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5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

--Manuscript Draft--

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Abstract:	Objectives: For children with Juvenile Idiopathic Arthritis (JIA) who fail to respond to methotrexate, the delay in identifying the optimal treatment at an early stage of disease can lead to long-term joint damage. Recent studies indicate that relevant variants to predict methotrexate response in JIA are those in 5-aminoimidazole-4-carboxamide ribonucleotide-transformylase (ATIC), inosine-triphosphate-pyrophosphatase (ITPA) and solute-liquid-carrier-19A1 (SLC19A1) genes. The purpose of the study was therefore to explore the role of these candidate genetic factors on methotrexate response in an Italian cohort of children with JIA. Methods: Clinical response to methotrexate was evaluated as clinical remission stable for a 6-months period, as ACRPed score and as change in JADAS score. The most relevant SNPs for each gene considered were assayed on patients' DNA. ITPA activity was measured in patients' erythrocytes. Results: 69 patients with JIA were analyzed: 52.2% responded to therapy (ACRPed70 score), while 37.7% reached clinical remission stable for 6 months. ATIC rs2372536 GG genotype was associated with improved clinical remission (adjusted p-value = 0.0090). For ITPA, rs1127354 A variant was associated with reduced clinical remission: (adjusted p-value = 0.028); this association was present even for patients with wild-type ITPA and low ITPA activity.

	Conclusions: These preliminary results indicate that genotyping of ATIC rs2372536 and ITPA rs1127354 variants or measuring ITPA activity could be useful to predict methotrexate response in children with JIA after validation by further prospective studies on a larger patient cohort.

Dear Prof. Loreto Carmona,

thank you for your evaluation of our manuscript. We appreciate all the reviewers' comments and we have edited the manuscript accordingly. Please find below the reviewers' comment, followed by our reply and by the changes done to our revised manuscript.

We hope that on the bases of these changes the manuscript will be now acceptable for publication and look forward to hear from you.

Best regards.

Reviewer #1: This is a small study in JIA that complements and supports previous observations.

In methods: clinical data were collected at baseline, after 6 months of methotrexate therapy, and then every 3 months during treatment. Clarify the number of months/study visits the patients were evaluated ? Also, clinical remission was defined as 6 months without active disease. If a patient was only followed for 6 months, do we assume that clinical remission was achieved immediately after starting MTX. More details need to be provided regarding the number of study follow-ups in this small cohort.

Reply: We appreciate the reviewer's comments. Methotrexate treatment lasted a minimum of 6 months. Clinical data were collected at baseline, after 6 months of methotrexate therapy and then every 3 months during treatment. Minimum follow up for each patient was 12 months. Therefore, each patients had at least 4 visits. We added a sentence specifying this in the methods (page 6, lines 78-79).

Reviewer #3: Dear Authors, I read with interest your manuscript on specific gene variants in predicting MTX response among children with JIA.

Indeed the task of optimizing treatment in children with JIA is crucial in everyday clinical activity, and any tool that could help the physician to find the best treatment approach in the single patient and in the shortest period of time since onset will be very useful.

Here are my major concerns on your manuscript:

_ Although your results are interesting, since you found some association with the explored genetic variants and different indicators of clinical response, the associations are quite heterogeneous (in terms of specific gene variants and specific clinical indicators of response). I think this is secondary to the relatively small number of patients recruited, that your results have to be interpreted as preliminary results and need further confirmatory studies on larger cohort of patients. This point need more discussion on the manuscript than what already stated

Reply: We agree with the reviewers' comment. We added the sentence "We acknowledge that the associations described in this study about explored genetic variants and different indicators of clinical response are heterogeneous: this is likely secondary to the relatively small number of patients recruited. The results therefore have to be interpreted as preliminary and need further confirmatory studies on larger cohort of patients" in the discussion of the revised manuscript (page 17, lines 325 – 329). Moreover, we added "these preliminary results indicate that" to the conclusion in the abstract (page 2, line 24).

_ I agree that the absence of a control population is a limitation of your study, please explain better why it is so

Reply: The lack of a validation cohort limits at this point the extensibility of the observation described in the paper to the general population. We have added a sentence specifying this to the discussion of the paper (page 17, lines 330 – 331).

_ In the discussion you stated low ITPA measured in erythrocytes was associated with reduced methotrexate response. I understand this conclusion comes from the observation that patients with higher ITPA activity had higher rates of response, but in fact you fail to find a statistically significant association between ITPA activity and clinical response. Please discuss this discrepancy in the discussion.

Reply: We acknowledge the reviewer's comment. Indeed, mean ITPA activity was not different in this study between responders and non responders to methotrexate. However, in this paper patients with variant ITPA genotype had lower remission rate that patients with wild-type ITPA; moreover, we observed that all patients with wild type ITPA genotype and an enzymatic activity comparable to that observed in patients with variant ITPA, did not respond to therapy as these patients. We decided to present this observation in the paper, supporting the role of low ITPA activity, besides ITPA variant genotype, as a determinant of lack of response to methotrexate. We acknowledge that the role of ITPA activity in methotrexate response in JIA needs to be further evaluated in larger studies. On these basis we edited the relevant sentence in the discussion of the revised manuscript (page 15-16, lines 299-306).

_ I personally do not agree on the possible outcome your results could have on deciding the treatment strategy in patients with JIA. In particular I do not agree that, if the results will be confirmed, we will be justified to skip the use of Methotrexate in children who will show a genotype predicting a low response to this drug, and starting biologics as first-line regimen. I agree that knowing the susceptibility to methotrexate in the single patients will be useful in switching more rapidly to a more aggressive treatment (i.e. MTX+biologics) in case of partial or no response, but, according to clinical practice and regulations, I think Methotrexate will remain the first-line treatment in children requiring DMARDs. Moreover I do not think that the genotype of a single patient for the explored gene variants will be used to predict the response to treatment in that patient.

Reply: We agree with the reviewer and we changed the discussion by underling that patients predisposed to lack of efficacy of methotrexate treatment could be switched more rapidly to a more aggressive therapy, maintaining methotrexate as a first line therapy (page 16, lines 322-324).

Minor concerns:

- Since the manuscript is directed towards pediatric rheumatologists I think the in-depth discussion of the core-set variables, the criteria for inactive disease and the JADAS are redundant

Reply: In the revised manuscript we erased the in-depth description of the clinical scores in the methods as requested by the reviewer (page 6).

- Even though well explained in the "material and methods", please specify in the results that ACRPed Score is evaluated at 6 months.

Reply: We have made the change suggested by the reviewer (page 11, lines 198-199).

- Page 1 line10 (abstract): "…methotrexate was evaluated [as] clinical remission…"
- Page 4 line 6: "...pharmacogenetics[,] published studies indicate…"
- Page 14 line 7: "…in terms [of] clinical remission…"
- Page 15 line 15:"…may be limited…"
- Page 16 line 2 :"…comparable to that measure[d in] patients…"

Reply: We have made all the edits suggested by the reviewer in the revised manuscript.

5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

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Conflict of interest statement

The authors declare that they have no conflict of interest

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Abstract

Objectives: For children with Juvenile Idiopathic Arthritis (JIA) who fail to respond to methotrexate, the delay in identifying the optimal treatment at an early stage of disease can lead to long-term joint damage. Recent studies indicate that relevant variants to predict methotrexate response in JIA are those in 5-aminoimidazole-4-carboxamide ribonucleotide-transformylase (ATIC), inosine-triphosphate-pyrophosphatase (ITPA) and solute-liquid-carrier-19A1 (SLC19A1) genes. The purpose of the study was therefore to explore the role of these candidate genetic factors on methotrexate response in an Italian cohort of children with JIA.

Methods: Clinical response to methotrexate was evaluated as clinical remission stable for a 6-months period, as ACRPed score and as change in JADAS score. The most relevant SNPs for each gene considered were assayed on patients' DNA. ITPA activity was measured in patients' erythrocytes.

Results: 69 patients with JIA were analyzed: 52.2% responded to therapy (ACRPed70 score), while 37.7% reached clinical remission stable for 6 months. ATIC rs2372536 GG genotype was associated with improved clinical remission (adjusted p-value = 0.0090). For ITPA, rs1127354 A variant was associated with reduced clinical remission: (adjusted p-value = 0.028); this association was present even for patients with wild-type ITPA and low ITPA activity.

Conclusions: These preliminary results indicate that genotyping of ATIC rs2372536 and ITPA rs1127354 variants or measuring ITPA activity could be useful to predict methotrexate response in children with JIA after validation by further prospective studies on a larger patient cohort.

Key words: juvenile idiopathic arthritis, methotrexate, pharmacogenetics, clinical remission, 5-aminoimidazole-4-carboxamide ribonucleotide-transformylase, inosine-triphosphate-pyrophosphatase

Introduction

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease of childhood and is an important cause of disability [1]. Methotrexate is the first choice disease-modifying anti-rheumatic drug in the JIA [2, 3], however, 35-45% of patients fail to respond, and the delay in identifying the optimal treatment in an early stage of disease can influence the long-term joint damage [4, 5].

Methotrexate is a folate analogue and enters the cell primarily via the reduced folate carrier (SLC19A1) [6]; pharmacological activity is increased by its enzymatic conversion to polyglutamated forms [7]. Methotrexate polyglutamates inhibit several key enzymes including thymidylate synthase (TYMS) that affects pyrimidine synthesis, dihydrofolate reductase (DHFR) that affects folate synthesis and 5-aminoimidazole-4-carboxamide ribonucleotide-transformylase (ATIC) that affects purine synthesis [6] . The latter is the pathway most potently inhibited by methotrexate polyglutamates, which results in a reduced conversion of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to formyl-AICAR by the enzyme ATIC [8]. Anti-inflammatory effects of methotrexate are thought to be related to accumulation of adenosine, a potent anti-inflammatory mediator, mainly consequent to ATIC inhibition [9].

Recent studies have evaluated effects of genetic variants in the complex pathway of candidate genes involved in methotrexate pharmacokinetics and pharmacodynamics on the response to the medication in adults with rheumatoid arthritis and children with JIA [8, 10, 11]. Hinks et al. presented an association of two SNPs in the ATIC gene and one SNP within the inosine-triphosphate-pyrophosphatase (ITPA) gene with reduced response to methotrexate in JIA; only one of the ATIC SNPs showed any trend towards MTX response in an independent cohort of North American JIA children (the ITPA SNP was not genotyped in

the validation cohort) [8]. Another study by De Rotte et al. identified an association between solute carrier 19A1 (SLC19A1) rs1051266 and response to methotrexate in 287 patients with JIA studied longitudinally [10].

Although fine mapping of genetic variants or genome-wide studies into MTX response in JIA or RA are lacking [12, 13] and despite inconsistencies in results from reports about MTX pharmacogenetics, published studies indicate that relevant variants to predict methotrexate response in JIA are those in ATIC, ITPA and SLC19A1 genes .

The aim of the present study was therefore to evaluate the role of these candidate genetic factors on the response to methotrexate in terms of clinical remission in an Italian cohort of children with JIA.

Materials and Methods

Patients and study design

Children who fulfilled International League of Associations for Rheumatology (ILAR) criteria for JIA, and who received methotrexate or were going to start methotrexate for active arthritis, were enrolled at Burlo Garofolo Children's Hospital. Children affected by systemic subtype of JIA were excluded. The study was carried out in compliance with the Helsinki Declaration and had full ethical approval by Burlo Garofolo Ethical Committee. Fully informed parental consent and child assent when appropriate was obtained. Demographic and clinical data were collected at baseline (up to 4 weeks before starting methotrexate) and after 6 months of methotrexate therapy, and then every 3 months during treatment. Methotrexate treatment lasted a minimum of 6 months. Minimum follow-up for each patient was 12 months. Weekly methotrexate was given by either oral or subcutaneous route at 10–15 mg/m^2. Data allowing assessment of clinical response to the drug were collected using the validated core set variables[14] and the definition of clinical remission on medication for JIA, according to Wallace criteria[15]. To declare achievement of clinical remission, the patient must have not received other medications (NSAID, oral steroids, intra-articular steroids) for a 6-month period of inactive disease on methotrexate [15]. The absolute disease activity at baseline and in follow-up is evaluated with Juvenile Arthritis Disease Score (JADAS) [16].

Venous blood samples were taken when the child required blood sampling for routine clinical care.

SNP selection

A total of 3 SNPs in 3 genes in the methotrexate pathway were selected for genotyping, on the basis of results from recent comprehensive studies [8, 9, 17]. In particular, analysis was performed for the coding non-synonymous SNPs rs2372536 in ATIC, rs1127354 in ITPA and rs1051266 in SLC19A1 [18].

Genotyping

Genomic DNA was extracted from peripheral blood samples using a commercial kit (Sigma, Milan, Italy) and stored at -20 °C until use. Genotyping for rs2372536 in ATIC and rs1127354 in ITPA was carried out using the TaqMan Pre-Developed Assay Reagents for genotyping (assay ID respectively: C_16218146_10 and C_27465000_10; Applied Biosystems, Foster City, CA), according to the manufacturer instructions. Genotyping of rs1051266 in SLC19A1 was done using a PCR-RFLP assay [19].

Measurement of ITPA activity

Red cell ITPA activity was measured by evaluation of the hydrolysis of ITP to IMP in lysates of patients' erythrocytes with HPLC, according to the method by Shipkova et al. [20]. The reaction mixture contains ITP and, 15 minutes after the addition of a fixed amount of lysates, the sample is extracted by addition of perchloric acid, neutralized and loaded on the HPLC for quantification of IMP. ITPA activity is expressed as units (U): 1 U corresponds to 1 µmol IMP / g hemoglobin / hour.

Statistical analysis

Statistical analysis was performed using the software R (version 3.0).

Each SNP was tested for conformance of genotype frequencies to those expected under Hardy-Weinberg equilibrium with a Chi-square goodness-of-fit test.

Analysis for association of clinical response, evaluated as a categorical variable (i.e, attaining an ACRPed score of at least 70 and clinical remission on methotrexate for a 6-month period), was performed by logistic regression. For these analyses, binomial models were generated using response to methotrexate as the dependent variable and the clinical, demographic or genetic covariate of interest (i.e., gender, age, disease duration at methotrexate start, methotrexate route of administration, methotrexate dose, frequency of homozygous variant genotype for ATIC, ITPA or SLC19A1, ITPA activity) as the independent variable. Odds ratios were calculated from estimates of the logistic regression models; for models with zero counts in a level of the variables, Haldane's modification was used to calculate odds ratio, which add 0.5 to all cells to accommodate for the zero count[21].

For the analysis considering continuous variables (disease activity as JADAS score, evaluated at the start of methotrexate therapy or after 6 months of treatment and ITPA enzymatic activity), generalized linear models of the Gaussian family were used. Before applying linear models, normality of the continuous variable was assessed by visual examination of the histogram and Shapiro test; if distribution resulted non-normal, Box-Cox transformation was applied to increase normality. Analysis of the effect of genotypes on the difference in JADAS score between 6 months of therapy and at methotrexate start, adjusted for baseline JADAS score, was done by linear models, with JADAS score at 6 months as the dependent variable and the candidate genotype and JADAS score at methotrexate start as the independent variables.

For all statistical test, adjustment for multiple testing was done by calculating adjusted p-values with Holm's method.

Results

Patients enrolled

Seventy three patients with JIA treated with methotrexate were considered. These are all consecutive patients treated with methotrexate at Burlo Garofolo Children's Hospital since 2000. Four patients have been excluded from the study: one with systemic subtype of JIA, two treated with a biologic drug (etanercept) in association with methotrexate and one with incomplete data available. We present therefore retrospective results from 69 children whose full core set variable data and DNA sample were available. Demographic and clinical data are reported in Table 1. Most patients have been treated with subcutaneous administration (43/69, 62.3 %) of the medication; oral administration has been used in the rest of the cohort (26/69, 37.7%).

Clinical response

Clinical Remission

Clinical remission was achieved by 37.7% of patients (26/69). Patients' gender, age at disease onset, age at methotrexate start, disease subtype, disease duration, methotrexate administration route and dose did not have a significant effect on response to therapy evaluated as clinical remission (Table 1).

ACRPed Score

The results for each response definition for patients considered are ACRPed30 for 79.7% (55/69) of patients, ACRPed50 for 73.9% (51/69) and ACRPed70 for 52.2% (36/69); 20.3%

(14/69) failed to reach even ACRPed30 score. Note that all children who reach ACRPed70 automatically also reach ACRPed30 and ACRPed50, while those who achieve ACRPed50 also achieve ACRPed30. Patients' gender, age at disease onset, age at methotrexate start, disease subtype, disease duration at methotrexate start, methotrexate administration route and dose did not have a significant effect on response to therapy evaluated as ACRPed score (Supplementary Table 1).

JADAS score for disease activity

Disease activity at the start of treatment with methotrexate, evaluated with the JADAS score, indicated a median value of 18.3 (range 3.0 – 49.8); after 6 months of treatment with methotrexate a pronounced reduction in the disease activity was achieved (p-value = 3.0×10^{-16}, linear model), with a median value of 5.3 (range 0 – 36.8).

Genotyping

SNPs genotyped in this cohort, rs2372536 in ATIC, rs1127354 in ITPA and rs1051266 in SLC19A1 were characterized in all patients (69/69). All polymorphisms considered follow Hardy-Weinberg equilibrium and their frequency is in agreement with the distribution of these SNPs in the Caucasian population, with minor allele frequency of 37.7%, 4.3% and 49.3% respectively for ATIC rs2372536 (C>G), ITPA rs1127354 (C>A) and SLC19A1 rs1051266 (A>G).

Clinical response and genotyping

Clinical remission

Considering clinical remission, results for significant associations are shown in Table 2. ATIC rs2372536 presented a significant effect (p-value adjusted for multiple testing 0.0090, logistic regression): homozygous variant G genotype was more frequent in patients achieving clinical remission (31% in patients with clinical remission vs 5% in patients with no clinical remission, odds ratio 9.11, 95% C.I. 1.76 – 47.23). ITPA rs1127354 also presented a significant effect (p-value adjusted for multiple testing = 0.028, logistic regression): no patient in clinical remission presented a variant CA or AA genotype, while these variants were present in 14.1% of patients that did not reach clinical remission. Multivariate logistic regression confirmed independent effects for SNPs ATIC rs2372536 and ITPA rs1127354 in terms of their association with response to methotrexate evaluated as induction of clinical remission (adjusted p-value respectively 0.0030 and 0.031, logistic regression).

ACRPed scores

Trends for an association with improved response evaluated as ACRPed70score, evaluated at 6 months, was identified for SLC19A1 rs1051266 and ATIC rs2372536, however these tendencies were not significant after adjusting for multiple testing (Table 3). No significant effect was identified for the variant in ITPA on response to methotrexate in terms of ACRPed70 score (Table 3).

JADAS scores

Considering disease activity evaluated by JADAS score at the start of treatment with methotrexate and after 6 months of therapy, SLC19A1 rs1051266 SNP demonstrated statistically significant effects (Figure 1). Patients with a variant GG genotype for SLC19A1 rs1051266 presented higher JADAS scores after 6 months of therapy in comparison to

patients with either AA or AG genotypes (p-value adjusted for multiple testing = 0.012, linear model). SLC19A1 rs1051266 had no significant effect on JADAS score at the beginning of methotrexate therapy. Analysis of the JADAS score after 6 months of therapy, adjusted for baseline JADAS score, showed that both baseline JADAS score and SLC19A1 genotype had a significant association with JADAS score after 6 months of therapy (Figure 1, p-value adjusted for multiple testing respectively < 0.0001 e 0.036, linear models).

No significant effect of the ATIC and ITPA SNPs considered on JADAS score was identified (Supplementary Figures 1 and 2).

ITPA activity

ITPA activity was measured successfully in erythrocytes from 62/73 patients. As expected, a highly significant association of the enzymatic activity with SNP rs1127354 in ITPA was detected, so that the variant A allele additively induced a reduction in the enzyme activity: indeed ITPA activity was 162.7, 52.4 and 0.75 U among patients with CC, CA and AA genotype respectively (Figure 2, p-value = 1.0×10^{-5}, linear model). ITPA activity was not associated with patients' gender, age, disease subtype. Moreover, no association of ITPA activity was detected with methotrexate dose or clinical response to methotrexate, measured either with ACRPed score or as clinical remission. However, all 9 patients with low ITPA activity (<92 U, highest value observed in patients with variant ITPA genotype) did not achieve clinical remission, while frequency of remission was 43.4% (23/53) among patients with ITPA activity higher than 92 U (Table 4, p-value = 0.0024, logistic regression).

Discussion

Polymorphisms in genes encoding for enzymes involved in methotrexate pharmacokinetics and pharmacodynamics have been associated with drug response. Recent studies have evaluated the effect of various candidate variants in adults with rheumatoid arthritis and children with JIA [8, 10, 17, 18]. Hinks et al. have characterized genetic variability in 13 candidate genes involved in methotrexate pharmacokinetic and pharmacodynamic pathways, using for each gene the tagSNPs, selected on the basis of the haplotype map [8]. This study considered two large cohorts of patients with JIA, one from UK and one from US. Results obtained have shown that two SNPs in the ATIC gene and one SNP within ITPA were significantly associated with methotrexate response in the discovery cohort from UK. One of the SNPs in ATIC, an intronic variant (rs12995526), showed a trend with association even in the validation cohort from US. Another study by De Rotte et al. identified an association between SLC19A1 rs1051266 and response to methotrexate in 287 patients with JIA studied longitudinally [10]. Moreover, two recent studies in adult patients with rheumatoid arthritis have used a similar approach [17, 18]. On these bases, we studied the effects of the main functional variant of ATIC, ITPA and SLC19A1 (respectively rs2372536, rs1127354 and rs1051266) on response to methotrexate in a cohort of Italian patients with JIA. ATIC and ITPA are two genes encoding enzymes involved in purines biosynthesis and metabolism, while SLC19A1 (known also as reduced folate carrier 1) is a transporter responsible for methotrexate influx in cells [6]. Our study considered a cohort of 69 patients, which constitutes all consecutive patients with JIA that have been treated with methotrexate at Burlo Garofolo Children's Hospital from 2000 to 2013.

Results show that frequency of response to methotrexate evaluated in this study is similar to that reported in the literature [1, 22]. As expected, the majority of patients enrolled are

254 females; however, demographic, clinical and pharmacological covariates had no significant
255 effects on response to therapy. In particular, route of methotrexate administration had no
256 significant effect on response to the medication; this observation is in agreement with
257 recent reports [10, 23].
258 As far as the effect of the candidate genotypes considered is concerned, our study showed a
259 significant effect of the functional variant in ATIC (rs2372536): variant GG genotype was
260 associated with better response in terms of clinical remission (odds ratio 9.1), with trends for
261 an effect on ACRPed70 (Table 2) and ACRPed30 scores (Supplementary Table 2). Even the
262 functional variant in ITPA (rs1127354) presented an effect on response to methotrexate:
263 variant A allele had a lower percentage of clinical remission in comparison to wild type C
264 allele. Multivariate analysis supported the view that the significant effects of ATIC and ITPA
265 variants on clinical remission were independent. Genetic polymorphisms of ATIC and ITPA
266 may impact MTX response independently since these enzymes are involved in different,
267 though interconnected, enzymatic pathways in cells (i.e., respectively de novo synthesis and
268 salvage pathways for purines) [18].
269 Previous reports in the literature investigated the pharmacogenetics of methotrexate in JIA
270 [8, 10, 11, 24]. Our study is in agreement with previous studies that consider efficacy of
271 methotrexate in patients with rheumatoid arthritis, indicating therefore that the ATIC
272 rs2372536 GG genotype may be associated with improved response even in children with JIA
273 [9, 17, 25]. This variant likely influences methotrexate efficacy since it predisposes the ATIC
274 enzyme to the inhibition induced by the methotrexate active metabolites [26], which results
275 in a more pronounced reduction of de novo purine synthesis and increased adenosine
276 release, the main molecular mechanisms underlying methotrexate efficacy in JIA [9].

A recent study by De Rotte et al. identified an association between SLC19A1 rs1051266 and response to methotrexate in 287 patients with JIA studied longitudinally [10]. In our study the SLC19A1 variant rs1051266 was not associated with response to methotrexate evaluated as clinical remission. However, a trend was present for ACRPed70 score (not significant after adjustment for multiple testing as in the study by De Rotte et al.); this observation is in agreement with many other studies reported in the literature, describing a controversial association of this variant with response to methotrexate in JIA and rheumatoid arthritis [8, 19, 27-31]. Interestingly, in our patients' population there seem to be an association of this SNP with the change in JADAS score between methotrexate start and after 6 months of therapy: patients homozygous for the variant GG genotype displayed a reduced improvement in JADAS score, in comparison with patients with either AA or AG genotype. However, this effect was not associated with modifications of the clinical remission rate. This is the first report about an effect of SCL19A1 rs1051266 genotype on JADAS score change and should be validated by other studies; moreover, clinical relevance of this observation may be limited, since this effect did not modify clinical remission induced by methotrexate [15].

Distribution of ITPA activity measured in erythrocytes of JIA patients enrolled in this study and its association with ITPA rs1127354 variant are consistent with previous reports in healthy subjects [20, 32]. To note, especially for the rs1127354 CC genotype, there is a large amount of variation in levels of enzymatic activity between individuals. This observation is consistent with previous reports in the literature [20] and may be related to the effect of genetic polymorphisms in genes different from ITPA on its enzymatic activity ("trans effect"), as it has been shown recently for TPMT [33]. Although average ITPA activity was not different between responders and non responders, low activity of ITPA measured in

301 erythrocytes was associated with reduced methotrexate response in this study, confirming
302 the role of ITPA rs1127354 variant; indeed even patients with normal ITPA genotype and low
303 ITPA activity, comparable to that measured in patients with variant ITPA, did not respond to
304 therapy. This observation and the role of ITPA activity on methotrexate response need to be
305 further evaluated by larger prospective studies, possibly considering even ITPA gene
306 expression [13, 34].
307 This is the first report considering the pharmacogenetics of response to methotrexate in JIA
308 in terms of clinical remission. This clinical phenotype may be more relevant to describe the
309 benefit induced by the treatment and to guide patient care, in comparison to the ACRPed
310 score or changes in JADAS score, since it represents a longer period in which the patient
311 does not present signs of disease activity, including uveitis, the most significant complication
312 of JIA [15].
313 Identification of patients who are likely to respond to methotrexate before treatment in JIA
314 would be very useful for the clinician and our study supports the development of multilocus
315 pharmacogenetic signatures to predict response to methotrexate in these patients.
316 Genotyping should be performed at diagnosis and patients with a genotype predisposing to
317 response, such as the ATIC rs2372536 GG genotype, should be treated with methotrexate,
318 given the high probability of response to this treatment. This study provides a rational for
319 reserving biologics to patients that will likely not benefit from less expensive but still
320 effective treatments such as methotrexate. On the contrary, patients with variants
321 associated with lack of efficacy of methotrexate (such as ITPA rs1127354 A allele and in
322 general those with low ITPA activity), should be switched more rapidly to a more aggressive
323 treatment (i.e., methotrexate + biologics or biologics alone). Methotrexate remains however
324 the first line treatment in children with JIA requiring DMARDs..

We acknowledge that the associations described in this study about explored genetic variants and different indicators of clinical response are heterogeneous: this is likely secondary to the relatively small number of patients recruited. The results therefore have to be interpreted as preliminary and need further confirmatory studies on larger cohort of patients.

A key limitation of this study is the lack of a validation cohort supporting its findings, limiting at this point the extensibility of this observation to the general population. Moreover, given the paucity of studies that comprehensively fine mapped candidate genes to identify the causal variants in each or genome-wide association studies into MTX response in JIA or RA, it is possible that additional genetic effects will be contained within other genomic regions not yet investigated [12]. If the results described in the present study will be validated by larger prospective trials, application of pharmacogenetic guided treatment of JIA may allow rationalization and reduction of costs associated with care, by directing and personalizing the use of methotrexate and biologics.

Acknowledgments

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Table 1: Demographics of patients with juvenile idiopathic arthritis (JIA)			
	Overall	Remission	No Remission
Number of cases	69	26	43
JIA subtype at the beginning of therapy with methotrexate			
Oligoarticular persistent	37 (54%)	15 (58%)	22 (51%)
Oligoarticular extended	7 (10%)	2 (2%)	5 (12%)
Polyarticular RF - *	23 (33%)	9 (35%)	14 (32%)
Enthesitis related arthritis	1 (1%)	0	1 (2%)
Psoriatic	1 (1%)	0	1 (2%)
Female	53 (77%)	21 (81%)	53 (77%)
Age at disease onset, median and range (years)	3, 1 – 16	3.5, 1 - 13	3, 1 – 16
Age at the start of methotrexate, median and range (years)	8, 1 – 22	9, 2 - 22	8, 1 – 19
Disease duration at the start of methotrexate, median and range (years)	1, 0 – 19	1, 0 - 19	1, 0 – 12
Physician's global assessment of disease activity, VAS score	6, 2 – 10	6.5, 2 - 9	6, 2 – 10
Patient/parent 's global assessment of disease activity, VAS score	7, 0 – 10	7, 0 - 10	6, 2- 10
CHAQ	0.6, 0 – 3	0.55,0-2.9	0.7, 0 – 3

Median and range of active joints at start of methotrexate	3, 0 – 26 **	2.5, 0 -16**	3, 1 – 26
Median and range of restricted joints at start of methotrexate	2, 0 – 28	2, 0 - 22	2, 0 -28
ESR (mm/h)	41, 2-120	40.5,2-106	45, 2-120
Administration route (subcutaneous vs oral)	43 (62%)	17 (65%)	26 (60%)
Median and range of methotrexate dose (mg/m^2)	15, 10 – 20	15,10-20	15,10-20
Concomitant treatment			
Intra-articular glucocorticoid	44 (64%)	20 (77%)	24 (56%)
NSAIDs	66 (96%)	23 (88%)	43 (100%)
Oral glucocorticoid	41 (59%)	14 (54%)	27 (63%)

CHAQ: Childhood Health Assessment Questionnaire; ESR: erythrocyte sedimentation rate; NSAIDs: non-steroidal anti-inflammatory drug; VAS: visual analogue scale.

*: no patient with polyarticular RF+ subset of disease was found.

**: one patient with 0 active joints but affected by dry synovitis (subset of RF- polyarticular JIA) with important stiffness and 8 joints with limitation of motion.

Table 2: Clinical response evaluated as remission and the considered genotypes in 69 patients with JIA. Analysis were performed according to a recessive inheritance model.

SNP	Gene	WT+het/var	Genotype Frequency Remission n = 26	Genotype Frequency No Remission n = 43	p-value	p-value adjusted	Odds ratio (95% CI)
rs2372536	ATIC	CC+CG/GG	0.69/0.31	0.95/0.05	0.0030	0.0090	9.11 (1.76-47.2)
rs1127354	ITPA	CC/CA+AA*	1/0	0.86/0.14	0.014	0.028	0.17 (0.012-2.53)
rs1051266	SLC19A1	AA+AG/GG	0.81/0.19	0.74/0.26	0.54	0.54	0.69 (0.21-2.28)

*: for ITPA rs1127354, analysis was done by grouping heterozygous CA patients with homozygous variant AA, given the low frequency of homozygous patients (1 in 69 patients) and the strong functional effect of the variant allele even in its heterozygous form (see Figure 2). P-values are from logistic regression and adjustment for multiple testing was done using Holm's method. CI: confidence interval; WT: wild-type; het: heterozygous; var = variant.

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Table 3: Clinical response evaluated as ACRPed70 score and the considered genotypes in 69 patients with JIA. Analysis were performed according to a recessive inheritance model.

SNP	Gene	WT+het/var	Genotype Frequency ACRPed70 n = 37	Genotype Frequency No ACRPed70 n = 32	p-value	p-value adjusted	Odds ratio (95% CI)
rs2372536	ATIC	CC+CG/GG	0.78/0.22	0.94/0.06	0.061	0.12	4.14 (0.81-21.15)
rs1127354	ITPA	CC/CA+AA*	0.95/0.05	0.88/0.12	0.30	0.30	0.40 (0.068-2.35)
rs1051266	SLC19A1	AA+AG/GG	0.86/0.14	0.66/0.34	0.039	0.12	0.30 (0.091-0.98)

481 *: for ITPA rs1127354, analysis was done by grouping heterozygous CA patients with
482 homozygous variant AA, given the low frequency of homozygous patients (1 in 69 patients)
483 and the strong functional effect of the variant allele even in its heterozygous form (see
484 Figure 2). P-values are from logistic regression; adjustment for multiple testing was done
485 using Holm's method. CI: confidence interval; WT: wild-type; het: heterozygous; var =
486 variant.

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Table 4: Clinical response to methotrexate evaluated as remission stable for 6 months and ITPA activity				
	Clinical response			
ITPA activity	Patients reaching remission	Non responder	Odds ratio (95% C.I.)	p-value
High (> 92 U)	23	30	14.64 (0.81–264.53)	0.0024
Low (< 92 U)	0	9		

489 One unit (U) of ITPA activity corresponds to 1 µmol IMP / g hemoglobin / hour. The cut-off
490 between patients with low and high ITPA activity was defined based on the highest value of
491 activity observed among patients with variant ITPA (i.e., 92 U, Figure 2).

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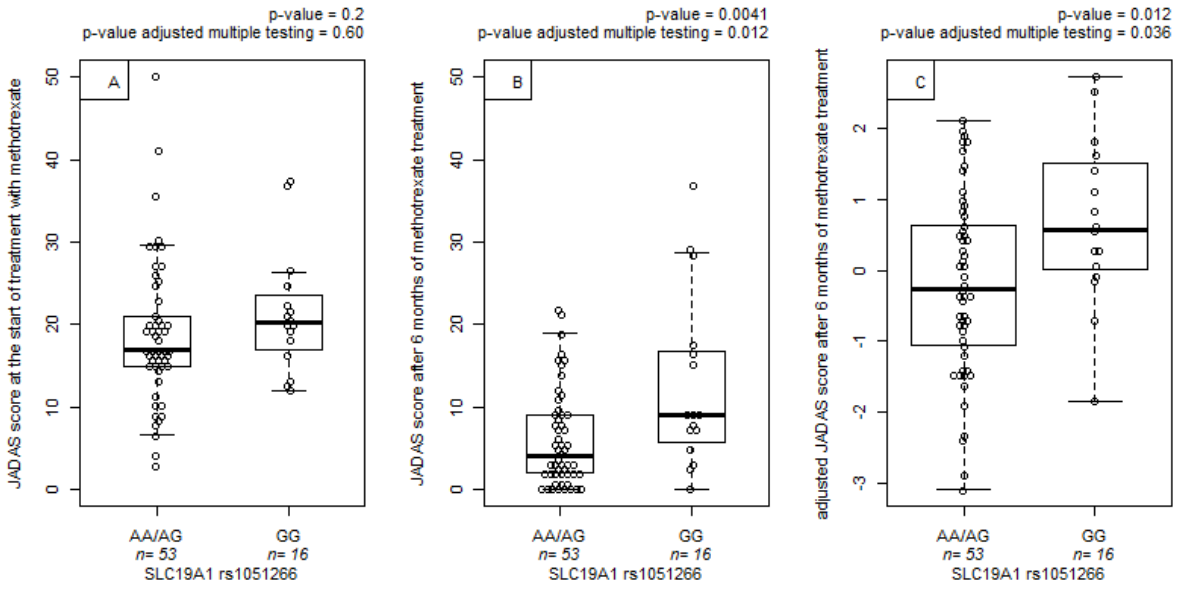

494

495 Figure 1: JADAS score before methotrexate treatment (panel A), after 6 months of therapy
496 with methotrexate (panel B), JADAS score after treatment adjusted for baseline JADAS value
497 (panel C) and SLC19A1 rs1051266 genotype. P-values are from linear models. Adjustment for
498 multiple comparison was done using Holm's method.

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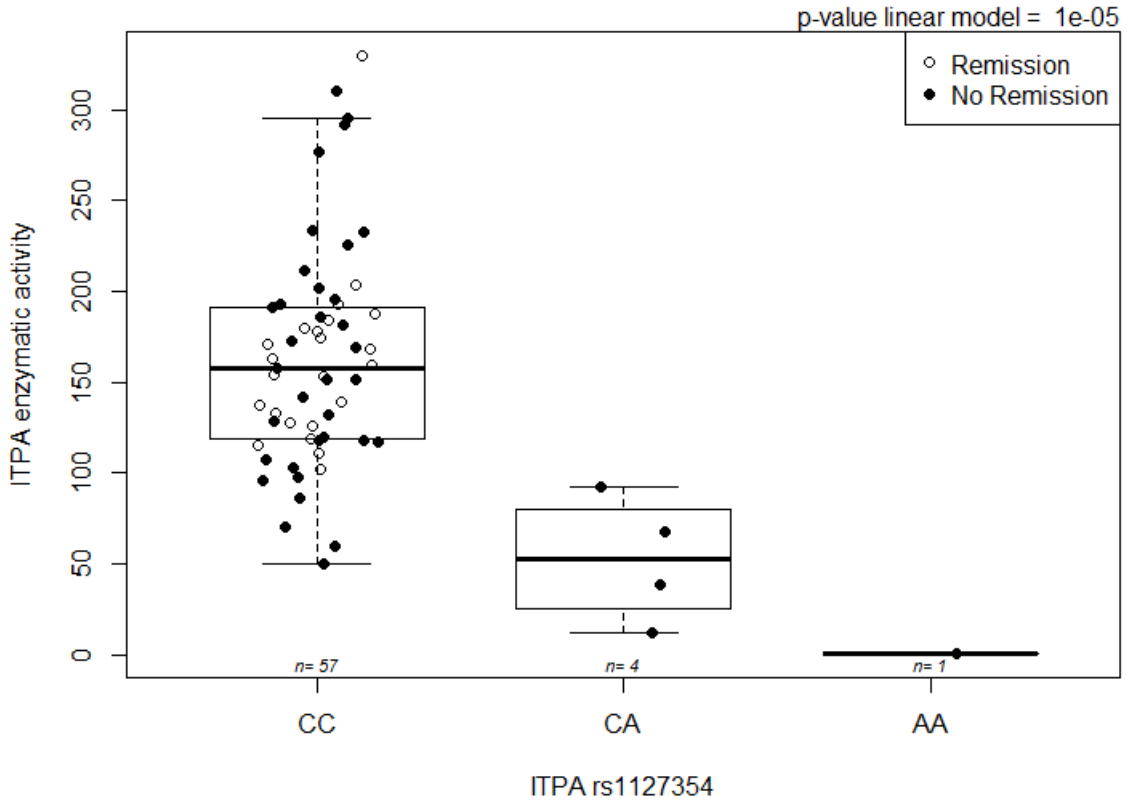

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501 Figure 2: Association between ITPA enzymatic activity in erythrocytes and ITPA rs1127354
502 SNP.

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Electronic Supplementary Material "Rheumatology International"

5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

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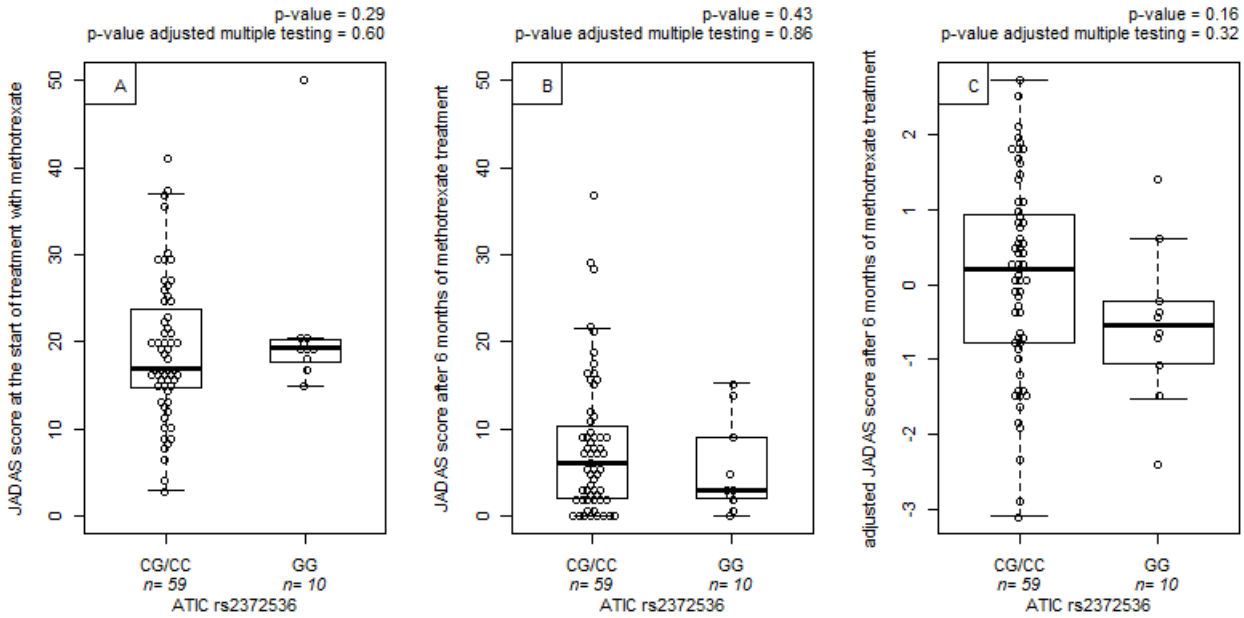
Supplementary Table 1: Demographics of patients with juvenile idiopathic arthritis (JIA) and response to methotrexate treatment (ACRPed70 vs no ACRPed70)			
	Overall	ACR70	No ACR70
Number of cases	69	37	32
JIA subtype at the beginning of therapy with methotrexate			
Oligoarticular persistent	37 (54%)	23 (62%)	14 (44%)
Oligoarticular extended	7 (10%)	1 (3%)	6 (19%)
Polyarticular RF - *	23 (33%)	13 (35%)	10 (31%)
Enthesitis related arthritis	1 (1%)	0	1 (3%)
Psoriatic	1 (1%)	0	1 (3%)
Female	53 (77%)	28 (76%)	25 (78%)
Age at disease onset, median and range (years)	3, 1 – 16	3, 1 - 16	4.5, 1 - 13
Age at the start of methotrexate, median and range (years)	8, 1 – 22	8, 1 - 22	8.5, 2– 19
Disease duration at the start of methotrexate, median and range (years)	1, 0 – 19	1, 0 - 19	1, 0 – 12
Physician's global assessment of disease activity, VAS score	6, 2 – 10	6, 2 - 10	7, 2 – 10
Patient/parent 's global assessment of disease activity, VAS score	7, 0 – 10	7, 0 - 10	6, 0 -10
CHAQ	0.6, 0 – 3	0.7, 0 - 3	0.6, 0 -3
Median and range of active joints at start of methotrexate	3, 0 – 26 **	2, 0 - 26	3, 1 – 10
Median and range of restricted joints at start of methotrexate	2, 0 – 28	2, 0 - 28	2, 0 – 16

ESR (mm/h)	41, 2-120	39, 2-120	45, 9-114
Administration route (subcutaneous vs oral)	43 (62%)	24 (65%)	19 (59%)
Median and range of methotrexate dose (mg/m^2)	15, 10 – 20	15, 10–20	15, 10–20
Concomitant treatment			
Intra-articular glucocorticoid	44 (64%)	28 (76%)	16 (50%)
FANS	66 (96%)	34 (92%)	32 (100%)
Oral glucocorticoid	41 (59%)	22 (59%)	19 (59%)

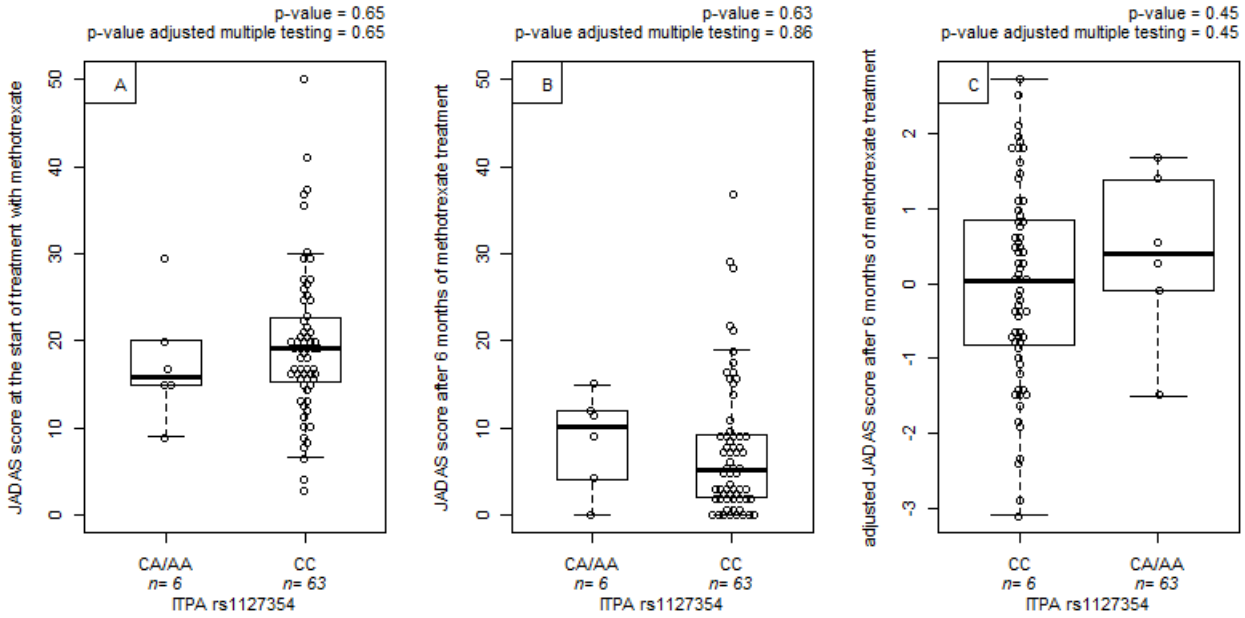
Supplementary Table 2: Clinical response evaluated as ACR30Ped score and the considered genotypes in 69 patients with JIA. Analysis were performed according to a recessive inheritance model.

SNP	Gene	WT+het/var	Genotype Frequency ACRPed30 n = 55	Genotype Frequency Non Responders n = 14	p-value	p-value adjusted	Odds ratio (95% CI)
rs2372536	ATIC	CC+CG/GG	1/0	0.82/0.18	0.026	0.080	5.32 (0.34-82.75)
rs1127354	ITPA	CC/CA+AA*	0.93/0.07	0.86/0.14	0.43	0.86	0.47 (0.077-2.88)
rs1051266	SLC19A1	AA+AG/GG	0.76/0.24	0.79/0.21	0.86	0.86	1.14 (0.27-4.70)

*: for ITPA rs1127354, analysis was done by grouping heterozygous CA patients with homozygous variant AA, given the low frequency of homozygous patients (1 in 69 patients) and the strong functional effect of the variant allele even in its heterozygous form (see Figure 2). P-values are from logistic regression; adjustment for multiple testing was done using Holm's method. CI: confidence interval; WT: wild-type; het: heterozygous; var = variant.



Supplementary Figure 1: JADAS score before (panel A), after 6 months of therapy with methotrexate (panel B) and JADAS score after treatment adjusted for baseline JADAS value (panel C) and ATIC rs2372536 genotype. P-values are from linear models. Adjustment for multiple comparison was done using Holm's method.



Supplementary Figure 2: JADAS score before (panel A), after 6 months of therapy with methotrexate (panel B) and JADAS score after treatment adjusted for baseline JADAS value (panel C) and ITPA rs1127354 genotype. P-values are from linear models. Adjustment for multiple comparison was done using Holm's method.

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Corresponding Author's Name: Gabriele Stocco

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Gabriele
2. Surname (Last Name): Stocco
3. Date: 30-May-2014

4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title
5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

6. Manuscript Identifying Number (if you know it)

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

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Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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Dr. Stocco has nothing to disclose.

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Section 1. Identifying Information

1. Given Name (First Name): Valentina
2. Surname (Last Name): Moressa
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No
Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

6. Manuscript Identifying Number (if you know it)
RHEI-D-14-00435R1

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Dr. Moressa has nothing to disclose.

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Section 1. Identifying Information

1. Given Name (First Name): Luigi
2. Surname (Last Name): Zandonà
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No
Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
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Dr. Zandonà has nothing to disclose.

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Diego
2. Surname (Last Name): Favretto
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No
Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
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Section 1. Identifying Information

1. Given Name (First Name): Noelia
2. Surname (Last Name): Malusà
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
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1. Given Name (First Name): Giuliana
2. Surname (Last Name): Decorti
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title: 5-aminoimidazole-4-carboxamide ribonucleotide-transformylase and inosine-triphosphate-pyrophosphatase genes variants predict remission rate during methotrexate therapy in patients with juvenile idiopathic arthritis

6. Manuscript Identifying Number (if you know it): RHEI-D-14-00435R1

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 3. Relevant financial activities outside the submitted work.

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1. Identifying information.

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1. Given Name (First Name): Loredana
2. Surname (Last Name): Lepore
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No
Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
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1. Given Name (First Name): Alessandro
2. Surname (Last Name): Ventura
3. Date: 28-August-2014

4. Are you the corresponding author? ☐ Yes ☑ No
Corresponding Author's Name: Gabriele Stocco

5. Manuscript Title
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