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Associations of novel genetic variations in the folate-related and *ARID5B* genes with the pharmacokinetics and toxicity of high-dose methotrexate in paediatric acute lymphoblastic leukaemia

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Summary

High-dose methotrexate (HD-MTX) plays an important role in the consolidation therapy of acute lymphoblastic leukaemia (ALL) in many treatment regimens worldwide. However, there is a large interpatient variability in the pharmacokinetics and toxicity of the drug. We investigated the influence of single nucleotide polymorphisms (SNPs) in genes of the folate metabolic pathway, transporter molecules and transcription proteins on the pharmacokinetics and toxicity of MTX and 7-hydroxy-methotrexate (7-OH-MTX). 63 SNPs of 14 genes were genotyped and a total of 463 HD-MTX courses (administered according to the ALL-BFM 95 and ALL IC-BFM 2002 protocols) were analysed. Haematological, hepatic and renal toxicities, estimated by routine laboratory parameters were evaluated. Random forest and regression trees were used for variable selection and model building. Linear mixed models were established to prove the significance of the selected variables. SNPs (rs4948502, rs4948496, rs4948487) of the *ARID5B* gene were associated with the serum levels of MTX ($P < 0{\cdot}02$), serum levels and area under the curve of 7-OH-MTX ($P < 0{\cdot}02$) and with hypoproteinaemia ($P = 0{\cdot}004$). *SLCO1B1* rs4149056 also showed a significant association with serum MTX levels ($P < 0{\cdot}001$). Our findings confirm the association of novel genetic variations in folate-related and *ARID5B* genes with the serum MTX levels and acute toxicity.

Keywords: methotrexate, 7-hydroxy-methotrexate, acute lymphoblastic leukaemia, *ARID5B*, pharmacogenetics.

Methotrexate (MTX) is a widely used anticancer agent that plays an important role in the treatment of childhood malignancies. Intravenous high-dose MTX (HD-MTX) with folinic acid rescue is a key component of the consolidation therapy of childhood acute lymphoblastic leukaemia (ALL) (Moricke *et al*, 2008). The pharmacokinetics and pharmacodynamics of the drug show large interpatient variability even with the same treatment protocol (Schmiegelow, 2009; Mikkelsen *et al*, 2011; Csordas *et al*, 2013). This diversity can be partly explained by genomic alterations (mainly single nucleotide polymorphisms, SNPs) in genes involved in the MTX absorption, metabolism, excretion, cellular transport and effector targets or target pathways (Schmiegelow, 2009; Mikkelsen *et al*, 2011). However, conflicting results exist as to whether there are unambiguous candidate pharmacogenetic predictors for MTX clearance and toxicity (de Jonge *et al*, 2005; Trevino *et al*, 2009a; Radtke *et al*, 2013).

Methotrexate is a folate analogue that enters the cells by both passive diffusion and the solute carrier 19A1 (SLC19A1, formerly known as the reduced folate carrier 1, RFC1) (Nersting & Schmiegelow, 2009; Mikkelsen *et al*, 2011). Inside the cell MTX is converted to its active polyglutamate forms (methotrexate polyglutamates, MTXPGs) by the enzyme folylpolyglutamate synthase (FPGS) (Mikkelsen *et al*, 2011). The primary action of MTX is the inhibition of the enzyme dihydrofolate reductase (DHFR), which is responsible for converting folate to its active, chemically reduced, tetrahydrofolate form (Mikkelsen *et al*, 2011). Several other enzymes

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involved in the folate pathway influence the effect of MTX, including methylenetetrahydrofolate reductase (MTHFR), methylenetetrahydrofolate dehydrogenase (MTHFD1), thymidylate synthetase (TYMS), serine hydroxymethyltransferase 1 (SHMT1), 5-methyltetrahydrofolate-homocisteine methyltransferase (MTR) and 5-methyltetrahydrofolate-homocisteine methyltransferase reductase (MTRR) (Mikkelsen *et al*, 2011). MTXPGs induce stronger inhibition of the target enzymes (TYMS and DHFR) and further inhibit key enzymes in the *de novo* purine synthesis pathway (Mikkelsen *et al*, 2011). The degradation of MTXPGs to MTX is catalysed by the enzyme gamma-glutamyl hydrolase (GGH) (Mikkelsen *et al*, 2011). Genetic variability in these metabolizing enzymes, transporters and targets has already extensively been studied because it can lead to an altered sensitivity to MTX and thus exert clinically significant effects on the efficacy and toxicity of MTX treatments in children with ALL (Cheok & Evans, 2006; Faganel Kotnik *et al*, 2011; Gervasini & Vagace, 2012). Hepatic uptake of MTX involves the solute carrier organic anion transporter family, member 1B1 (SLCO1B1). SNPs in the *SLCO1B1* gene have recently been identified as important determinants of MTX pharmacokinetics (Trevino *et al*, 2009a; Mikkelsen *et al*, 2011; Radtke *et al*, 2013; Ramsey *et al*, 2013). Inside the hepatocytes MTX is converted to 7-hydroxy-methotrexate (7-OH-MTX), a metabolite that contributes to the effect and toxicity of MTX (Walling, 2006; Yarlagadda & Perazella, 2008). Systemic MTX is eliminated primarily by renal excretion. The solute carrier family 22 (organic anion transporter), member 8 (SLC22A8) is one of the renal transporters that have an affinity for MTX (Mikkelsen *et al*, 2011). MTX is also a substrate of ABCB1, which is a member of the ATP-binding cassette transporter family [ATP-binding cassette, sub-family B (MDR/TAP), member 1] and mediates the efflux of MTX across the cell membrane (Mikkelsen *et al*, 2011).

The AT rich interactive domain 5B gene (*ARID5B*, also termed *MRF2*) encodes a member of the ARID family of transcription factors and is a novel susceptibility factor for childhood B-cell ALL (Trevino *et al*, 2009b; Healy *et al*, 2010; Lautner-Csorba *et al*, 2012). A relationship between *ARID5B* and ALL treatment response in the context of a frontline ALL clinical trial and an association between *ARID5B* SNP genotypes and greater MTXPGs accumulation of the leukaemic cells have already been observed (Trevino *et al*, 2009b; Xu *et al*, 2012), but no association between *ARID5B* and the pharmacokinetics or toxicity of MTX has previously been reported.

The aim of the present study was to investigate the influence of SNPs in genes of the folate metabolic pathway, transporter molecules and transcription proteins on the pharmacokinetics and toxicity of MTX and 7-OH-MTX in children with ALL. A total of 63 SNPs of 14 genes were selected and evaluated in an integrated approach, including all relevant covariates and factors in a paediatric point of view.

Methods

Patients, pharmacokinetics and toxicity

The DNA and clinical data were collected retrospectively from 118 children with ALL who were treated with HD-MTX at the 2nd Department of Paediatrics, Semmelweis University (Budapest, Hungary) between 1998 and 2011. All children who underwent HD-MTX treatment and whose clinical data and DNA could be obtained were included. The mean patient age was 6·4 years (range: 1·1–18 years). All patients were of Hungarian (Caucasian) ethnicity. The study was approved by the Hungarian Scientific and Research Ethics Committee of the Medical Research Council. Informed consent was obtained from all participants and their parents or legal guardians before inclusion in the study.

Each patient was treated according to two Berlin-Frankfürt-Münster (BFM) ALL protocols [ALL-BFM 95 (25 patients – 5 g/m^2) or ALL intensive chemotheraphy (IC)-BFM 2002 (23 patients – 5 g/m^2 and 70 patients – 2 g/m^2)] (ALL IC-BFM Trial Steering Committee, 2002; Moricke *et al*, 2008). Intravenous HD-MTX infusions were administered in the consolidation phase of chemotherapy. According to the protocols, HD–MTX was administered as a 24-h continuous intravenous infusion four times during the consolidation phase of chemotherapy. Pre-hydration with alkalinized intravenous fluids was applied at 1500 ml/m^2/12 h before HD-MTX treatment, concurrent hydration was applied at 3000 ml/m^2/24 h and post-hydration was applied at 3000 ml/m^2/24 h in the 48–72 h following HD-MTX treatment. According to the protocols, calcium-leucovorin rescue was initiated with a 15 mg/m^2 dose at 42 h after the start of the HD-MTX infusion, and given twice more, at 48 and 54 h after the infusion. If the serum MTX level had not decreased below 0·25 μmol/l at 48 h, extended leucovorin rescue was given. We documented those cases in which the MTX levels at 48 h were >0·25 or >0·5 μmol/l.

Complete sets of pharmacokinetic data on four courses of MTX treatment were available for 113 patients and pharmacokinetic data on two courses of MTX treatment were available for an additional four patients, while three courses of MTX treatment were available from one patient. A total of 463 MTX courses were included in the analysis.

Patients' characteristics and data regarding the pharmacokinetics and toxicity of MTX were collected from the patients charts (Table I).

The MTX and 7-OH-MTX serum levels were measured from blood samples at 24, 36 and 48 h after the beginning of the infusion (24, 36 and 48 h). Additional MTX serum measurements were performed at 66, 72, 96 and 120 h if the MTX level was measurable after 48 h. The cerebrospinal fluid (CSF) MTX levels were measured 24 h after the start of the infusion. The MTX and 7-OH-MTX levels were estimated either by high-performance liquid chromatography (HPLC) in the Pharmacokinetic Laboratory of the National Oncology Institute (Budapest, Hungary) or by enzyme immunoassay

Table I. Patient characteristics and toxicity* of HD-MTX treatments.

Patient characteristics	Value
Total number of patients	118
Gender *n* (%)	
Male	74 (62·7)
Female	44 (37·3)
Mean age at diagnosis in years (range)	6·4 (1·1–18)
Protocol *n* (%)	
ALL-BFM 95	25 (21·2)
ALL IC-BFM 2002	93 (78·8)
Risk group *n* (%)	
Standard	38 (32·2)
Medium	60 (50·8)
High	20 (17)
Immunophenotype *n* (%)	
B-ALL	95 (80·5)
T-ALL	21 (17·8)
Biphenotype	2 (1·7)
MTX dose *n* (%)	
5 g/m^2	
Number of patients treated	48 (40·7)
Number of MTX courses	178 (38·4)
2 g/m^2	
Number of patients treated	70 (59·3)
Number of MTX courses	285 (61·6)
Total number of MTX courses	463
Hepatotoxicity* *n* (%) (*serum bilirubin level >50 μmol/l on days 1–7*)	
5 g/m^2	
Number of MTX courses	7/172 (4·1)
2 g/m^2	
Number of MTX courses	5/279 (1·8)
Nephrotoxicity* *n* (%) (*serum creatinine level >100 μmol/l on day 7*)	
5 g/m^2	
Number of MTX courses	5/155 (3·2)
2 g/m^2	
Number of MTX courses	4/208 (1·9)
Granulocytopenia* *n* (%) (*absolute neutrophil count <1 × 10^9/l on day 7*)	
5 g/m^2	
Number of MTX courses	60/153 (39·2)
2 g/m^2	
Number of MTX courses	73/267 (27·3)
Hypoproteinaemia* *n* (%) (*serum total protein level <60 g/l on day 7*)	
5 g/m^2	
Number of MTX courses	22/149 (14·8)
2 g/m^2	
Number of MTX courses	18/206 (8·7)

HD, high dose; MTX, methotrexate; ALL, acute lymphoblastic leukaemia.

*Toxicity is shown only if it was associated with the investigated genetic variants.

(EIA) at the 2nd Department of Paediatrics, Semmelweis University (Budapest, Hungary). As serum MTX or 7-OH-MTX measurements were available at only 3 time points per MTX course in most of the patients, MTX or 7-OH-MTX clearance could not be calculated appropriately. Hence we investigated the simultaneous alteration of the MTX or 7-OH-MTX levels at 24 h + 36 h + 48 h. The area under the concentration-time curve (AUC) of MTX and 7-OH-MTX was calculated according to the trapezoidal rule (Hegyi *et al*, 2012; Csordas *et al*, 2013). MTX and 7-OH-MTX levels at 24 h + 36 h + 48 h represented simultaneously as a multivariate response variable (MTX 24 h + 36 h + 48 h and 7-OH-MTX 24 h + 36 h + 48 h) and the AUC of MTX and 7-OHMTX were used for characterizing the pharmacokinetics of MTX and 7-OH-MTX.

The evaluation of toxicity was performed as previously reported (Csordas *et al*, 2013). Serum haemoglobin levels, platelet counts, white blood cell counts, granulocyte counts, transaminase levels [alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT)], creatinine levels, total protein levels and bilirubin levels were measured from the blood samples before the HD-MTX infusion (day 0) and on days 1, 2 and 7 after each HD-MTX infusion. Toxicity values were evaluated by applying the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 scoring system (http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf) and the most severe (grade 3–4) toxicity was analysed.

Candidate gene selection and genotyping

The process of gene selection and genotyping were conducted according to our previous report (Lautner-Csorba *et al*, 2012). We selected 14 candidate genes from the literature, based on the results of genome-wide association studies, candidate gene association studies and other studies in which the investigated pathways could also be important for MTX. We searched for SNPs in these 14 genes in online databases. The selection criterion was: minor allele frequency >10%. The SNPs were prioritized according to their published role in the pharmacokinetics of MTX, and their estimated functionality. In some cases tag SNPs were selected from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). A total of 63 SNPs were selected. Detailed information regarding the selected genes and SNPs is shown in Table SI.

Genomic DNA from the children was obtained retrospectively from whole peripheral blood samples taken in remission using QIAmp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was carried out by Sequenom iPLEX Gold MassARRAY technology at the McGill University and Génome Québec Innovation Centre, Montréal, Canada. Only SNPs with a genotyping call rate over 90% were included in the analysis.

Statistical analysis

Allele frequencies were calculated by allele counting (Table SII, which contains the genotype and allele frequency of the

studied SNPs). Hardy-Weinberg equilibrium was tested by using a chi-square goodness-of-fit test with an acceptable cut-off value of $P \leq 0{\cdot}05$ (http://www.oege.org/software/hwe-mr-calc.shtml). A SNP was excluded from the statistical analysis if the frequencies of the wild type and variant alleles deviated from the Hardy-Weinberg equilibrium requirement (a total of 4 SNPs were excluded).

The relationships between the SNPs and the pharmacokinetics or toxicity of MTX were analysed by the R (version 2.15) program (R Development Core Team, 2010). Patient characteristics, prognostic factors (gender, age, ALL immunophenotype, risk group and treatment protocol), the administered HD-MTX dose (2 or 5 g/m^2), the number (1–4) of sequential HD-MTX courses of each patient, the method of MTX measurement (HPLC or EIA) and the SNPs were all included as explanatory variables in the analysis.

In the first step, random forest (RF) method with the *cforest* function of the package *party* (version 1.0-6.) implemented in the R software was applied to calculate variable importance measures because of the large number of explanatory variables and the relatively small sample size (Strobl *et al*, 2008; Hothorn *et al*, 2013). The number of randomly preselected variables (*mtry*) was set to the square root of the number of observations ($\sqrt{n}$). The number of trees (*ntree*) was 1000. The value of the test statistic or $1 - P$ value that must be exceeded in order to implement a split (*mincriterion*) was set to 0·8. The RF was used to select the important variables from all explanatory variables for further analyses. Variables were considered informative or important if their value was above the absolute value of the lowest negative-scoring variable (Strobl *et al*, 2009).

In the second step, we built classification and regression trees (CART) with the *ctree* function of the package *party* (Hothorn *et al*, 2006). The value of test statistic ($1 - P$ value) that must be exceeded in order to implement a split (*mincriterion*) was set to 0·95. The minimum sum of weights in a node in order to be considered for splitting (*minsplit*) was 2. The minimum sum of weights in a terminal node (*minbucket*) was 1. The CARTs were built with the selected explanatory variables to generate clinical decision rules and to explore the relationship between the response and the explanatory variables (Strobl *et al*, 2009).

In the last step, the general linear mixed model (GLMM, testing the relationship between SNPs and the logarithmic transformed serum levels of MTX and 7-OH-MTX) and generalized linear mixed model (GzLMM) with logit link function for binomial data (testing the relationship between SNPs and toxicity) were applied to prove the significance of the selected variables and their interactions by the CART and to estimate effect sizes. GLMM and GzLMM enabled the correlated nature of the data (four measurements for each patient) to be taken into account (Pinheiro & Bates, 2000; Bates *et al*, 2012). The proportion of variation in the response variable that is explained by the mixed models [marginal and conditional R^2, $R^2_{\mathrm{GLMM(m)}}$ and $R^2_{\mathrm{GLMM(c)}}$] was calculated according to Nakagawa and Schielzeth (2013). Relationships between SNPs and toxicity were analysed separately according to the administered MTX dose (in patients who received 5 g/m^2 and in patients who received 2 g/m^2 MTX). Model effects were tested together based on their F values (GLMM) and likelihood ratio tests in case of generalized models. All factors and potential interactions were evaluated, with the cut-off for inclusion being $P < 0{\cdot}05$. The 95% confidence intervals (CIs) of odds ratios (OR) were calculated by the Wald's method (Agresti, 2002).

The statistical power of the linear mixed-effect model analyses was calculated with the *Pwr* function of the package *nlmeU* (version 0.70-3., Galeczki & Burzykowski, 2013). The power of our models ranged between 62% and 86%.

Results

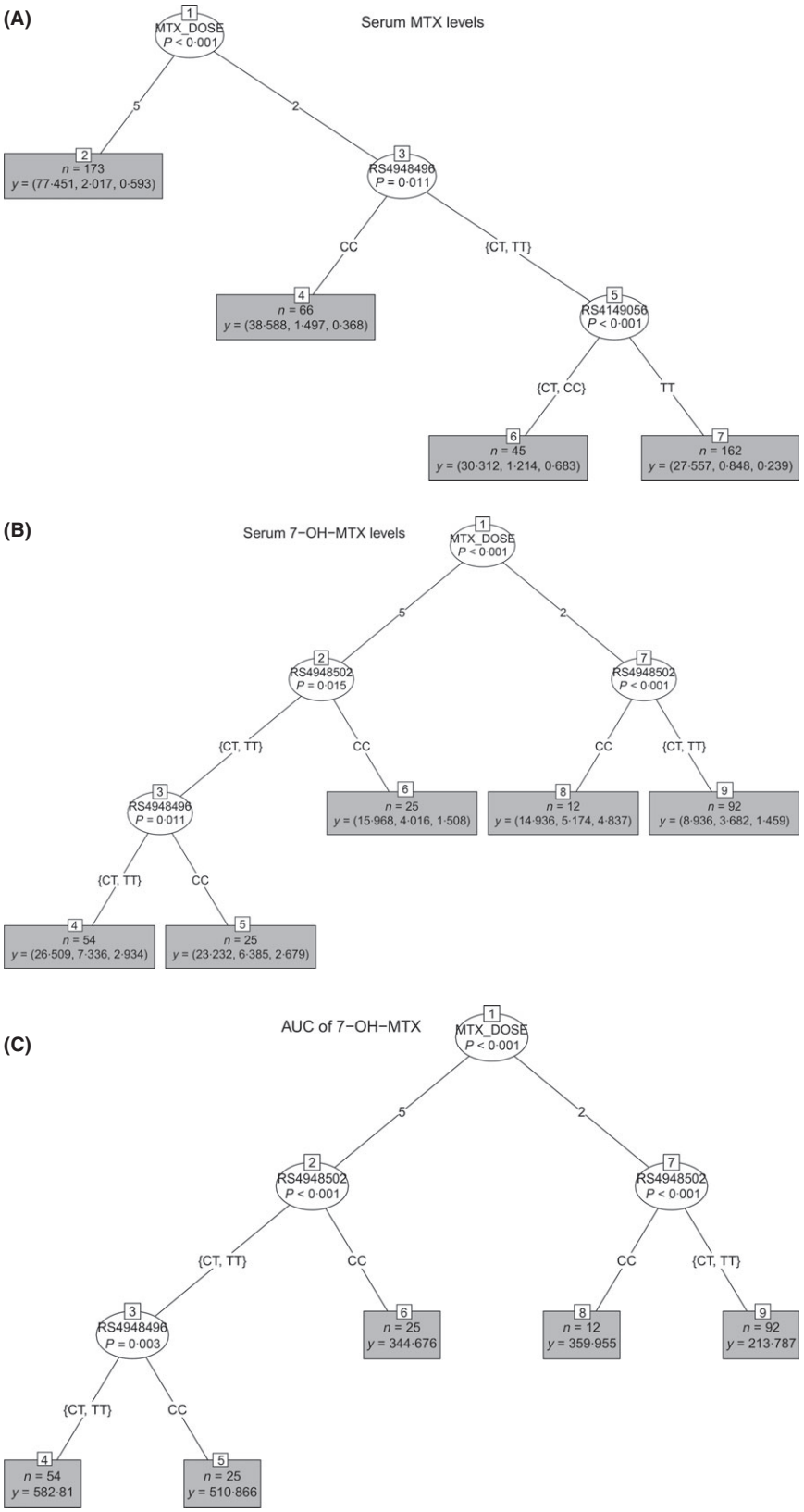
MTX and 7-OH-MTX levels

In the case of the serum MTX and 7-OH-MTX levels, RF analysis was conducted according to the mean MTX and 7-OH-MTX levels at the defined time points (24, 36 and 48 h separately).

In the case of serum MTX levels or AUC of MTX, the administered MTX dose, ALL phenotype, risk group, patient age, rs2612100, rs2236225, rs1004474, rs9967368, rs7499, rs7089424, rs10821936, rs4506592, rs1076991, rs745686, rs4948496, rs1801394, rs4363657, rs4149056 and rs1202179 seemed to be associated with one or more of the mean serum MTX levels (24 and/or 36 and/or 48 h) and/or with the AUC of MTX.

The classification and regression trees (CARTs) of the serum MTX levels and the AUC of MTX were built with the use of these selected explanatory variables. The MTX levels at the defined time points were represented simultaneously as a multivariate response variable (MTX 24 h + 36 h + 48 h).

The CART of the serum MTX levels (24 h + 36 h + 48 h) shows that the administered MTX dose (5 or 2 g/m^2) had the strongest effect on the serum MTX levels ($P < 0{\cdot}001$, Fig 1A). In patients who received 2 g/m^2, *ARID5B* rs4948496 showed a significant association with the serum MTX levels ($P = 0{\cdot}01$). Patients with the CC genotype were found to have higher serum MTX levels than patients with the CT+TT genotype. In patients who received 2 g/m^2 MTX and had the *ARID5B* rs4948496 CT+TT genotype, the *SLCO1B1* rs4149056 also showed a significant association with the serum MTX levels ($P < 0{\cdot}001$, Fig 1A). Patients with the TT genotype had lower serum MTX levels than patients with CT+CC genotype. In patients who received 2 g/m^2 MTX associations between both SNPs and serum MTX levels were confirmed with the general linear mixed models (GLMM) (rs4948496: $P = 0{\cdot}039$, rs4149056: $P = 0{\cdot}004$). In the GLMM the rs4948496 + rs4149056 genotypes explained 6%, 10·7% and 9% interindividual variability [$R^2_{\mathrm{GLMM(m)}}$] in the MTX levels at 24, 36 and 48 h [$R^2_{\mathrm{GLMM(c)}}$ was 25%, 37·1% and 22·7% at 24, 36 and 48 h].



In patients who received 2 g/m^2 MTX and had the *ARID5B* rs4948496 CC genotype, high (>0·25 μmol/l) MTX levels at 48 h were found more frequently (CC *versus* CT+TT genotype: 61% vs. 39%, $P = 0·044$). However, in the case of the calculated AUC of MTX, the administered MTX dose (5 or 2 g/m^2) showed the strongest and only effect according to the CART and the GLMM ($P < 0·001$).

In the case of the CSF MTX levels only patient age and the rs4948502 seemed to be associated with the CSF MTX levels according to the RF, however none of the explanatory variables was proved to be associated with the CSF MTX levels according to the CART and GLMM.

Serum 7-OH-MTX levels or AUC of 7-OH-MTX seemed to be associated with the administered MTX dose, ALL phenotype, patient age, rs9967368, rs10841769, rs7499, rs2235013, rs4451422, rs1202179, rs4948487, rs4948496, rs2518463, rs4948502, rs12759827, rs10106 and rs1801394 according to the RF. The CARTs of the AUC of 7-OH-MTX and serum 7-OH-MTX levels were built with the use of these explanatory variables. The serum 7-OH-MTX levels at the defined time points were represented simultaneously as a multivariate response variable (7-OH-MTX 24 h + 36 h + 48 h).

The CART of the serum 7-OH-MTX levels (24 h + 36 h + 48 h) showed similar results to the CART of the serum MTX levels (Fig 1B). The administered MTX dose (5 or 2 g/m^2) had the strongest effect on the serum 7-OH-MTX levels ($P < 0·001$). In both groups *ARID5B* rs4948502 showed significant associations with the serum levels ($P = 0·015$ and $P < 0·001$), which was also confirmed by the GLMM (5 and 2 g/m^2, $P = 0·003$ and $P = 0·033$).

In the patients who received 5 g/m^2 MTX the rs4948502 accounted for 12·6%, 14% and 16·3% of interindividual variability [$R^2_{\mathrm{GLMM(m)}}$] in the 7-OH-MTX levels at 24, 36 and 48 h [$R^2_{\mathrm{GLMM(c)}}$ was 48·4%, 58% and 45·3% at 24, 36 and 48 h]. In those patients who received 2 g/m2 MTX the the rs4948502 accounted for 15%, 9·3% and 12·9% of interindividual variability [$R^2_{\mathrm{GLMM(m)}}$] in the 7-OH-MTX levels at 24, 36 and 48 h [$R^2_{\mathrm{GLMM(c)}}$ was 56%, 61·2% and 51·7% at 24, 36 and 48 h].

However, the associations between the SNP and serum levels in the two groups are not unambiguous. Higher serum 7-OH-MTX levels were found in patients who received 2 g/m^2 MTX and had the *ARID5B* rs4948502 CC genotype, whereas in patients who received 5 g/m^2 MTX the CC genotype was associated with lower serum 7-OH-MTX levels (in comparison with patients with the CT+TT genotype). In the patients who received 5 g/m^2 MTX and had the *ARID5B* rs4948502 CT or TT genotype an additional SNP, *ARID5B* rs4948496, showed a significant association with the 7-OH-MTX levels according to the CART ($P = 0·011$) but this was not confirmed by the GLMM.

The result of the CART of the calculated AUC of 7-OH-MTX was similar to the CART of the serum 7-OH-MTX levels (Fig 1C). The *ARID5B* rs4948502 showed a significant association with the AUC of 7-OH-MTX in both groups (5 and 2 g/m^2: $P < 0·001$ separately), which was confirmed by the GLMM (5 and 2 g/m^2: $P < 0·001$ and $P = 0·013$).

In the patients who received 5 g/m^2 MTX the rs4948502 accounted for 14·1% interindividual variability [$R^2_{\mathrm{GLMM(m)}}$] in the AUC of 7-OH-MTX [$R^2_{\mathrm{GLMM(c)}}$ was 52%]. In those patients who received 2 g/m^2 MTX the rs4948502 accounted for 18·9% interindividual variability [$R^2_{\mathrm{GLMM(m)}}$] in the AUC of 7-OH-MTX [$R^2_{\mathrm{GLMM(c)}}$ was 61·5%].

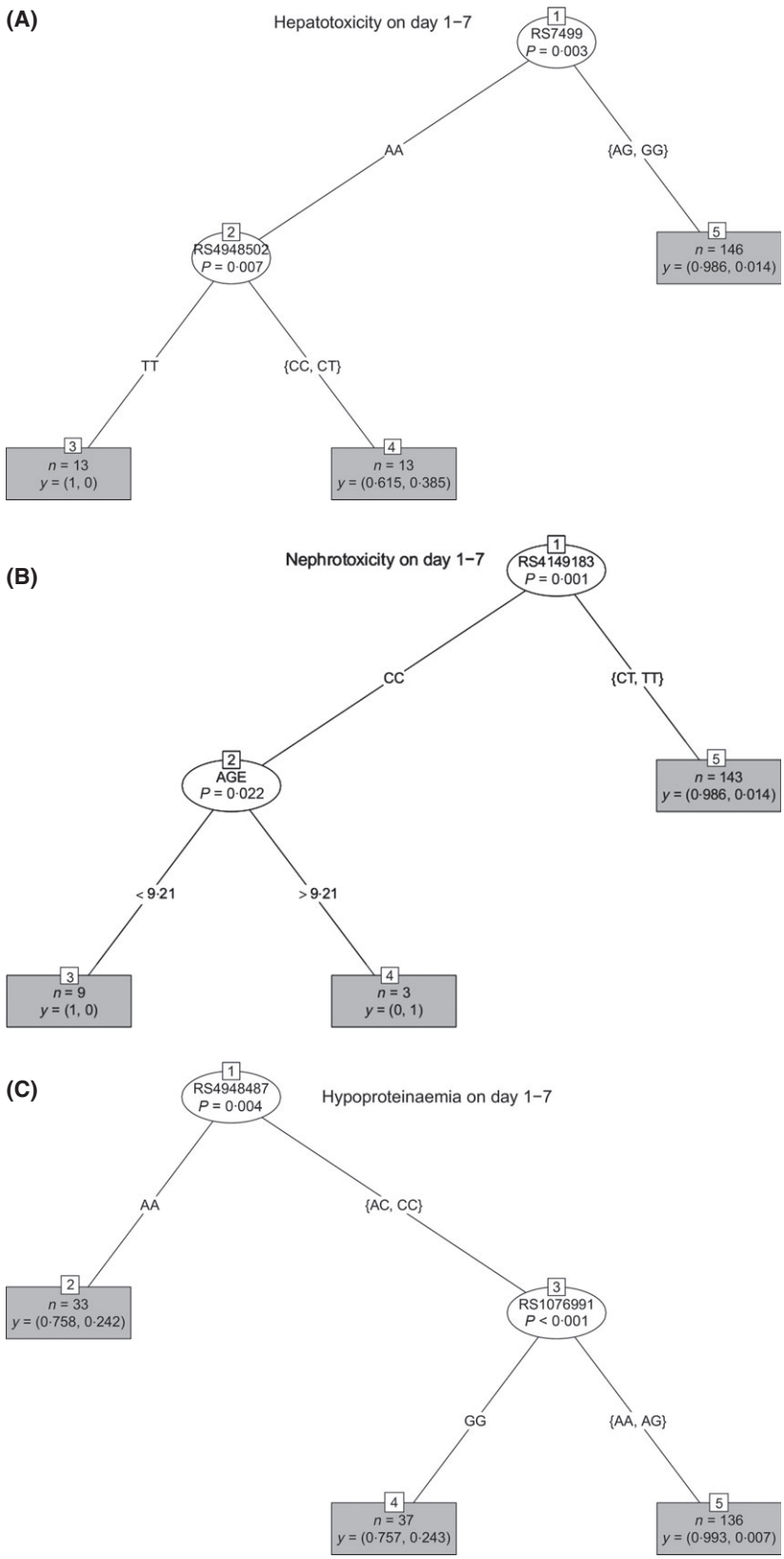
The *ARID5B* rs4948496 showed a significant association with the AUC 7-OH-MTX in patients who received 5 g/m^2 MTX and had the *ARID5B* rs4948502 CT or TT genotype ($P = 0·003$), but this was confirmed by the CART only.

Toxicity of HD-MTX treatments

Hepatotoxicity (serum bilirubin level >50 μmol/l) on days 1–7 seemed to be associated with the administered MTX dose, patient age, rs3768142, rs11045819, rs7499, rs1544105, rs17328763, rs11045818, rs2853523, rs10106, rs4451422, rs745686, rs4948502, rs2276299 and rs2853533 according to the RF analysis. The CART showed significant associations between hepatotoxicity and the *SLC19A1* rs7499 ($P = 0·003$) and *ARID5B* rs4948502 ($P = 0·007$) in patients who received 5 g/m^2 MTX (Fig 2A). Toxic bilirubin levels did not occur in cases ($n = 13$) with the *SLC19A1* rs7499 AA genotype and the *ARID5B* rs4948502 TT genotype.

Nephrotoxicity (serum creatinine level >100 μmol/l) on days 1–7 seemed to be associated with the MTX levels at 36 h, rs4149183, rs1544105, rs1004474 and rs3212713 according to

Fig 1. (A) Classification and regression tree of the serum MTX levels (24 h + 36 h + 48 h). Circles (1, 3, 5) represent the explanatory variables that are significantly associated with the serum methotrexate (MTX) levels. The different subgroups of the explanatory variables are shown along the lines. Rectangles (2, 4, 6, 7) represent the mean MTX levels at 24 h + 36 h + 48 h simultaneously: y = (mean MTX level at 24, 36, 48 h), and the number of the observations in the adequate group (n = number of MTX cycles). Results are specified in the text. (B) Classification and regression tree of the serum 7-OH-MTX levels (24 h + 36 h + 48 h). Circles (1, 2, 3, 7) represent the explanatory variables that are significantly associated with the serum 7-hydroxy-methotrexate (7-OH-MTX) levels. The different subgroups of the explanatory variables are shown along the lines. Rectangles (4, 5, 6, 8, 9) represent the mean 7-OH-MTX levels at 24 h + 36 h + 48 h simultaneously: y = (mean 7-OH-MTX level at 24, 36, 48 h) and the number of the observations in the adequate group [n = number of methotrexate (MTX) cycles]. Results are specified in the text. (C) Classification and regression tree of the AUC of 7-OH-MTX. Circles (1, 2, 3, 7) represent the explanatory variables that are significantly associated with the area under the concentration-time curve (AUC) of 7-hydroxy-methotrexate (7-OH-MTX). The different subgroups of the explanatory variables are shown along the lines. Rectangles (4, 5, 6, 8, 9) represent the mean AUC of 7-OH-MTX: y = (mean AUC of 7-OH-MTX) and the number of the observations in the adequate group [n = number of methotrexate (MTX) cycles]. Results are specified in the text.



the RF analysis. The CART showed a significant association between the *SLC22A8* rs4149183 and nephrotoxicity in patients who received 5 g/m^2 MTX (Fig 2B). Nephrotoxicity was found significantly more frequently in patients with CC genotype *versus* CT+TT genotype (25% vs. 1%, $P = 0{\cdot}001$). According to the CART, among patients with CC genotype,

Table II. Contingency table of the hypoproteinaemia developed according to the rs4948487 and rs1076991* genotypes.

	rs4948487 (*ARID5B*)		rs1076991 (*MTHFD1*)	
Hypoproteinaemia (<60 g/l)	AA [*n* (%)]	AC+CC [*n* (%)]	GG [*n* (%)]	AG+AA [*n* (%)]
Yes	8 (24)	7 (4)	9 (23)	8 (5)
No	25 (76)	156 (96)	31 (77)	151 (95)

HD, high dose; MTX, methotrexate.
n (%) = number (percent) of MTX courses.
*In patients who received 2 g/m^2 HD-MTX.

those aged >9·2 years had a 100% occurrence of nephrotoxicity *versus* a 0% occurrence in patients aged <9·2 years ($P = 0{\cdot}022$). In the cases of hepatotoxicity and nephrotoxicity, generalized linear mixed models (GzLMM) were not carried out, because the occurrences of these side-effects were <5%.

Hypoproteinaemia (serum total protein level <60 g/l) on days 1–7 seemed to be associated with the risk group, the rs4948487, rs1076991 and the serum 7-OH-MTX levels at 36 h according to the RF. The CART showed significant associations between hypoproteinaemia and the *ARID5B* rs4948487 ($P = 0{\cdot}004$) and the *MTHFD1* rs1076991 ($P < 0{\cdot}001$) in patients who received 2 g/m^2 MTX (Fig 2C). GzLMM was not carried out because hypoproteinaemia occurred in 18 cases only. However the trend that hypoproteinaemia occurred more frequently in patients with the *ARID5B* rs4948487 AA genotype and in patients with the *MTHFD1* rs1076991GG genotype is represented in Table II.

Granulocytopenia (absolute neutrophil granulocyte count $<1 \times 10^9$/l) on days 1–7 seemed to be associated with the risk group, administered MTX dose, rs1979277, rs2235013, rs9967368, rs10821936, rs4948502, rs4506592, rs7089424, rs1801394, rs1544105, rs4948496 and rs3768142 according to the RF analysis. The CART and the GzLMM showed a significant association between granulocytopenia and the *MTR* rs3768142 in patients who received 2 g/m^2 MTX [GG *versus* GT+TT genotype: 56% vs. 23%, OR: 5·92 (95% CI: 2·03–17·27), $P = 0{\cdot}001$]. Granulocytopenia occurred significantly more frequently after the last (4th) MTX cycle than after the others (cycles 1–3); OR: 3·48 (95% CI: 1·40–8·64), $P = 0{\cdot}007$.

Discussion

The therapy of ALL is based on risk-adapted protocols with a 5-year survival rate of 84-94% in developed countries (Pui *et al*, 2011). Current ALL trials focus on improving not only the efficacy of the treatment but also the quality of life of the patients (Pui *et al*, 2011). Patients with higher risk of relapse are treated more intensively, with the potential risk for toxic side effects. The major clinical challenge is to balance the efficacy of HD-MTX therapy against its toxicity (Schmiegelow, 2009; Faganel Kotnik *et al*, 2011). HD-MTX plays an important role in the consolidation therapy of ALL in many treatment regimens worldwide. However, there is a large interpatient variability in the pharmacokinetics and toxicity of the drug (Schmiegelow, 2009; Mikkelsen *et al*, 2011; Csordas *et al*, 2013). If we had reliable biomarkers we could identify those patients with increased risk of treatment failures or specific toxicities and could adjust the HD-MTX treatment individually.

In the present study we investigated whether SNPs in genes of the folate metabolic pathway, transporter molecules and transcription proteins modify the pharmacokinetics or toxicity of MTX and 7-OH-MTX in children with ALL. A total of 63 SNPs of 14 genes were selected and evaluated in a multivariate analysis.

The SNPs of the *ARID5B* gene (rs4948502, rs4948496 and rs4948487) showed significant associations with the serum MTX (in patients who received 2 g/m^2 MTX) and 7-OH-MTX levels and with the development of hypoproteinaemia in our study. The CC genotype of the rs4948496 was associated with higher serum MTX levels; however the associations between the CC genotype of the rs4948502 and the 7-OH-MTX levels were not unambiguous. In patients who received 2 g/m^2 MTX it was associated with higher serum 7-OH-MTX levels, whereas lower serum 7-OH-MTX levels were found in patients who received 5 g/m^2 MTX and had the CC genotype. The *ARID5B* gene encodes a transcriptional factor that plays a role in the cell-growth and the differentiation of B-lymphocyte progenitors (Trevino *et al*, 2009b). SNPs of this gene

Fig 2. (A) Classification and regression tree of hepatotoxicity on days 1–7 in patients who received 5 g/m^2 MTX. Circles (1, 2) represent the explanatory variables that are significantly associated with the development of hepatotoxicity. The different subgroups of the explanatory variables are shown along the lines. Rectangles (3, 4, 5) represent the frequency of developed toxicity y = (frequency of no toxicity, frequency of toxicity) in the different groups, and the number the observations in the adequate group [n = number of methotrexate (MTX) cycles]. Results are specified in the text. (B) Classification and regression tree of nephrotoxicity on days 1–7 in patients who received 5 g/m^2 MTX. Circles (1, 2) represent the explanatory variables that are significantly associated with the development of nephrotoxicity. The different subgroups of the explanatory variables are shown along the lines. Rectangles (3, 4, 5) represent the frequency of developed toxicity: y = (frequency of no toxicity, frequency of toxicity) in the different groups, and the number the observations in the adequate group [n = number of methotrexate (MTX) cycles]. Results are specified in the text. (C) Classification and regression tree of hypoproteinaemia on days 7 in patients who received 2 g/m^2 MTX. Circles (1, 3) represent the explanatory variables that are significantly associated with the development of hypoproteinaemia. The different subgroups of the explanatory variables are shown along the lines. Rectangles (2, 4, 5) represent the frequency of developed toxicity: y = (frequency of no toxicity, frequency of toxicity) in the different groups and the number the observations in the adequate group [n = number of methotrexate (MTX) cycles]. Results are specified in the text.

have previously been associated with higher susceptibility to ALL and B-hyperdiploid subtype of ALL and to contribute to racial disparities in the incidence and treatment outcome of ALL (Trevino *et al*, 2009b; Lautner-Csorba *et al*, 2012; Xu *et al*, 2012). Genetic variations of *ARID5B* have also been linked to greater MTXPGs accumulation in patients with ALL (Trevino *et al*, 2009b). However this is the first study to investigate the relationship between the *ARID5B* gene and the pharmacokinetics and toxicity of MTX. The exact role of this gene on MTX levels and toxicity needs further investigations.

The rs4149056 of the *SLCO1B1* gene showed significant association with serum MTX levels in patients who received 2 g/m^2 MTX according to our study. Lower MTX levels were found in patients with the *SLCO1B1* rs4149056 TT genotype than in patients with CT+CC genotype. The *SLCO1B1* gene encodes an organic anion transporter that is localized at the sinusoidal membrane of hepatocytes and is responsible for the hepatic uptake of the drug (Trevino *et al*, 2009a; Radtke *et al*, 2013). Trevino *et al* (2009a) described that the *SLCO1B1* rs4149056 was associated with MTX clearance and gastrointestinal toxicity. More recently, the association between the *SLCO1B1* rs4149056 and MTX clearance was also clearly replicated (Radtke *et al*,2013; Ramsey *et al*, 2013). Other SNPs of the *SCLO1B1* gene (rs11045879 and rs4149081) have also been associated with high MTX plasma levels in former studies (Lopez-Lopez *et al*, 2011, 2013). Our results confirm the studies that suggest *SLCO1B1* is a novel predictor for MTX clearance and toxicity (Trevino *et al*, 2009a; Lopez-Lopez *et al*, 2011, 2013; Ramsey *et al*, 2013).

The most common side effects following HD-MTX therapy are hepato-, nephro- and myelotoxicity. Conflicting results exist whether genetic variants in genes that encode proteins of the folate pathway contribute to the development of MTX toxicity.

Our results indicate that hepatotoxicity was significantly associated with the *SLC19A1* rs7499. This is the first study to show an association between this genetic variant of the *SLC19A1* gene and HD-MTX toxicity. However, the most frequently studied polymorphism of *SLC19A1*, rs1051266, showed no association with either the MTX levels or the developed toxicity in the present study. The relationship between the genetic variants of the *SLC19A1* gene and the serum MTX levels has previously been shown (Laverdiere *et al*, 2002; Lopez-Lopez *et al*, 2013). In former studies the rs1051266 was associated with gastrointestinal, bone marrow and hepatotoxicity (Kishi *et al*, 2007; Gregers *et al*, 2010; Yee *et al*, 2010). A former study also reported an association between the rs1051266 and a decreased risk for leucopenia in ALL/malignant lymphoma patients (Faganel Kotnik *et al*, 2011). On the other hand, some studies have not confirmed any association between *SLC19A1* and HD-MTX toxicity (Kishi *et al*, 2003; Huang *et al*, 2008; Faganel Kotnik *et al*, 2010).

Our CART analysis showed significant association between the development of nephrotoxicity and the *SLC22A8* rs4149183 in patients who received 5 g/m^2 MTX. Nephrotoxicity occurred in each patient aged >9·2 years with the *SLC22A8* rs4149183 CC genotype. Higher MTX levels, slower clearance and higher occurrence of MTX toxicity have been reported in older patients in the majority of the published studies, including a previous one from our group (Groninger *et al*, 2004; Plard *et al*, 2007; Csordas *et al*, 2013). SLC22A8 is mainly expressed in the kidneys and to a lesser extent in the brain (Rizwan & Burckhardt, 2007). Genetic variants of *SLC22A8* were significantly associated with MTX clearance in a previous study (Lopez-Lopez *et al*, 2013).

In the present study, granulocytopenia was associated with the *MTR* rs3768142 in patients who received 2 g/m^2 MTX. This is the first study to report a significant association between this genetic variant of the *MTR* gene and the development of HD-MTX toxicity. Genetic variants of the *MTR* gene are less commonly studied, but the rs1805087 SNP of this gene has previously been associated with gastrointestinal and haematologic toxicity of HD-MTX in osteosarcoma patients (Patino-Garcia *et al*, 2009). However, controversial results also exist; in another study the same SNP showed no significant association with the toxicity of HD-MTX therapy in patients with ALL (Faganel Kotnik *et al*, 2011).

Beside the SNP of the *ARID5B* gene, the rs1076991 of the *MTHFD1* gene was associated with the development of hypoproteinaemia after the HD-MTX treatments in patients who received 2 g/m^2 MTX. *MTHFD1* plays a key role in the folate metabolism (de Jonge *et al*, 2005). Another genetic variant (rs2236225) of the *MTHFD1* gene has been shown to reduce the odds of hepatotoxicity, but was also related to anaemia after HD-MTX treatments in ALL/osteosarcoma patients (Erculj *et al*, 2012; Windsor *et al*, 2012). The *MTHFD1* rs1076991 has been associated with congenital neural tube defect (Greene *et al*, 2009), but to date no association with HD-MTX toxicity has been reported.

Some limitations of our study need to be considered. It was a retrospective study with a modest sample size. Data were collected from patients who had received HD-MTX according to their risk group. However, every possible influential variable was taken into account in our multivariate analysis. The SNPs of the *MTHFR* and *GGH* genes were not included in the present study. However, some publications have referred to the effect of the different genetic variations of these genes on MTX pharmacokinetics and toxicity (Panetta *et al*, 2010; Faganel Kotnik *et al*, 2011).

The strength of our study is that variable importance measures were made with RF analysis, which is a type of recursive partitioning method, particularly well-suited when a small sample size with large number of observations is given (small n and large p problems) (Strobl *et al*, 2008, 2009). RF variable importance measures, as compared to univariate screening methods, cover the impact of each predictor variable individually as well as in multivariate interactions with other predictor variables. We used classification and regression trees to demonstrate our results. The results were validated by linear mixed models in each case.

Our results should be interpreted as exploratory findings that might serve for hypothesis generation. We believe that our results might be useful in designing future pharmacogenetic studies. Our ongoing international collaboration is now developing a MTX pharmacogenetic study (ALL IC-BFM Trial Steering Committee 2009) in which we focus on exploring the influence of different genetic variations on MTX pharmacokinetics in a larger, randomized cohort.

Conclusion

Polymorphisms of the *ARID5B* gene were significantly associated with the serum levels and toxicity of MTX and its metabolite, 7-OH-MTX. The present study confirms the possible role of *SLCO1B1* as a pharmacogenetic predictor for MTX pharmacokinetics. Novel genetic variants of the *SLC19A1*, *SLC22A8*, *MTR* and *MTHFD1* genes were shown to be associated with the development of acute toxicity after HD-MTX treatment. Further analysis and validation in a larger cohort is necessary to determine the predictive value of these associations. An international collaboration is now exploring this issue in a new ALL trial.

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Author contributions

K.C. reviewed medical records, participated in the statistical analysis, evaluated the results and wrote the manuscript. O.L.C. and A.F.S. carried out the molecular genetic elements of the study. A.H. designed and conducted the statistical analysis. M.H. and D.J.E reviewed medical records and evaluated the results O.T.E. reviewed medical reports. C.S. designed the molecular genetic analysis. G.K. organized the study, evaluated the results. All authors contributed to revising the paper.

Disclosure of conflict of interest

The authors declare no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes and SNPs included in the analyses.

Table S2. Genotype and allele frequency of the studied SNPs.

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