNPs involved in Folate Pathway with MTX Therapeutic Outcome (cont.)

HAPLOTYPES								
***DHFR* T>A (rs7387)**	***DHFR* G>A (rs1232027)**	***DHFR* A>G (rs1643657)**	***DHFR* T>C (rs10072026)**	**% ***	**Clinical Response**		**Overall Toxicity**	
					p	**OR (95%CI)**	***p***	**OR (95%CI)**
T	G	A	T	50.9		1.00		1.00
A	A	G	T	29.0	0.310	1.34 (0.76-2.35)	0.380	1.32 (0.71-2.43)
T	G	A	C	10.7	0.110	0.50 (0.21-1.17)	0.150	2.01 (0.79-5.12)
T	A	A	T	5.9	0.990	1.01 (0.33-3.04)	0.770	1.19 (0.37-3.84)
A	G	G	T	2.8	0.230	2.40 (0.51-15.47)	0.260	2.56 (0.50-12.99)
***MTHFR* A>C (rs1801131)**		***MTHFR* C>T (rs1801133)**						
A		C		38.9		1.00		1.00
A		T		33.4	0.390	1.27 (0.73-2.21)	0.770	0.91 (0.49-1.70)
C		C		27.4	0.210	0.70 (0.40-1.22)	0.890	1.05 (0.55-1.99)

GENETIC RISK INDEXES					
Non-RespGRI		**Clinical Response**			
		Response	**Non-response**	***p***	**OR (95%CI)**
Index 0	rs1801131 – AC+CC **AND** rs1801133 – CC+CT	57 (53.3)	50 (46.7)		1.00
Index 1	rs1801131 – AA **OR** rs1801133 – TT	41 (42.3)	56 (57.7)	0.197	1.58 (0.79-3.17)
Index 2	rs1801131– AA **AND** rs1801133 – TT	7 (24.1)	22 (75.9)	**0.009**	5.23 (1.51-18.14)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). ToxGRI was not performed because genotypes from studied SNPs for folate pathway did not revealed to be statistically significant with MTX-related toxicity.*Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. [a)] When reference was C carriers: OR=1.96, 95%CI: 1.01-3.79. A: adenine; C: cytosine; CI: confidence interval; DHFR: dihydrofolate reductase; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methylenetetrahydrofolate synthetase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine; ToxGRI: genetic risk index for MTX-toxicity.

Methionine pathway: Table 4 represents the relation between MTX therapeutic outcome and polymorphisms in *MS* and *MTRR* by genotype- and genetic risk index-based approaches. The *MS* rs1805087 A homozygotes and *MTRR* rs1801394 A carriers were associated with about 2-fold increased risk for a non-response profile to MTX but demonstrated a non-significant association with MTX-related toxicity. Non-RespGRI from methionine pathway demonstrated that patients with Index 2 (*MS* rs1805087 AA + *MTRR* rs1801394 A carriers) presented more than 5-fold increased risk for MTX non-response then those with Index 0.

Table 4. Association of SNPs involved in Methionine Pathway with MTX Therapeutic Outcome

	Clinical Response				Overall Toxicity			
	Response	**Non-response**	***p***	**OR (95%CI)**	**Non-toxicity**	**Toxicity**	***p***	**OR (95%CI)**
GENOTYPES								
***MS* A>G (rs1805087)**								
AA+AG	102 (44.7)	126 (55.3)		1.00	153 (67.1)	75 (32.9)		1.00
GG	3 (60.0)	2 (40.0)	0.247	0.27 (0.03-2.51)	3 (60.0)	2 (40.0)	0.316	2.86 (0.36-22.47)
AA	66 (41.8)	92 (58.2)		1.00	108 (68.4)	50 (31.6)		1.00
AG+GG	39 (52.0)	36 (48.0)	**0.017**	0.42 (0.20-0.86) [a)]	48 (64.0)	27 (36.0)	0.066	2.04 (0.95-4.39)
***MTRR* G>A (rs1801394)**								
GG+GA	84 (46.7)	96 (53.3)		1.00	123 (68.3)	57 (31.7)		1.00
AA	21 (39.6)	32 (60.4)	**0.046**	2.36 (1.01-5.52)	33 (62.3)	20 (37.7)	0.903	1.05 (0.45-2.48)
GG	33 (53.2)	29 (46.8)		1.00	38 (61.3)	24 (38.7)		1.00
GA+AA	72 (42.1)	99 (57.9)	**0.041**	2.16 (1.03-4.53)	118 (69.0)	53 (31.0)	0.316	0.67 (0.30-1.47)

GENETIC RISK INDEXES					
Non-RespGRI		**Clinical Response**			
		Response	**Non-response**	***p***	**OR (95%CI)**
Index 0	rs1805087 – AG+GG **AND** rs1801394 – GG	10 (55.6)	8 (44.4)		1.00
Index 1	rs1805087 – AA **OR** 1801394 – GA+AA	52 (51.5)	49 (48.5)	0.753	1.24 (0.34-4.67)
Index 2	rs1805087 – AA **AND** rs1801394 – GA+AA	43 (37.7)	71 (62.3)	**0.018**	5.68 (1.35-23.87)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). ToxGRI was not performed because genotypes from studied SNPs for methionine pathway did not revealed to be statistically significant with MTX-related toxicity [a)] When reference was G carriers: OR=2.40, 95%CI: 1.17-4.96. A: adenine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; MS: methionine synthase; MTRR: methionine synthase reductase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; ToxGRI: genetic risk index for MTX-toxicity.

***De novo* purines synthesis pathway:** Table 5 represents the relation between MTX therapeutic outcome and SNPs in *ATIC* and *GART* by genotype-, haplotype- and genetic risk index-based approaches. Considering genotype approach, 4 SNPs in *ATIC* were associated with about 3-fold increased risk for MTX non-response: rs2372536 C carriers, rs4673993 T carriers, rs7563206 T carriers and rs12995526 T carriers. For haplotype approach, CGTTT haplotype for *ATIC* combination 1 was associated with an increased risk for MTX non-response when compared to GGCCC haplotype. Non-RespGRI from *de novo* purines synthesis pathway demonstrated that patients with Index 4 (*ATIC* rs2372536 C carriers + *ATIC* rs4673993 T carriers + *ATIC* rs7563206 T carriers + *ATIC* rs12995526 T carriers) presented more than 5-fold increased risk for MTX non-response when compared to those with Index 0. Four SNPs in *ATIC* were associated with about 2-fold increased risk for MTX-related toxicity: rs2372536 G carriers, rs3821353 T carriers, rs7563206 C homozygotes and rs12995526 C homozygotes. Regarding haplotype approach, CTTCC haplotype for *ATIC* combination 1 presented an increased risk for MTX-related toxicity when compared to CGTTT haplotype. No statistically significant associations were observed for *ATIC* rs16853834 and *GART* rs8971 with MTX therapeutic outcome. ToxGRI demonstrated that patients with Index 4 (*ATIC* rs2372536 G carriers + *ATIC* rs3821353 T carriers + *ATIC* rs7563206 CC + *ATIC* rs12995526 CC) presented more than 15-fold increased risk for ADRs occurrence then those with Index 0.

Table 5. Association of SNPs involved in *De Novo* Purines Synthesis Pathway with MTX Therapeutic Outcome

	Clinical Response				Overall Toxicity			
	Response	**Non-response**	***p***	**OR (95%CI)**	**Non-toxicity**	**Toxicity**	***p***	**OR (95%CI)**
				GENOTYPES				
***ATIC* C>G (rs2372536)**								
CC+CG	88 (42.1)	121 (57.9)		1.00	141 (67.5)	68 (32.5)		1.00
GG	17 (70.8)	7 (29.2)	**0.036**	0.27 (0.08-0.92) [a)]	15 (62.5)	9 (37.5)	0.580	0.71 (0.21-2.41)
CC	47 (42.7)	63 (57.3)		1.00	77 (70.0)	33 (30.0)		1,00
CG+GG	58 (47.2)	65 (52.8)	0.568	0.83 (0.43-1.59)	79 (64.2)	44 (35.8)	**0.050**	2.09 (1.00-4.38)
***ATIC* G>T (rs3821353)**								
GG+GT	96 (45.3)	116 (54.7)		1.00	142 (67.0)	70 (33.0)		1.00
TT	9 (42.9)	12 (57.1)	0.868	0.91 (0.29-2.86)	14 (66.7)	7 (33.3)	0.766	0.83 (0.25-2.79)
GG	72 (43.4)	94 (56.6)		1.00	116 (69.9)	50 (30.1)		1.00
GT+TT	33 (49.3)	34 (50.7)	0.071	0.51 (0.25-1.06)	40 (59.7)	27 (40.3)	**0.046**	2.18 (1.01-4.71)
***ATIC* T>C (rs4673993)**								
TT+TC	88 (42.1)	121 (57.9)		1.00	141 (67.5)	68 (32.5)		1.00
CC	17 (70.8)	7 (29.2)	**0.036**	0.27 (0.08-0.92) [b)]	15 (62.5)	9 (37.5)	0.580	0.71 (0.21-2.41)
TT	48 (43.6)	62 (56.4)		1.00	76 (69.1)	34 (30.9)		1.00
TC+CC	57 (46.3)	66 (53.7)	0.950	0.98 (0.51-1.89)	80 (65.0)	43 (35.0)	0.194	1.61 (0.78-3.31)
***ATIC* T>C (rs7563206)**								
TT+TC	66 (37.9)	108 (62.1)		1.00	125 (71.8)	49 (28.2)		1.00
CC	39 (66.1)	20 (33.9)	**<0.001**	0.20 (0.09-0.46) [c)]	31 (52.5)	28 (47.5)	**0.010**	3.06 (1.31-7.12)
TT	29 (44.6)	36 (55.4)		1.00	45 (69.2)	20 (30.8)		1.00
TC+CC	76 (45.2)	92 (54.8)	0.558	0.81 (0.40-1.65)	111 (66.1)	57 (33.9)	0.147	1.84 (0.81-4.18)
***ATIC* T>C (rs12995526)**								
TT+TC	69 (39.0)	108 (61.0)		1.00	126 (71.2)	51 (28.8)		1.00
CC	36 (64.3)	20 (35.7)	**0.001**	0.23 (0.10-0.53) [d)]	30 (53.6)	26 (46.4)	**0.013**	2.96 (1.26-6.95)
TT	29 (43.3)	38 (56.7)		1.00	47 (70.1)	20 (29.9)		1.00
TC+CC	76 (45.8)	90 (54.2)	0.413	0.74 (0.37-1.51)	109 (65.7)	57 (34.3)	0.106	1.97 (0.87-4.49)
***ATIC* C>T (rs16853834)**								
CC+CT	102 (44.7)	126 (55.3)		1.00	154 (67.5)	74 (32.5)		1.00
TT	3 (60.0)	2 (40.0)	0.595	1.76 (0.22-14.30)	2 (40.0)	3 (60.0)	0.902	1.14 (0.13-9.68)
CC	74 (46.2)	86 (53.8)		1.00	105 (65.6)	55 (34.4)		1.00
CT+TT	31 (42.5)	41 (57.5)	0.438	1.32 (0.65-2.68)	51 (69.9)	22 (30.1)	0.084	0.48 (0.21-1.10)
***GART* A>G (rs8971)**								
AA+AG	95 (44.4)	119 (55.6)		1.00	144 (67.3)	70 (32.7)		1.00
GG	10 (52.6)	9 (47.4)	0.540	0.69 (0.21-2.26)	12 (63.2)	7 (36.8)	0.336	0.52 (0.14-1.96)
AA	54 (42.2)	74 (57.8)		1.00	85 (66.4)	43 (33.6)		1.00
AG+GG	51 (48.6)	54 (51.4)	0.164	0.62 (0.32-1.21)	71 (67.6)	34 (32.4)	0.513	1.27 (0.62-2.61)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). *Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. [a)] When reference was GG genotype, OR=3.66, 95%CI: 1.09-12.33. [b)] When reference was CC genotype, OR=3.66, 95%CI: 1.09-12.33. [c)] When reference was CC genotype, OR=4.93, 95%CI: 2.15-11.29. [d)] When reference was CC genotype, OR=4.29, 95%CI: 1.89-9.76. A: adenine; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; C: cytosine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; GART: glycineamide ribonucleotide formyl transferase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine; ToxGRI: genetic risk index for MTX-toxicity.

Table 5. Association of SNPs involved in *De Novo* Purines Synthesis Pathway with MTX Therapeutic Outcome (cont.)

HAPLOTYPES

ATIC C>G (rs2372536)	*ATIC* G>T (rs3821353)	*ATIC* T>C (rs4673993)	*ATIC* T>C (rs7563206)	*ATIC* T>C (rs12995526)	% *	Clinical Response		Overall Toxicity	
						p	OR (95%CI)	*p*	OR (95%CI)
C	G	T	T	T	48.1		1.00		1.00
G	G	C	C	C	31.1	**0.023**	0.46 (0.23-0.89)	0.150	1.56 (0.85-2.85)
C	T	T	C	C	15.9	0.062	0.51 (0.26-1.03)	**0.019**	2.38 (1.16-4.88)
C	T	T	T	T	3.0	1.000	1.00 (0.23-4.31)	0.390	0.48 (0.09-2.49)
***ATIC* C>G (rs2372536)**	***ATIC* T>C (rs4673993)**	***ATIC* T>C (rs7563206)**	***ATIC* T>C (rs12995526)**	***ATIC* C>T (rs16853834)**					
C	T	T	T	C	34.6		1.00		1.00
G	C	C	C	C	31.1	0.076	0.58 (0.31-1.96)	0.200	1.53 (0.80-2.93)
C	T	T	T	T	16.5	0.810	0.92 (0.44-1.89)	0.610	0.81 (0.36-1.80)
C	T	C	C	C	16.1	0.063	0.58 (0.33-1.03)	0.054	2.04 (0.99-4.21)

GENETIC RISK INDEXES

Non-RespGRI		Clinical Response			
		Response	Non-response	*p*	OR (95%CI)
Index 0	rs2372536 – GG **AND** rs4673993 – CC **AND** rs7563206 – CC **AND** rs12995526 – CC	17 (70.8)	7 (29.2)		1.00
Index 1	rs2372536 – CC+CG **OR** rs4673993 – TT+TC **OR** rs7563206 – TT+TC **OR** rs12995526 – TT+TC	0 (0.0)	0 (0.0)	-	-
Index 2	[rs2372536 – CC+CG **AND** rs4673993 – TT+TC] **OR** [rs2372536 – CC+CG **AND** rs7563206 – TT+TC] **OR** [rs2372536 – CC+CG **AND** rs12995526 – TT+TC] **OR** [rs4673993 – TT+TC **AND** rs7563206 – TT+TC] **OR** [rs4673993 **AND** rs12995526 – TT+TC] **OR** [rs7563206 – TT+TC **AND** rs12995526 – TT+TC]	19 (59.4)	13 (40.6)	0.219	0.25 (0.03-2.28)
Index 3	[rs2372536 – CC+CG **AND** rs4673993 – TT+TC **AND** rs7563206 – TT+TC] **OR** [rs2372536 – CC+CG **AND** rs4673993 – TT+TC **AND** rs12995526 – TT+TC] **OR** [rs2372536 – CC+CG **AND** rs7563206 – TT+TC **AND** rs12995526 – TT+TC] **OR** [rs4673993 – TT+TC **AND** rs7563206 – TT+TC **AND** rs12995526 – TT+TC]	3 (100.0)	0 (0.0)	0.999	1.00 (1.00-1.00)
Index 4	rs2372536 – CC+CG **AND** rs4673993 – TT+TC **AND** rs7563206 – TT+TC **AND** rs12995526 – TT+TC	66 (37.9)	108 (62.1)	**0.013**	5.23 (1.41-19.30)
ToxGRI		**Overall Toxicity**			
		Non-toxicity	**Toxicity**	***p***	**OR (95%CI)**
Index 0	rs2372536 – CC **AND** rs3821353 – GG **AND** rs7563206 – TT+TC **AND** rs12995526 – TT+TC	47 (70.1)	20 (29.9)		1.00
Index 1	rs2372536 – CG+GG **OR** rs3821353 – GT+TT **OR** rs7563206 – CC **OR** rs12995526 – CC	78 (72.9)	29 (27.1)	0.548	1.32 (0.53-3.26)
Index 2	[rs2372536 – CG+GG **AND** rs3821353 – GT+TT] **OR** [rs2372536 – CG+GG **AND** rs7563206 – CC] **OR** [rs2372536 – CG+GG **AND** rs12995526 – CC] **OR** [rs3821353 – GT+TT **AND** rs7563206 – CC] **OR** [rs3821353 – GT+TT **AND** rs12995526 – CC] **OR** [rs7563206 – CC **AND** rs12995526 – CC]	1 (33.3)	2 (66.7)	0.999	1.00 (1.00-1.00)
Index 3	[rs2372536 – CG+GG **AND** rs3821353 – GT+TT **AND** rs7563206 – CC] **OR** [rs2372536 – CG+GG **AND** rs3821353 – GT+TT **AND** rs12995526 – CC] **OR** [rs2372536 – CG+GG **AND** rs7563206 – CC **AND** rs12995526 – CC] **OR** [rs3821353 – GT+TT **AND** rs7563206 – CC **AND** rs12995526 – CC]	15 (62.5)	9 (37.5)	0.261	2.40 (0.52-11.02)
Index 4	rs2372536 – CG+GG **AND** rs3821353 – GT+TT **AND** rs7563206 – CC **AND** rs12995526 – CC	15 (46.9)	17 (53.1)	**0.001**	15.60 (3.06-79.48)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). *Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. [a)] When reference was GG genotype, OR=3.66, 95%CI: 1.09-12.33. [b)] When reference was CC genotype, OR=3.66, 95%CI: 1.09-12.33. [c)] When reference was CC genotype, OR=4.93, 95%CI: 2.15-11.29. [d)] When reference was CC genotype, OR=4.29, 95%CI: 1.89-9.76. A: adenine; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; C: cytosine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; GART: glycineamide ribonucleotide formyl transferase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine; ToxGRI: genetic risk index for MTX-toxicity.

Adenosine pathway: Table 6 represents the relation between MTX therapeutic outcome and SNPs in *ADORA2A* and *AMPD1* by genotype- and haplotype-based approaches. Considering MTX non-response, no statistically significant associations were observed. Nevertheless, *ADORA2A* rs2267076 T homozygotes was associated with about 3-fold increased risk for MTX-related toxicity. Regarding haplotype approach, TC haplotype for *ADORA2A* rs2267076 and rs2298383 demonstrated almost 2-fold increased risk for MTX-related toxicity when compared to CT haplotype.

Table 6. Association of SNPs involved in Adenosine Pathway with MTX Therapeutic Outcome

	Clinical Response				Overall Toxicity			
	Response	Non-response	*p*	OR (95%CI)	Non-toxicity	Toxicity	*p*	OR (95%CI)
GENOTYPES								
***ADORA2A* C>T (rs2267076)**								
CC+CT	90 (44.3)	113 (55.7)		1.00	141 (69.5)	62 (30.5)		1.00
TT	15 (50.0)	15 (50.0)	0.552	0.73 (0.26-2.04)	15 (50.0)	15 (50.0)	**0.025**	3.26 (1.16-9.15)
CC	39 (42.9)	52 (57.1)		1.00	61 (67.0)	30 (33.0)		1.00
CT+TT	66 (46.5)	76 (53.5)	0.962	1.02 (0.52-2.00)	95 (66.9)	47 (33.1)	0.126	1.83 (0.84-3.99)
***ADORA2A* T>C (rs2298383)**								
TT+TC	84 (44.9)	103 (55.1)		1.00	127 (67.9)	60 (32.1)		1.00
CC	21 (45.7)	25 (54.3)	0.525	0.76 (0.33-1.75)	29 (63.0)	17 (37.0)	0.270	1.64 (0.68-3.98)
TT	34 (44.2)	43 (55.8)		1.00	50 (64.9)	27 (35.1)		1.00
TC+CC	71 (45.5)	85 (54.5)	0.883	0.95 (0.47-1.91)	106 (67.9)	50 (32.1)	0.124	1.90 (0.84-4.28)
***AMPD1* C>T (rs17602729)**								
CC+CT	104 (45.6)	124 (54.4)		1.00	151 (66.2)	77 (33.8)		1.00
TT	1 (20.0)	4 (80.0)	0.674	1.76 (0.13-24.58)	5 (100.0)	0 (0.0)	0.999	1.00 (1.00-1.00)
CC	78 (43.3)	102 (56.7)		1.00	121 (67.2)	59 (32.8)		1,00
CT+TT	27 (50.9)	26 (49.1)	0.517	0.77 (0.36-1.68)	35 (66.0)	18 (34.0)	0.583	0.79 (0.35-1.80)
HAPLOTYPES								
***ADORA2A*C>T (rs2267076)**		***ADORA2A* T>C (rs2298383)**	**% ***	**Clinical Response**		**Overall Toxicity**		
				p	**OR (95%CI)**	***p***	**OR (95%CI)**	
C		T	56.7		1.00		1.00	
T		C	36.9	0.770	0.93 (0.56-1.54)	**0.036**	1.84 (1.05-3.23)	
C		C	6.4	0.660	0.82 (0.34-1.98)	0.360	0.55 (0.15-2.01)	

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). Non-RespGRI and ToxGRI were not performed because only one genotype was statistically significant for MTX non-response and no statistically significant associations were obtained regarding MTX-related toxicity. *Rare haplotypes (estimated haplotype frequency <2.0%) were excluded from analysis. A: adenine; ADORA2A: adenosine receptor A2A; AMPD1: adenosine deaminase 1; C: cytosine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; GRI: genetic risk index; MTX: methotrexate; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine.

Phase II reactions: Table 7 represents the relation between MTX therapeutic outcome and *GSTP1* A>G (rs1695) by genotype-based approach. No statistically significant associations were observed for this SNP and MTX therapeutic outcome.

Table 7. Association of SNPs involved in Phase II Reactions with MTX Therapeutic Outcome

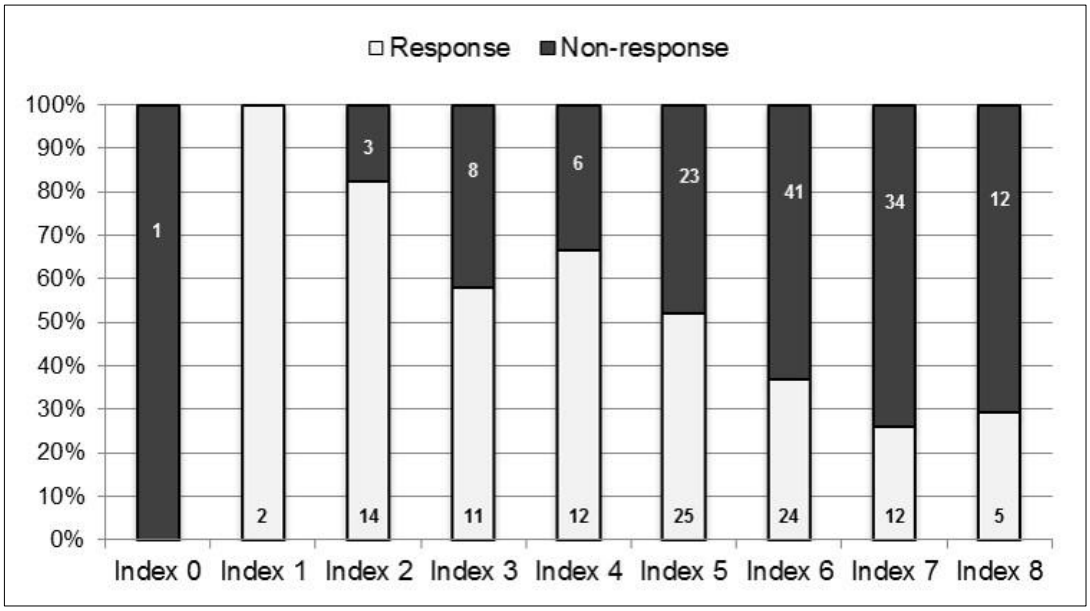
	Clinical Response				Overall Toxicity			
	Response	Non-response	*p*	OR (95%CI)	Non-toxicity	Toxicity	*p*	OR (95%CI)
GENOTYPES								
***GSTP1* A>G (rs1695)**								
AA+AG	93 (44.9)	114 (55.1)		1.00	138 (66.7)	69 (33.3)		1.00
GG	12 (46.2)	14 (53.8)	0.817	0.88 (0.32-2.48)	18 (69.2)	8 (30.8)	0.123	0.39 (0.12-1.29)
AA	44 (42.7)	59 (57.3)		1.00	75 (72.8)	28 (27.2)		1.00
AG+GG	61 (46.9)	69 (53.1)	0.288	0.70 (0.36-1.35)	81 (62.3)	49 (37.7)	0.862	1.07 (0.51-2.21)

Results are expressed in n (%). $p \leq 0.05$ was considered as statistically significant (highlighted in bold). Multivariate analysis was adjusted to following variables: 1) patient-related: age, gender, smoking status and renal function; 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). Haplotypes and GRIs were not performed because only one SNP related with Phase II reactions was studied. A: adenine; CI: confidence interval; DMARDs: disease modifying anti-rheumatic drugs; G: guanine; GSTP1: human glutathione S-transferase P1; GRI: genetic risk index; MTX: methotrexate; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; T: thymine.

Overall Genetic Risk Indexes & Methotrexate Therapeutic Outcome

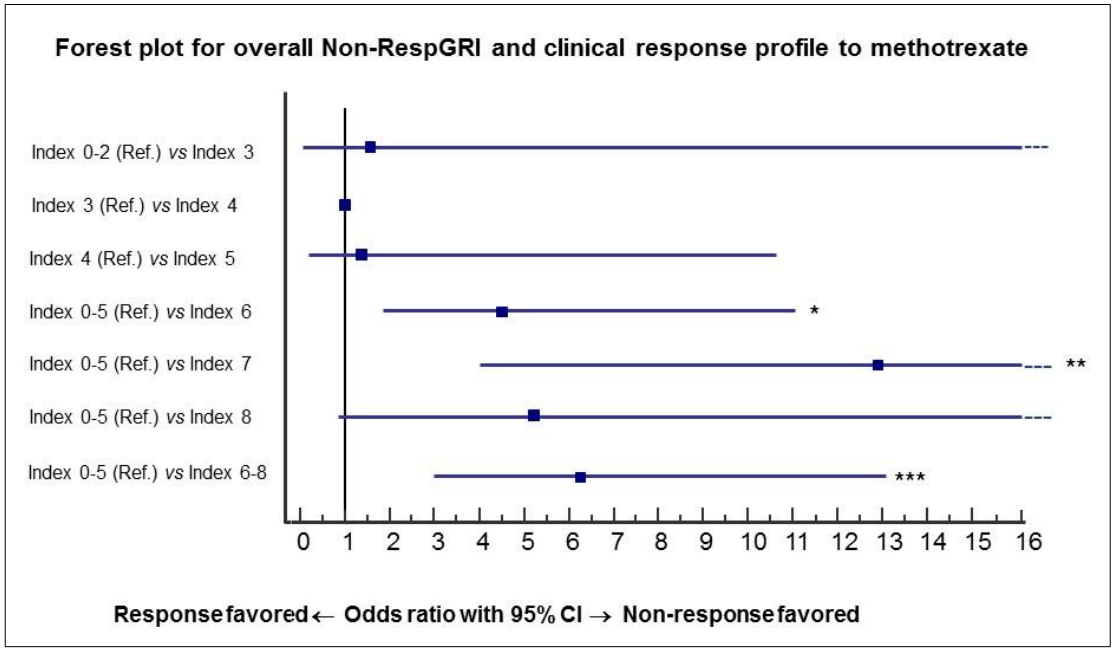
Pertaining to MTX non-response risk genotypes, Non-RespGRI consisted in 8 possible combinations: *MTHFR* rs1801131 AA, *MTHFR* rs1801133 TT, *MS* rs1805087 AA, *MTRR* rs1801394 A carriers, *ATIC* rs2372536 C carriers, *ATIC* rs4673993 T carriers, *ATIC* rs7563206 T carriers and *ATIC* rs12995526 T carriers. Distribution of overall Non-RespGRI demonstrated an increasingly risk for MTX non-response with an increased Non-RespGRI Index (p<0.001) **(Figure 2A)**. Patients with Index 6 to 8, i.e. patients with more than 5 risk genotypes for MTX non-response, demonstrated more than 5-fold increased risk for developing a non-response profile to MTX when compared to those with Index up to 5 **(Figure 2B)**. Regarding MTX-related toxicity risk genotypes, ToxGRI consisted in 5 possible combinations: *ATIC* rs2372536 G carriers, *ATIC* rs3821353 T carriers, *ATIC* rs7563206 CC, *ATIC* rs12995526 CC and *ADORA2A* rs2267076 TT. Distribution of Overall ToxGRI demonstrated an increasingly risk for MTX-related toxicity occurrence with an increased ToxGRI Index (p=0.002) **(Figure 2C)**. Patients with Index 3/4, i.e. patients with more than 2 risk genotypes, demonstrated more than 7-fold increased risk for developing MTX-related toxicity when compared to those with Index 1/2 **(Figure 2D)**.

A)

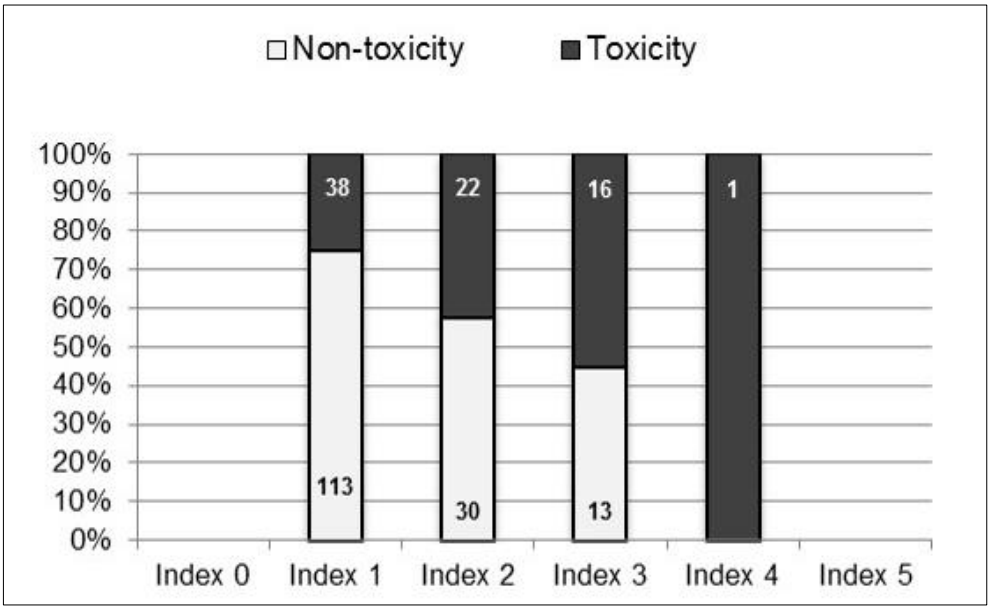


Overall Non-RespGRI for MTX clinical response profile

B)



C)



Overall ToxGRI for MTX-related toxicity

D)

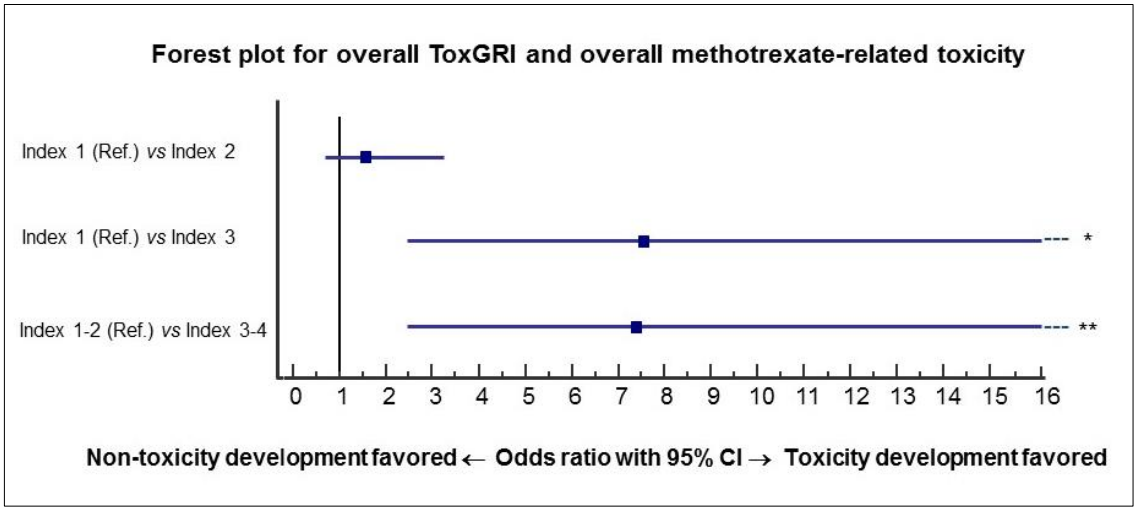


Figure 2. Association of overall genetic risk indexes and methotrexate therapeutic outcome. A) Distribution of overall Non-RespGRI, constituted by 8 combinations of risk genotypes for MTX non-response: *MTHFR* rs1801131 AA, *MTHFR* rs1801133 TT, *MS* rs1805087 AA, *MTRR* rs1801394 A carriers, *ATIC* rs2372536 C carriers, *ATIC* rs4673993 T carriers, *ATIC* rs7563206 T carriers and *ATIC* rs12995526 T carriers, by clinical response profile to MTX. B) Forest plot representing multivariate analysis of the association between overall Non-RespGRI and clinical response profile to MTX: *p=0.001, OR=4.58, 95%CI: 1.87-11.20. **p<0.001, OR=13.42, 95%CI: 4.00-44.96. ***p<0.001, OR=6.23, 95%CI: 2.91-13.31. C) Distribution of overall ToxGRI, constituted by 5 combinations of risk genotypes for MTX-related toxicity: *ATIC* rs2372536 G carriers, *ATIC* rs3821353 T carriers, rs7563206 CC, *ATIC* rs12995526 CC and *ADORA2A* rs2267076 T, by overall MTX-related toxicity. D) Forest plot representing multivariate analysis of the association between the overall ToxGRI and overall MTX-related toxicity: *p<0.001, OR=7.65, 95%CI: 2.46-23.75. **p<0.001, OR=7.38, 95%CI: 2.42-22.50.
ADORA2A: adenosine receptor A2A; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; CI: confidence interval; MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; MTRR: methionine synthase reductase; MTX: methotrexate; Non-RespGRI: genetic risk index for non-response; OR: odds ratio; ToxGRI: genetic risk index for MTX-toxicity.

DISCUSSION

The present work aims to evaluate selected SNPs in genes encoding for proteins involved in MTX pathways as potential predictors of MTX therapeutic outcome in Portuguese RA patients, based on genotype-, haplotype- and genetic risk index-based approaches. Haplotype-based approach was performed to incorporate allelic heterogeneity, since different genetic polymorphisms may lead to a similar phenotype, which is a blind spot for a coherent genotype-based approach [56].

For polyglutamation pathway, results showed that genotypes were not associated with MTX therapeutic outcome, in accordance to other studies [57, 58]. Yet, CC haplotype for *GGH* rs3758149 and rs12681874 were associated with non-response when compared to TC haplotype. Literature describes no significant associations for rs3758149 and clinical response profile to MTX [19, 58, 59], prompting

more studies to elucidate this, while for rs12681874 the C allele was associated with MTX non-response in RA [46], in accordance to the tendency of CC haplotype.

Considering folate pathway, *MTHFR* rs1801131 A homozygotes and *MTHFR* rs1801133 T homozygotes were associated with a non-response profile to MTX. MTHFR is responsible for the conversion of 5,10-MTHF to 5-MTHF, a carbon donor for the remethylation of homocysteine into methionine [60]. *MTHFR* A1298C (rs1801131) and C677T (rs1801133) were described as the most important *MTHFR* polymorphisms because of their influence in MTHFR activity and, consequently, in MTX action mechanism [15, 61]. The rs1801131 A allele has been associated with an increased MTHFR activity [62], leading to an increased pool of folates, resulting in a non-response profile to MTX, in accordance to our study and literature in RA [45, 63]. Nevertheless, one study associated A allele with better response to MTX in RA [64] while others demonstrated non-significant results [59, 65, 66]. For MTX-related toxicity, no significant results were observed for this SNP in this study and were described in literature for RA [59, 63, 66-70], still some studies demonstrated significant results [57, 71-73]. Also, T allele for rs1801133 has been described as responsible for a thermolabile form of MTHFR with reduced activity [74]. Despite this functional impact, all studies with significant results concerning this SNP in RA demonstrated an association of T allele with non-response to MTX [4, 19, 59, 64], were in accordance to present study. Concerning MTX-related toxicity, we demonstrated non-significant results for this SNP, as demonstrated in other studies in RA [57, 70]. Moreover, no significant associations were observed for *DHFR* polymorphisms (rs7387, rs1232027, rs1643657 and rs10072026) and *MTHFS* rs8923 with MTX therapeutic outcome. For *DHFR* polymorphisms, literature confirms our results for rs1232027 [64] while for rs1643657 and rs10072026, studies are in agreement only regarding to clinical response profile to MTX [46]. For rs7387, our results are different from those of Sharma *et al.* [41], probably due to ethnic differences of population. Regarding haplotype approach, associations were not statistically significant, prompting for more studies to elucidate *DHFR* and *MTHFR* polymorphisms role in MTX therapeutic outcome. Also, to the best of our knowledge, this is the first study to analyze the association of *MTHFS* rs8923 with MTX therapeutic outcome.

In relation to methionine pathway, *MS* rs1805087 A homozygotes and *MTRR* rs1801394 A carriers were associated with a non-response profile to MTX. MS (also known as methyltransferase - MTR) mediates the conversion of 5-MTHF to THF in the presence of methyl(III)cobalamin, which occurs simultaneously with the addition of a methyl group to homocysteine, forming methionine [60, 75]. The MTRR maintains adequate levels of methylcob(III)alamin for the reaction mediated by MS occurs [76]. Despite the A alleles for *MS* rs1805087 (A2756G) and *MTRR* rs1801394 (A66G) have been described as responsible for increasing enzyme activity [77, 78], literature demonstrated no significant associations of these polymorphisms with MTX therapeutic outcome in RA [39, 40, 42, 45]. Since we demonstrated associations of these SNPs with clinical response profile to MTX, more studies are necessary to elucidate this.

Considering *de novo* purine synthesis, *ATIC* rs2372536 C carriers, rs4673993 T carriers, rs7563206 T carriers and rs12995526 T carriers were associated with a non-response profile to MTX. ATIC is responsible for the conversion of AICAR into FAICAR [4]. The rs2372536 is the most studied *ATIC* polymorphism. Although the effect of this SNP on ATIC activity still unknown, one study in RA associated C homozygotes with better response [39] while other more recent studies demonstrated non-significant associations [40-42, 44-46, 79], prompting for more studies to address this issue. Despite unknown functional impact, T alleles for rs4673993 and rs7563206 were associated with non-response in RA [4, 46, 47], confirmed by our results, while T allele for rs12995526 was associated with better response [46]. Nevertheless, haplotype approach followed the same tendency of genotype approach, where CGTTT haplotype for *ATIC* combination 1 was associated with a non-response profile to MTX when compared to GGCCC haplotype. This renders more relevance of our results regarding *ATIC* and MTX therapeutic outcome, but confirmation from other studies is needed. Regarding MTX-related toxicity, *ATIC* rs2372536 G carriers, rs3821353 T carriers, rs7563206 C homozygotes and rs12995526 C homozygotes were associated with about 2-fold risk for MTX-related toxicity. For rs2372536, all studies with significant results concerning this SNP in RA demonstrated an association of G allele with MTX-related toxicity [19, 39, 43, 80], confirmed by our results. Only one study for rs3821353, rs7563206 and rs12995526 demonstrated a non-significant association of these SNPs with MTX-related toxicity [46], Once more, haplotype approach followed the same tendency of genotype approach, where CTTCC haplotype for *ATIC* combination 1 was associated with MTX-related toxicity when compared to CGTTT haplotype. Also, no significant associations were observed for *ATIC* rs16853834 and *GART* rs8971 with MTX therapeutic outcome. The rs16853834 was previously studied by Owen *et al*., which demonstrated a significant association with clinical response to MTX but not with MTX-related toxicity [46] while for *GART* rs8971, to the best of our knowledge, this is the first study to analyze its association with MTX therapeutic outcome.

For adenosine pathway both genotype- and haplotype-based approaches demonstrated non-significant associations for *ADORA2A* rs2267076, *ADORA2A* rs2298383 and *AMPD1* rs17602729 with clinical response profile to MTX. ADORA2A is a member of adenosine receptor group of G-protein-coupled receptors, responsible for adenosine transport [30]. Hider *et al.* studied rs2267076 and rs2298383 and demonstrated no association with clinical response to MTX [81], in accordance to our results. AMPD1 catalyzes the conversion of AMP to IMP [39] and rs17602729 (C34T) seems to generate an AMPD1 enzyme with lower activity due to the non-synonymous amino acid change at codon 12 [82]. Thus, deficiency of AMPD1 could enhance adenosine release [39]. Nevertheless, we and other investigators demonstrated no associations with MTX clinical response [41, 42, 44, 46], even though some authors demonstrated a better response for T allele [43, 83], prompting for an elucidation of this polymorphism impact in MTX clinical response profile. In addition, *ADORA2A* rs2267076 T homozygotes were associated with about 3-fold increased risk for developing overall toxicity to MTX, in accordance to literature [81]. Haplotype approach followed the same tendency of genotype approach, where TC

haplotype for *ADORA2A* rs2267076 and rs2298383 demonstrated almost 2-fold increased risk for MTX-related toxicity when compared to CT haplotype.

Regarding Phase II reactions, *GSTP1* rs1695 was not associated with MTX therapeutic outcome. GSTP1 is a phase II reaction enzyme involved in MTX inactivation [31] and, despite the functional impact of *GSTP1* rs1695 (A313G) [48], studies in RA demonstrated no significant associations of this SNP with MTX therapeutic outcome [84, 85], in accordance to our results.

In order to improve the characterization of the impact of relevant studied SNPs associated with MTX non-response and MTX-related toxicity, genetic risk indexes for each pathway and overall genetic risk indexes were created. Regarding MTX non-response, Overall Non-RespGRI revealed that patients with Indexes 6 to 8 had more than 6-fold increased risk for a non-response profile when compared to patients with Indexes 0 to 5. Accordingly to MTX-related toxicity, Overall ToxGRI revealed that patients with Indexes 3 and 4 had more than 7-fold increased risk for MTX-related toxicity.

Besides the potential of our results, we are aware of possible study limitations such sample size and study design (single-center retrospective study). In order to minimize potential false positive results, multivariate analyses adjusted to variables that could influence the measured outcome were performed. Beyond multivariate analyses, other strengths of this study can be highlighted such: 1) studied population was homogenous relatively to ethnic origin; 2) population characteristics regarding disease gender epidemiology and to diagnosis age were in accordance with other reported studies [86, 87]; 3) all genotyping results were manually inspected, 10% of the samples were randomly selected for a second analysis and results were 100% concordant; 4) polymorphisms were included only if genotyping call rates were superior to 95% and the minor allele frequency was greater than 10.0%; and, 5) having included a good number of polymorphisms (thirty-five) in fourteen different genes that encode for proteins involved in intracellular pathways and Phase II reactions, many of which had never been studied before or present conflicting results regarding therapeutic outcome to MTX compared to other studies in RA. To the best of our knowledge, this is the first report regarding the study of the association of such amount of polymorphisms with MTX therapeutic outcome in Portuguese RA patients.

As future perspectives, due to lack or conflicting studies analyzing the impact of the studied SNPs in proteins function and/or MTX therapeutic outcome, further evidence is necessary to support the interpretation of our results. Since we could not genotype all the selected SNPs due to technical limitations of genotyping technique, other techniques should be considered due to the possible importance and lack of studies analyzing the SNPs that were excluded. Finally, further studies should consider that a possible synergetic effect between MTX and other DMARDs could influence the associations between genetic polymorphisms and MTX therapeutic outcome.

CONCLUSIONS

From a total of 35 SNPs only 26 were studied to evaluate the influence in MTX therapeutic outcome in RA Portuguese patients. From these 26 SNPs, 8 genotypes (*MTHFR* rs1801131 AA, *MTHFR* rs1801133 TT, *MS* rs1805087 AA, *MTRR* rs1801394 A carriers, *ATIC* rs2372536 C carriers, *ATIC* rs4673993 T carriers, *ATIC* rs7563206 T carriers and *ATIC* rs12995526 T carriers) and 3 haplotypes (CC for *GGH* rs3758149 and rs12681874; CGTTT for *ATIC* combination 1 and CTTTC for *ATIC* combination 2) could be predictors of MTX non-response. In addition, 5 genotypes (*ATIC* rs2372536 G carriers, *ATIC* rs3821353 T carriers, *ATIC* rs7563206 CC, *ATIC* rs12995526 CC and *ADORA2A* rs2267076 T) and 2 haplotypes (CTTCC for *ATIC* combination 1 and TC for *ADORA2A* rs2267076 and rs2298383) could be predictors of MTX-related toxicity. Interestingly, from the 14 studied genes encoding for crucial proteins involved in MTX action mechanism, 6 genes demonstrated to be important predictors of MTX therapeutic outcome. Moreover, Non-RespGRI and ToxGRI revealed the importance of integrating different risk genotypes to predict MTX therapeutic outcome of each RA patient, which was highlighted when Overall Non-RespGRI and ToxGRI were performed for all risk genotypes from different genes and pathways. Consequently, genotyping patients according to these genetic markers, and analysis by genotype- and haplotype-based approaches, may be helpful to identify which patients will not benefit from MTX treatment. Furthermore, proposed Non-RespGRI and ToxGRI highlight the importance of using genome patients' information to develop the field of personalized medicine/therapeutics. Nevertheless, and despite the potential of these findings, translation into clinical practice requires larger and multicentric studies in order to clearly endorse the utility of the obtained results.

SUMMARY POINTS

- MTX therapeutic outcome is a complex phenotype, partly modulated by SNPs (e.g. in genes implicated in key MTX pathways).
- Identifying genetic predictors of MTX therapeutic outcome has great importance for moving towards personalized medicine in RA.
- Eight genotypes and 3 haplotypes could be predictors of MTX non-response: *MTHFR* rs1801131 AA, *MTHFR* rs1801133 TT, *MS* rs1805087 AA, *MTRR* rs1801394 A carriers, *ATIC* rs2372536 C carriers, *ATIC* rs4673993 T carriers, *ATIC* rs7563206 T carriers and *ATIC* rs12995526 T carriers; CC for *GGH* rs3758149 and rs12681874; CGTTT for *ATIC* combination 1 and CTTTC for *ATIC* combination 2.
- Five genotypes and 2 haplotypes could be predictors of MTX-related toxicity: *ATIC* rs2372536 G carriers, *ATIC* rs3821353 T carriers, *ATIC* rs7563206 CC, *ATIC* rs12995526 CC and

ADORA2A rs2267076 T; CTTCC for *ATIC* combination 1 and TC for *ADORA2A* rs2267076 and rs2298383.

- Genotyping patients and analysis by genotype- and haplotype-based approaches may be helpful to identify which patients will not benefit from MTX treatment.
- Proposed genetic risk indexes (Non-RespGRI, ToxGRI and Overall GRIs) highlight the importance of use genome patients' information to develop the field of personalized medicine/therapeutics. Further studies, larger and multicentric, should be develop in order to clearly endorse the utility of the obtained results.

ABBREVIATIONS LIST

ACR: American College of Rheumatology; ADR: adverse drug reactions; ADOR: G-coupled adenosine receptors; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; CD: cluster of differentiation; CTCAE: Common Terminology Criteria for Adverse Events; DAS28: Disease Activity Score in 28 joints; DHFR: dihydrofolate reductase; DMARDs: disease modifying anti-rheumatic drugs; eGFR: estimated glomerular filtration rate; EULAR: European League Against Rheumatism; FPGS: folylpolyglutamate synthetase; GART: glycineamide ribonucleotide formyl transferase; GGH: γ-glutamyl hydrolase; GSTP1: glutathione S-transferase P1; HWE: Hardy-Weinberg equilibrium; LD: Linkage disequilibrium; MDRD: Modification of Diet in Renal Disease; Non-RespGRI: genetic risk index for non-response; MS: methionine synthase; MTHFD1: methylenetetrahydrofolate dehydrogenase 1; MTHFR: methylenetetrahydrofolate reductase; MTHFS: methenyltetrahydrofolate synthetase; MTRR: methionine synthase reductase; MTX: methotrexate; MTXPGs: MTX polyglutamates; NTs: nucleoside transporters; NSAIDs: non-steroidal anti-inflammatory drugs; PO: *per os*; PPAT: phosphoribosyl pyrophosphate amidotransferase; RA: rheumatoid arthritis; SOC: System Organ Class; ToxGRI: genetic risk index for MTX-toxicity.

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*** Interesting original research about the association of SNPs in genes encoding for folate pathway enzymes with methotrexate efficacy and toxicity in early rheumatoid arthritis patients.**

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*** Original research about the association of *MTHFR* C677T polymorphism with methotrexate toxicity in a relatively large cohort of rheumatoid arthritis patients.**

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*** Original study with patients in methotrexate monotherapy analyzing 27 genetic variants in *DHFR*, *TYMS*, *MTHFR*, *ATIC* and *CCND1* with methotrexate outcome by multivariate analyses.**

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ANEXO

Descrição da População Alvo de Estudo

Table A1. 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 (1^{st} 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)
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.