

## VARIANTS IN TPMT, ITPA, ABCC4 AND ABCB1 GENES AS PREDICTORS OF 6-MERCAPTOPYRINE INDUCED TOXICITY IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

POLIMORFIZMI U GENIMA TPMT, ITPA, ABCC4 I ABCB1 KAO PREDIKTORI TOKSIČNOSTI UZROKOVANE PRIMENOM 6-MERKAPTOPYRINA KOD DECE OBOLELE OD AKUTNE LIMFOBLASTNE LEUKEMIJE

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### Summary

**Background:** Acute lymphoblastic leukemia is the most common childhood malignancy. Optimal use of antileukemic drugs has led to less toxicity and adverse reactions, and a higher survival rate. Thiopurine drugs, including 6-mercaptopurine, are mostly used as antileukemic medications in the maintenance phase of treatment for children with acute lymphoblastic leukemia. For those patients, TPMT genotype-tailored 6-mercaptopurine therapy is already implemented in the treatment protocols. We investigated the role of TPMT, ITPA, ABCC4 and ABCB1 genetic variants as predictors of outcome and 6-mercaptopurine induced toxicity during the maintenance phase of treatment in pediatric acute lymphoblastic leukemia.

**Methods:** Sixty-eight children with acute lymphoblastic leukemia were enrolled in this study. Patients have been treated according to ALL IC-BFM 2002 or ALL IC-BFM 2009 protocols. Toxicity and adverse events have been monitored via surrogate markers (off-therapy weeks, episodes of leukopenia and average 6-mercaptopurine dose) and a prob-

### Kratak sadržaj

**Uvod:** Akutna limfoblastna leukemija je najčešća maligna bolest dečjeg doba. Optimalna upotreba antileukemijskih lekova dovela je do smanjenja toksičnosti i neželjenih događaja, kao i do povećanja stope preživljavanja. Tiopurinski lekovi, uključujući 6-merkaptopurin, predstavljaju najčešće korišćene antileukemijske lekove u lečenju dece obolele od akutne limfoblastne leukemije, koji se koriste u toku faze održavanja. Personalizovana terapija 6-merkaptopurinom, prilagođena TPMT genotipu svakog pacijenta, već je implementirana u terapijske protokole. Cilj ove studije je bio da ispita ulogu varijanti u genima TPMT, ITPA, ABCC4 i ABCB1 kao prediktora ishoda i toksičnosti uzrokovane 6-merkaptopurinom u toku faze održavanja u lečenju pedijatrijske akutne limfoblastne leukemije.

**Metode:** U studiju je bilo uključeno 68 dece obolele od akutne limfoblastne leukemije. Pacijenti su lečeni primenom ALL IC-BFM 2002 ili ALL IC-BFM 2009 protokola. Toksičnost i neželjeni događaji su posmatrani i beleženi upotrebom surogat markera (broj nedelja bez terapije, broj

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*List of abbreviations:* ALL, acute lymphoblastic leukemia; 6-MP, 6-mercaptopurine; TPMT, thiopurine S-methyltransferase; ITPA, inosine triphosphate pyrophosphatase; MRP4, Multidrug Resistance Protein 4; ABCC4, ATP-binding cassette sub-family C member 4; MDR1, Multidrug Resistance 1; ABCB1, ATP-binding cassette sub-family B member 1; PCR-RFLP, polymerase chain reaction – Restriction Fragment Length Polymorphism; ROC, receiver operating characteristic; AUC, area under the curve; 6-TG, 6-thioguanine; SNPs, single-nucleotide polymorphisms; ATP, adenosine triphosphate; GWAS, genome-wide association study, SR, standard risk; IR, intermediate risk; HR, high risk; WBC, white blood cell; bp, base pair; ANOVA, analysis of variance.

abilistic model was employed to predict overall 6-mercaptopurine related toxicity.

**Results:** We confirmed that patients with acute lymphoblastic leukemia that carry inactive *TPMT* allele(s) require 6-mercaptopurine dose reduction. *ITPA* and *ABCC4* genetic variants failed to show an association with 6-mercaptopurine induced toxicity during the maintenance phase. Carriers of *ABCB1* variant allele experienced greater hepatotoxicity. The probabilistic model Neural net which considered all the analysed genetic variants was assessed to be the best prediction model. It was able to discriminate ALL patients with good and poor 6-mercaptopurine tolerance in 71% of cases (AUC=0.71).

**Conclusions:** This study contributes to the design of a panel of pharmacogenetic markers for predicting thiopurine-induced toxicity in pediatric ALL.

**Keywords:** childhood acute lymphoblastic leukemia (ALL), 6-mercaptopurine (6-MP), *TPMT*, *ITPA*, *ABCC4*, *ABCB1*

## Introduction

Acute lymphoblastic leukemia (ALL) is a malignant clonal disease of the hematopoietic tissue. It is the most common cancer in childhood, accounting for approximately 30% of all pediatric malignancies. The optimal use of known antileukemic drugs in the context of multicentric clinical trials and improvements in the field of supportive therapy have led to higher survival rates (1).

Thiopurine drugs are mostly used as immunosuppressive and anticancer therapy for hematological malignancies, inflammatory bowel disease and renal transplant patients. Excessive accumulation of their active metabolites inside hematopoietic cells leads to increased toxicity. On the contrary, increased efflux of active thiopurine metabolites can lead to diminished efficacy of the therapy (2). 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are key immunosuppressive thiopurine medications in the therapy protocols for childhood ALL. The role of 6-MP is especially important during the maintenance phase of treatment (1).

Single nucleotide polymorphisms (SNPs) in the genes encoding proteins involved in 6-MP metabolism, cellular transport and mechanism of action can influence drug efficacy and toxicity (Figure 1) (3). Thiopurine S-methyltransferase (*TPMT*) is an important enzyme that inactivates thiopurine drugs. It is well known that individuals carrying two inactive *TPMT* alleles (homozygous *TPMT* deficiency) experience severe myelosuppression when treated with conventional doses of thiopurine drugs. Heterozygous deficient *TPMT* carriers (one inactive *TPMT* allele) show moderate to severe myelosuppression in contrast to homozygous carriers of the wild-type *TPMT* allele (*TPMT*\*1) and have a lower risk of myelosuppression when on conventional 6-MP doses (4). SNPs

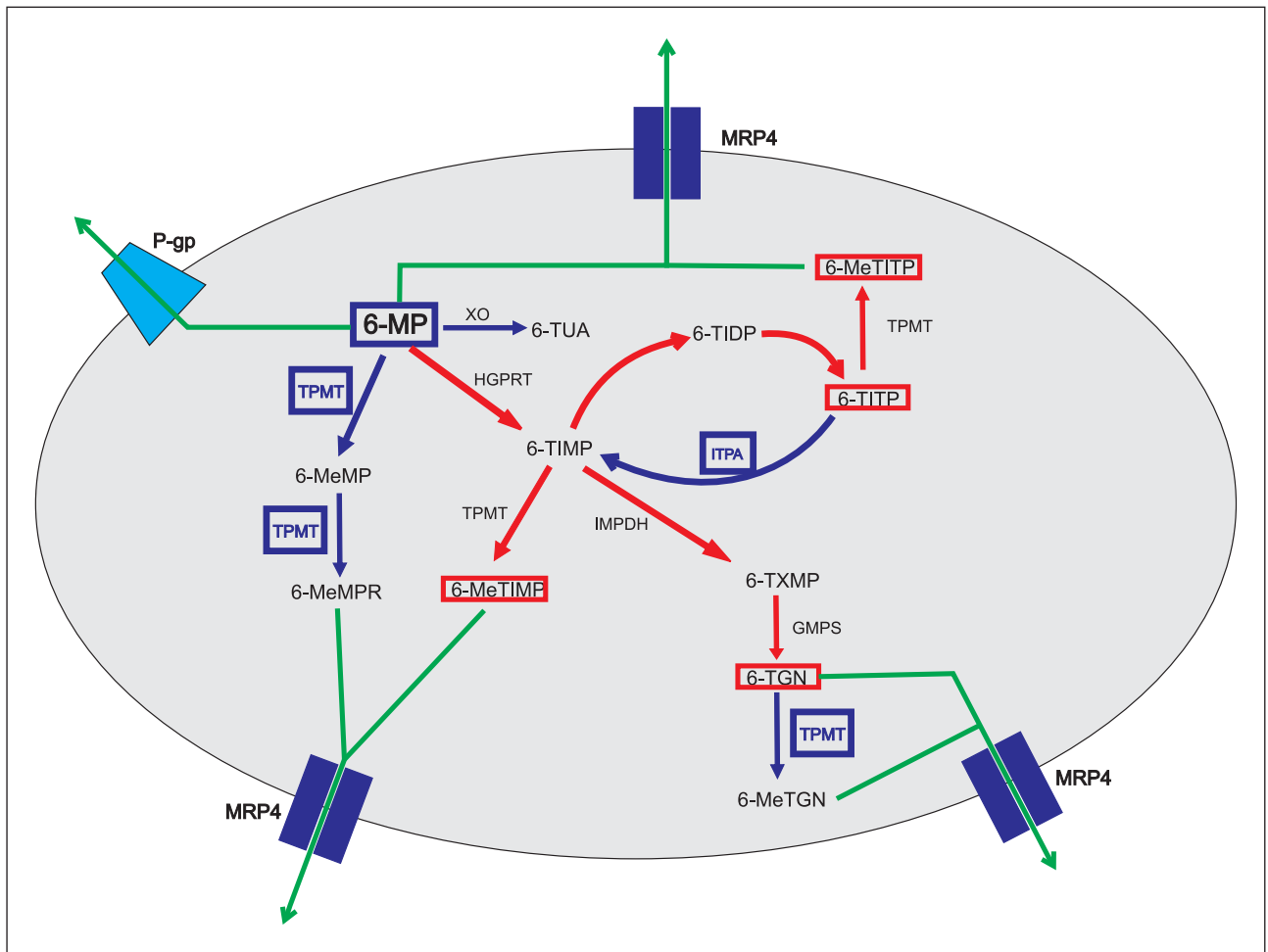
epizoda leukopenije i prosečna doza 6-merkaptopurina) a probabilistički modeli su korišćeni za predikciju ukupne toksičnosti uzrokovane primenom 6-merkaptopurina.

**Rezultati:** Studija je potvrdila da pacijenti oboleli od akutne limfoblastne leukemije koji imaju neaktivni *TPMT* alel zahtejavaju redukciju doze 6-merkaptopurina. Varijante u genima *ITPA* i *ABCC4* nisu bile povezane sa toksičnošću koja je uzrokovana primenom 6-merkaptopurina u toku faze održavanja. Nosioci varijante u genu *ABCB1* su ispoljili veću hepatotoksičnost. Probabilistički model *Neural net* kojim su posmatrane sve analizirane genske varijante se pokazao kao najbolji predikcioni model. Primenom ovog modela bilo je moguće razlikovati ALL pacijente sa lošom i dobrom tolerancijom 6-merkaptopurina u 71% slučajeva (AUC=0,71).

**Zaključak:** Ova studija doprinosi dizajniranju panela farmakogenetskih markera koji bi se koristili za predikciju toksičnosti uzrokovane primenom tiopurinskih lekova kod dece obolele od akutne limfoblastne leukemije.

**Ključne reči:** akutna limfoblastna leukemija (ALL), 6-merkaptopurin (6-MP), *TPMT*, *ITPA*, *ABCC4*, *ABCB1*

in the inosine triphosphate pyrophosphatase (*ITPA*) gene, which modulates the ITPase activity, have been shown to influence the accumulation of the above-mentioned toxic thiopurine metabolites. Thus, carriers of these SNPs are prone to severe myelosuppression (5–8). The Multidrug Resistance Protein 4 (*MRP4*) belongs to the family of ATP-binding cassette transporters and serves as a transmembrane efflux-pump for various organic anions. The physiological role of *MRP4* protein includes the detoxification of drugs and various endogenous toxic molecules (7). *MRP4* protein exports methylated 6-MP nucleotides – among other molecules – from cells, thus protecting them from 6-MP toxicity. The *ABCC4* (ATP-binding cassette sub-family C member 4) gene that encodes for *MRP4* protein is highly polymorphic, with more than 20 missense genetic variants. Less functional *ABCC4* alleles may be responsible for more severe myelosuppression during 6-MP treatment. Furthermore, more active *ABCC4* alleles could be responsible for 6-MP resistance (9). Notably, nucleotide T in *ABCC4* c.\*1372 T>G has been pointed-out by Lopez et al. (10) in a GWAS study as the genetic variant that results in decreased *ABCC4* expression and thus, 6-MP induced toxicity. The ATP-binding cassette sub-family B member 1 (*ABCB1*) gene encodes for P-glycoprotein, an ATP-dependent transmembrane efflux-pump. It transports xenobiotics out of cells, but also prevents the entry of toxic metabolites (11). Some tumours that are resistant to chemotherapy have *ABCB1* overexpressed. In more than half of the relapsed acute myeloid leukemia incidents, P-glycoprotein is more active than in the non-relapsed cases, thus making it a suitable candidate marker of poor prognosis. P-glycoprotein serves as an efflux-pump for various chemotherapeutics used in the treatment of childhood ALL, such as doxorubicin, etoposide, vincristine, and 6-MP (12). The overexpression of *ABCB1* could



**Figure 1** Schematic representation of 6-mercaptopurine metabolism.

Blue lines represent inactivation pathways; red lines represent pathways that generate active metabolites; green lines represent elimination (efflux) out of the cell. 6-MP: 6-mercaptopurine, XO: xanthine oxidase, 6-TUA: 6-thiouric acid, TPMT: thiopurine S methyltransferase, 6-MeMP: 6-methylmercaptopurine, 6-MeMPR: 6-methylmercaptopurine ribonucleotide, HGPRT: hypoxanthine phosphoribosyl transferase, 6-TIMP: 6-thioinosine 5'-monophosphate, 6-TIDP: 6-thioinosine 5'-diphosphate, 6-TITP: 6-thioinosine 5'-triphosphate, 6-MeTITP: 6-methylthioinosine 5'-triphosphate, ITPA: inosine triphosphate pyrophosphatase, 6-MeTIMP: 6-methylthioinosine 5'-monophosphate, IMPDH: inosine monophosphate dehydrogenase, 6-TXMP: 6-thioxanthosine 5'-monophosphate, GMPS: guanosine monophosphate synthetase, 6-TGN: 6-thioguanine nucleotide, 6-MeTGN: 6-methylthioguanine nucleotide, MRP4: multidrug resistance protein 4, P-gp: P-glycoprotein.

be responsible for therapy failure or relapse (13) while the reduced activity of P-glycoprotein can lead to more severe 6-MP toxicity.

Herein, we aim to explore the role of *TPMT*, *ITPA*, *ABCC4* and *ABCB1* genetic variants in the occurrence and intensity of 6-MP induced toxicity during the maintenance phase of childhood ALL treatment.

## Materials and Methods

Sixty-eight children with ALL, diagnosed and treated at University Children's Hospital, Belgrade in the period between January 2003 and January 2013

were enrolled in this study. Informed consent has been obtained from parents or legal guardians. The study has been approved by the Ethics Committee of University Children's Hospital, University of Belgrade. Children have been treated according to either ALL IC-BFM 2002 or ALL IC-BFM 2009 protocols, depending on the time of diagnosis: those diagnosed before April 1, 2010 were treated according to the ALL IC BFM 2002 protocol, whereas those diagnosed after that date were treated according to the currently used protocol (14). Patients were classified into risk groups based on their risk for relapse (standard risk group – SR, intermediate risk group – IR and high risk group – HR) according to the current protocol. All patients have received maintenance therapy that

included daily oral 6-MP (50 mg/m<sup>2</sup>) and weekly oral methotrexate (20 mg/m<sup>2</sup>). The maintenance phase lasted between 13 and 17 months (60–77 weeks). The target white blood cell (WBC) count was monitored to be between 2.0 and 3.0 × 10<sup>9</sup>/L. Patients had regular check-ups every 2 weeks during the entire maintenance phase. If the WBC count fell below 2.0 × 10<sup>9</sup>/L, the 6-MP dose was reduced in 25% weakly increments. Instead, if the WBC count increased above 3.0 × 10<sup>9</sup>/L, the 6-MP dose was also increased in 25% weakly increments. If the WBC count was below 1.0 × 10<sup>9</sup>/L, 6-MP administration was discontinued, until WBC recovery. The *TPMT* genotype was determined for all patients before the beginning of the maintenance therapy and 6-MP doses were adjusted accordingly (4, 15). Toxicity and adverse events during 6-MP treatment have been monitored using surrogate markers (off-therapy weeks, episodes of leukopenia and average dose, calculated considering 50 mg/m<sup>2</sup> as 100%). Side effects, such as hepatotoxicity (elevated levels of transaminases), rash, acute pancreatitis and flu-like symptoms, have also been recorded for all patients.

The analysis of *TPMT* c.460G>A (rs1800460), *TPMT* c.719A>G (rs1142345), *ITPA* c.94C>A (rs1127354), *ABCC4* c.\*1372T>G (rs9516519) and *ABCB1* c.2677G>T (rs2032582) was performed by PCR-RFLP in the Laboratory for Molecular Biomedicine, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade. In particular, the *TPMT*, *ITPA* and *ABCB1* genetic variants of interest were analysed as described previously (18). The region of *ABCC4* gene that contains c.\*1372 T>G was amplified using 5'-GCTTTTTAAGGCT-TCACTCAAT-AAAACAGC-3' as the forward primer and 5'-GTGTC-ACCTCCCTGAAATTGC-3' (reverse primer). Restriction digestion with *PvuII* enzyme followed by 3% agarose gel electrophoresis was used to distinguish T nucleotide (118 + 29 bp) from G nucleotide carriers (147 bp). Since both *TPMT* c.460 G>A and *TPMT* c.719 A>G cause major decreases in *TPMT* enzyme activity, presence of any or both SNPs was considered to have the same effect. Genotyping for *ITPA*, *ABCC4* and *ABCB1* was performed retrospectively, using bone marrow samples taken on the day of diagnosis.

To explore any association of our genotyping findings and toxicity, Mann-Whitney test, Student t-test, Kruskal-Wallis test and ANOVA were used when appropriate.

In order to predict 6-MP induced toxicity in ALL patients based on the studied variants in *TPMT*, *ITPA*, *ABCB1* and *ABCC4* genes, probabilistic classifier models were designed using Elastic net, Neural net and Random forest machine learning algorithms. These models were used to discriminate patients whose mean dose of 6-MP was below (poor tolerance) or above average (good tolerance). Repeated 10-fold cross-validation method was applied to avoid

model overfitting. The area under the ROC curve (AUC) values were used to assess performance of the classifying models. AUC values range from 0.5 (random classifications) to 1.0 (perfect classifications) (19). R software (v.3.4.3) was used to apply predictive algorithms and measure their performance using following packages: *glmnet* (20), *nnet* (21), *randomForest* (22), *ROCR* (23), *PresenceAbsence* (24).

## Results

A total number of 68 patients with ALL have been evaluated in this study. Median age at diagnosis was 5.2 years, ranging from 11 months up to 17.6

**Table I** Patients characteristics.

|                       |          |    |
|-----------------------|----------|----|
| (No=68 patients)      |          |    |
| Age                   | Years    |    |
| Average               | 6.5      |    |
| Median                | 5.2      |    |
| Range                 | 0.9–17.6 |    |
| Gender                | N°       | %  |
| Male                  | 38       | 56 |
| Female                | 30       | 44 |
| Immunophenotype       |          |    |
| B lineage             | 58       | 85 |
| T lineage             | 10       | 15 |
| Molecular genetics    |          |    |
| Negative              | 47       | 69 |
| BCR/ABL               | 3        | 4  |
| MLL/AF4               | 2        | 3  |
| TEL/AML1              | 14       | 21 |
| E2A/PBX1              | 2        | 3  |
| Applied protocol      |          |    |
| ALL IC BFM 2002       | 39       | 57 |
| ALL IC BFM 2009       | 29       | 43 |
| Prednisolone response |          |    |
| Good response         | 61       | 90 |
| Poor response         | 7        | 10 |
| Day 33 BM             |          |    |
| M1                    | 67       | 98 |
| M2                    | 0        | 0  |
| M3                    | 1        | 2  |
| Risk group            |          |    |
| Standard risk         | 11       | 16 |
| Intermediate risk     | 47       | 69 |
| High risk             | 10       | 15 |
| Outcome               |          |    |
| CR                    | 59       | 87 |
| Relapse               | 5        | 7  |
| Death due to relapse  | 4        | 6  |

Good prednisolone response: <1000 leukoblasts per mm<sup>3</sup>, poor prednisolone response: >1000 leukoblasts per mm<sup>3</sup>, BM: bone marrow, M1:<5% leukoblasts in BM, M2: ≥5% <25% leukoblasts, M3: ≥25% leukoblasts, CR: complete remission

**Table II** Genotype frequency of genetic variants in *TPMT*, *ABCC4*, *ITPA* and *ABCB1* genes in childhood ALL patients and surrogate myelotoxicity parameters and hepatotoxicity.

| Gene                     | n (%)     | Median average dose of 6-MP % | P-value | Longer than 10% off-therapy | P-value | Average number of leukopenic episodes | P-value | Hepatotoxicity n (%) | P-value |
|--------------------------|-----------|-------------------------------|---------|-----------------------------|---------|---------------------------------------|---------|----------------------|---------|
| <i>TPMT</i>              |           |                               |         |                             |         |                                       |         |                      |         |
| <i>TPMT*1/TPMT*1</i>     | 64 (94.2) | 111.0                         | 0.003   | 10                          | 0.521   | 2.3                                   | 0.155   | 42 (65.6)            | 0.584   |
| Variant allele† carriers | 4 (5.8)   | 72.0                          |         | 0                           |         | 3.5                                   |         | 3 (75)               |         |
| <i>ABCC4</i> c.*1372T>G  |           |                               |         |                             |         |                                       |         |                      |         |
| TT – wild type           | 41 (61.0) | 104                           | 0.836†† | 7                           | 0.729†† | 2.6                                   | 0.165†† | 27 (65.8)            | 0.945†† |
| TG                       | 24 (35.0) | 95                            |         | 3                           |         | 1.8                                   |         | 16 (66.7)            |         |
| GG                       | 3 (4.0)   | 89                            |         | 0                           |         | 2                                     |         | 2 (66.7)             |         |
| <i>ITPA</i> c.94C>A      |           |                               |         |                             |         |                                       |         |                      |         |
| CC – wild type           | 63 (93.0) | 100                           | 0.814†† | 9                           | 0.560†† | 2.3                                   | 0.737†† | 40 (63.5)            | 0.159†† |
| CA                       | 5 (7.0)   | 91                            |         | 1                           |         | 2.2                                   |         | 5 (100)              |         |
| AA                       | 0         | /                             |         | /                           |         | /                                     |         | 0                    |         |
| <i>ABCB1</i> c.2677G>T   |           |                               |         |                             |         |                                       |         |                      |         |
| GG – wild type           | 29 (43.0) | 99                            | 0.843†† | 5                           | 0.733†† | 2.5                                   | 0.613†† | 15 (51.7)            | 0.030†† |
| GT                       | 26 (26.0) | 100                           |         | 2                           |         | 2.5                                   |         | 21 (80.7)            |         |
| TT                       | 13 (19.0) | 95                            |         | 3                           |         | 1.5                                   |         | 9 (69.2)             |         |

† Detected *TPMT* variant alleles include *TPMT\*3A* (c.460A and c.719G in cis) and *TPMT\*3C* (c.719G)

†† Dominant genetic model was used.

years. Minor predominance of male patients was observed (56%). Patient characteristics are summarized in *Table I*. The genotype frequencies (%) for the *TPMT*, *ABCC4*, *ITPA* and *ABCB1* genomic variants of interest are shown in *Table II*. Average and median duration of maintenance therapy for patients was both 16 months, ranging from 9 to 21 months. The median dose and average dose of 6-MP during maintenance therapy was approximately 100% and 101%, respectively. The lowest 6-MP dose administered was 34% and the highest 197%. Ten patients with ALL (15%) have spent more than 10% of time skipping therapy due to leukopenia. Only 21 patients (30.5%) had no episodes of leukopenia during maintenance therapy. The remaining 47 patients had at least one episode of leukopenia. Hepatotoxicity of various degrees was detected in 45 patients (66%) at some point during the maintenance phase of therapy.

#### *TPMT*

The median average dose of 6-MP was 72% in the group of patients with ALL that carried at least one *TPMT* variant allele, c.460A or c.719G, and was found to be significantly lower ( $p=0.003$ ) than that of the ALL patients carrying wild type *TPMT* (111%). In *TPMT* positive patients with ALL, there were no patients that spent more than 10% of time skipping

therapy and the number of episodes of leukopenia was not higher than that of the patients carrying wild type *TPMT* (*Table II*). The absence of common therapy complications in *TPMT* positive patients is most likely a result of the fact that 6-MP doses were adjusted according to the *TPMT* genotype (determined for all patients before beginning of maintenance therapy).

#### *ITPA*

Five patients (7%) were carriers of the *ITPA* c.94CA allele and their median average dose for 6-MP was 91%. The patients with ALL carrying wild type *ITPA* had a median average dose of 100%. No statistically significant difference was noted between those groups and analysis failed to show any relationship between the *ITPA* c.94CA genotype and increased 6-MP toxicity using all three surrogate markers for myelotoxicity (*Table II*). Also, we could not establish any association among the variant *ITPA* alleles and increased occurrence of hepatotoxicity (*Table II*).

#### *ABCC4*

The *ABCC4* c.\*1372TT genotype (wild type) was detected in 61% of our patients with ALL, where heterozygote and homozygote carriers of variant



allele represented 35% and 4% of the cohort, respectively. In *ABCC4* c.\*1372GG and *MRP4* c.\*1372TG genotype carriers, the median average doses of 6-MP were 95% versus 104% in wild type allele carriers. Using all three surrogate markers for myelotoxicity, we did not confirm that *ABCC4* variant allele carriers experience greater 6-MP induced toxicity during the maintenance phase of therapy nor correlation with increased hepatotoxicity (Table II).

### *ABCB1*

The *ABCB1* c.2677TT genotype was detected in 19% of our patients and the *ABCB1* c.2677GT genotype in 38% of the cohort (Table II). The median average dose of 6-MP in wild type, heterozygote and homozygote carriers of variant alleles was 99%, 100% and 95%, respectively. Neither homozygote nor heterozygote carriers of *ABCB1* variant alleles experienced greater 6-MP induced myelotoxicity during treatment (Table II). However, patients that were variant allele carriers showed increased frequency of hepatotoxicity ( $p=0.030$ ).

### *Pharmacogenetic predictive model for 6-MP induced toxicity in treatment of childhood ALL*

Eleven patients with ALL, carriers of wild type *TPMT* alleles, could tolerate the same or even lower average dose of 6-MP (similarly to) as the carriers of *TPMT* variant alleles. Among these 11 patients, there were 4 heterozygous carriers of *ABCC4* variant allele, *ABCC4* c.\*1372TG, as well as 4 heterozygotes, *ABCB1* c.2677GT, and 4 homozygotes, *ABCB1* c.2677TT, for *ABCB1* variant allele. This finding suggests that, besides *TPMT* variant alleles, the above-mentioned genetic variants should be considered as additional pharmacogenetic markers towards patient-tailored treatment of childhood ALL.

Different prediction models (Elastic net, Neural net and Random forest) were used to classify patients whose mean dose of 6-MP for the entire duration of maintenance therapy was below average (poor tolerance) or above average (good tolerance). After evaluating performance of these prediction models using AUC values as measurement of classification accuracy, it was found that the best predictive model performance was achieved when all analyzed genetic variants (*TPMT* c.460G>A, *TPMT* c.719A>G, *ITPA* c.94C>A, *ABCC4* c.\*1372T>G and *ABCB1* c.2677G>T) were used, even though not all genetic variants showed association with 6-MP tolerance. Out of 3 predictive algorithms, Neural net algorithm showed the best performance for 6-MP tolerance prediction: AUC = 0.71 [95% CI: 0.59–0.83]. This means that according to our results, this model can accurately discriminate between pairs of ALL patients that have good and poor tolerance in 71% of cases.

## Discussion

This is the first study of the effect of pharmacogenetic variants in *ABCC4*, *ITPA* and *ABCB1* genes in Serbian childhood ALL patients. Pharmacogenetic variants in *TPMT* gene have already been studied in the Serbian childhood ALL patients (15). The *ITPA* variant genotype frequency in our group of patients was 7.0%. The genotype frequency of the same variant, *ITPA* c.94CA, in Malaysian children with ALL has been shown to be 28% (25). It was also shown that carriers of *ITPA* variant alleles were at increased risk of developing fever and liver toxicity during 6-MP treatment in Malaysian patients (25). In particular, carriers of *ITPA* variant alleles have a higher incidence of febrile neutropenia, but only if 6-MP treatment has been already modified according to patients' *TPMT* genotype (26). An increased intracellular concentration of 6-MP toxic metabolites was also monitored in patients carrying *ITPA* variant alleles (27). However, some studies did not confirm any correlation among *ITPA* variant alleles and 6-MP toxicity (28). Our results did not support the findings of others (27–29) that *ITPA* variant allele can be an independent predictor of 6-MP myelotoxicity or other adverse reactions, like flu-like symptoms, rash and pancreatitis, to thiopurine drugs. In our study group, those adverse reactions were not detected.

Studies in animal models have shown that low *ABCC4* expression was responsible for thiopurine sensitivity and increased myelotoxicity, whereas high gene expression could be responsible for treatment failure (9). Also, *ABCC4* variants were correlated to a decreased event free survival in children with ALL (30). Today, available data on myelotoxicity and outcome correlation with *ABCC4* genetic variants are scarce. Herein, the frequency of *ABCC4* c.\*1372TG and *ABCC4* c.\*1372GG in our group was 34.7% and 4.3%, respectively. No association of *ABCC4* c.\*1372T>G variant with myelotoxicity was found.

Genotype frequencies of *ABCB1* c.2677GT and *ABCB1* c.2677TT carriers in Korean children with ALL were 36% and 9%, respectively. In our patient group the same genotype frequencies were 38% and 19%. Also, the same study has failed to show correlation of variant *ABCB1* alleles with increased myelotoxicity or outcome (2). Our results have not confirmed that *ABCB1* variant allele could be an independent predictor of myelotoxicity. Increased occurrence of hepatotoxicity in our patients that were variant allele carriers is probably related to methotrexate use, rather than 6-MP (10). Gregers et al. have explored polymorphisms in *ABCB1* gene in relation to myelotoxicity and hepatotoxicity and failed to show correlation of *ABCB1* c.2677GT and *ABCB1* c.2677TT variants with hepatotoxicity, but did reveal correlation with increased myelotoxicity which was not the case in our study (31). Our study failed to demonstrate univariate association of any analysed genetic marker, except in *TPMT* gene, with 6-MP induced toxicities.

One of the aims of this study was to develop a prediction model based on genetic data which could potentially estimate 6-MP tolerance. To the best of our knowledge, no study has dealt with 6-MP tolerance or toxicity by employing predictive modeling with selected genetic markers as input variables. We used 3 classification algorithms and the best prediction model was assessed using Neural net which could discriminate ALL patients that have good and poor tolerance in 71% of cases. This model took into account all analysed genetic variants, not only those that showed significant association in univariate analysis, because liberal inclusion of variables could still contribute to model performance (32).

In our study group, we did not have all the possible combinations for the investigated genetic markers. This is a consequence of the relatively small cohort of patients we could collect in Serbia. Anyway, the probabilistic model predicts that not only variants in *TPMT* gene, but also variants in other genes relevant for 6-MP metabolism and transport (*ITPA*, *ABCC4* and *ABCB1*) could be used as predictors of 6-MP induced toxicity during the maintenance phase of treatment in pediatric ALL. We suggest that *TPMT* c.460G>A, *TPMT* c.719A>G, *ITPA* c.94C>A, *ABCC4* c.\*1372T>G and *ABCB1* c.2677G>T variants serve as markers towards patient stratification, identifying those patients at a higher risk of developing myelotoxicity and therefore, discontinuing therapy.

Children with ALL suffer from a rare, but highly curable disease. However, there is still around 10% of patients with complications and unfavourable outcome. As such, a therapeutic strategy that is efficacious and continuous (devoid of side effects) is of fundamental importance. This strategy has to include a treatment approach which is personalized, based on the individual genomic profile of each patient. Association studies on the pharmacogenomic profile of patients and data on the toxicity of drugs are the most promising directions on the road to personalized medicine. The high throughput methodology, such as next generation sequencing, would facilitate analysis of all known genes relevant for 6-MP metabolism and transport in each childhood ALL patient. The study of association of these genomic data and the occurrence and intensity of 6-MP induced toxicity during the maintenance phase of childhood ALL treatment, would enable the design of a panel of pharmacogenetic markers for predicting thiopurine-induced toxicity in pediatric ALL.

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### Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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