Polymorphisms in the ABCB1 gene and effect on outcome and toxicity in childhood acute lymphoblastic leukemia

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The membrane transporter P-glycoprotein, encoded by the ABCB1 gene, influences the pharmacokinetics of anti-cancer drugs. We hypothesized that variants of ABCB1 affect outcome and toxicity in childhood acute lymphoblastic leukemia (ALL). We studied 522 Danish children with ALL, 93% of all those eligible. Risk of relapse was increased 2.9-fold for patients with the 1199GA variant versus 1199GG (P = 0.001), and reduced 61% and 40%, respectively, for patients with the 3435CT or 3435TT variants versus 3435CC (overall P = 0.02). The degree of bone marrow toxicity during doxorubicin, vincristine and prednisolone induction therapy was more prominent in patients with 3435TT variant versus 3435CT/3435CC (P = 0.01/ P < 0.0001). We observed more liver toxicity after high-dose methotrexate in patients with 3435CC variant versus 3435CT/TT (P = 0.03). In conclusion, there is a statistically significant association between ABCB1 polymorphisms, efficacy and toxicity in the treatment of ALL, and ABCB1 1199GA may be a new possible predictive marker for outcome in childhood ALL.

INTRODUCTION
The 10-year overall survival rate for childhood acute lymphoblastic leukemia (ALL) is approaching 80% or higher, with many contemporary treatment programs.1–3 The majority of treatment failures are due to leukemic relapses. However, many of the treatment failures reflect not only the chemo-sensitivity to the different antileukemic drugs used in the protocols but may also depend on inherited single-nucleotide polymorphisms (SNPs) in genes affecting drug metabolism, transport and binding site affinity.4,5 Owing to the complex combination of chemotherapy in childhood ALL protocols, individual SNPs are unlikely to have measurable effects on drug disposition and cure rates unless they either affect antileukemic agents used extensively in the protocols such as 6-mercaptopurine5 or methotrexate (MTX),6 or when the gene in question affects several anticancer agents, such as the cytochrome P450 family7 or gluthatione S-transferases,8 and potentially the ABCB1 gene. The membrane transporter P-glycoprotein (P-gp), encoded by the ABCB1 gene, works both as a functional barrier and as an efflux transporter in a variety of tissues, and it can influence the pharmacokinetics of several anti-cancer drugs.9–11 Variants in the ABCB1 gene have been shown to alter expression and/or function of P-gp.12 Overexpression of P-gp in tumor cells leads to multidrug resistance13–16 and a number of antileukemic drugs (for example, glucocorticosteroids, anthracyclines and vincristine) are substrates for P-gp. Even though MTX is not regarded as a P-gp substrate, studies of patients in MTX monotherapy showed that the silent ABCB1 polymorphism 3435C>T may affect outcome and toxicity after MTX therapy.17,18 Studies exploring the clinical impact of ABCB1 SNPs in childhood ALL are few. We have therefore performed a Danish population-based study of the impact of ABCB1 polymorphisms 1199G>A, 1236C>T, 2677G>T/A and 3435C>T on incidence of ALL and risks of relapse and toxicity.

MATERIALS AND METHODS
Patients
Two hundred and forty-six girls and 317 boys, 1.0–14.9 of age (median 4.5 years) were diagnosed with precursor B-cell or T-cell ALL in Denmark from January 1992 to January 2007. Of these, 41 patients were excluded as a result of incomplete genotyping for all polymorphisms due to a lack of DNA material or poor quality of DNA. The remaining 522 patients included in this study, that is, 93% of those eligible during the study period, were treated according to the NOPHO ALL92 (n = 307) or ALL2000 (n = 215) protocols. Of these, 357 patients were classified as low-risk ALL and 165 as high-risk ALL.3 More than 95% of the patients were Nordic Caucasians. Blood samples from 200 healthy donors; 94 women and 106 men were genotyped to compare ABCB1 variant frequencies for patients and healthy volunteers (Table 1).

Toxicity studies were conducted on 233 children treated at Rigshospitalet, Copenhagen. For the three-drug (doxorubicin, prednisolone and vincristine) induction-therapy toxicity study, all patients with retrievable laboratory data before treatment day 22 were included. To ensure steady-state measuring of MTX, patients were only included in the MTX pharmacokinetic and toxicity studies if end-of-fusion MTX plasma values were
Genotyping

The ABCB1 1199G > A (rs2292109), 1236C > T (rs1128503), 2677G > A/T (rs2032582) and 3435C > T (rs1045642) genotypes were determined using pyrosequencing. Genomic DNA was extracted and purified by NaCl-ethanol-precipitation from 1 to 5 ml EDTA-stabilized blood. HotStar-Taq master mixture (WVR International, Stockholm, Sweden) was used for PCR amplification and reactions were carried out on a Mastercycler gradient instrument (Eppendorf, Hamburg, Germany) in a total volume of 25 µl. The SNPs were analyzed by a Pyrosequencing PSQ96MA instrument (Qiagen, Nordic, Sweden) according to the manufacturer’s protocol and as previously described.5,6 Five hundred and fourteen patients were successfully analyzed for all four polymorphisms. The reduced folate carrier polymorphism RFC80G > A in SLC19A1 was analyzed as previously described.6

Pharmacokinetics and toxicity

To quantify the degree of myelosuppression, we used the nadir hemoglobin, platelet and absolute neutrophil counts within the first 3 weeks of induction therapy or within 4 weeks after the first HD-MTX. The maximum plasma alanine aminotransferase level within the first 3 weeks of induction therapy or within 4 weeks after the first HD-MTX was used as marker of liver toxicity. Samples drawn 20–26 h after initiation of HD-MTX were considered to represent plasma MTX steady-state levels.

Statistics

SAS software (version 9.2, SAS Institute, Cary, NY, USA) was used for statistical analysis. Two-sided P-values < 0.05 were considered significant. Survival analyses were performed with a basic time scale defined by the date of diagnosis. The duration of event-free survival (EFS) was defined as the time from diagnosis until the date of relapse, death, or the development of a second malignancy (whichever first) or the last known follow-up for event-free survivors. When relapse was considered an event, then death, second malignancy, bone marrow transplantation, protocol failure and changes of protocol were classified as censored events. Patients in first remission were followed until 30 July 2008. Relapse probabilities were estimated using the Kaplan–Meier method. Univariate Cox regression and multivariate Cox regression analysis, stepwise backward selection with stratification by risk group, were used to identify potential risk factors for an event. Model assumptions, including the proportionality assumption, were assessed using Schoenfeld and martingale residual. A general linear model was used for HD-MTX toxicity and pharmacokinetic analyses, and a general linear mixed model with repeated measures was used for induction therapy. In the statistical tests, all data were log transformed. In multivariate analyses, adjustment variables were gender, protocol (ALL92/ALL2000), risk group (high/low) and immunophenotype (pre-B/T). Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated when appropriate. χ²-test was applied to test for risk of ALL in the ABCB1 polymorphisms. As the ABCB1 polymorphisms are in linkage disequilibrium and thus inter-dependent (Table 2); no correction for multiple testing was done.

Table 1. Variations of 1199G > A, 3435C > T, 2677G > A/T and 1236C > T polymorphisms in the ABCB1 gene

|    | GG | GA | GT | AA | AT | CC | CT | TT |
|----|----|----|----|----|----|----|----|----|
| All patients | 1199G > A | 477 (92) | 41 (8) | — | 0 | — | — | — |
| 1236C > T | — | — | 170 (33) | 248 (48) | 100 (19) | — | — | — |
| 2677G > A/T | 247 (47) | 0 | 8 (2) | — | — | — | — | — |
| Donors | 1199G > A | 188 (94) | 12 (6) | — | 0 | — | — | — |
| 94 Women | — | — | — | — | — | 62 (31) | 93 (46.5) | 45 (22.5) |
| 106 Men | 2677G > A/T | 62 (31) | 3 (1.5) | 86 (43) | 0 | 5 (2.5) | — | — | — |
| 3435C > T | — | — | — | — | — | — | — | — |
| HDM 1 | 1199G > A | 115 (93) | 9 (7) | — | 0 | — | — | — |
| 124 Patients | 2677G > A/T | 41 (33) | 3 (3) | 60 (48) | 0 | 0 | — | — | — |
| Induction | 2677G > A/T | 70 (30) | 9 (4) | 101 (44) | 0 | 2 (1) | — | — | — |
| 3435C > T | — | — | — | — | — | — | — | — |
| (doxo, vinc., prednisolone) | — | — | — | — | — | — | — | — |

Percentages in brackets (%). The number of patients in each study from the top, where all four polymorphisms were successfully analyzed, was 514, 200, 124 and 230.

between 20 and 26 h after the first high-dose MTX (HD-MTX) course were retrievable (n = 124).

The Ethics Committee of Copenhagen (J.nr. 01-259108) and the Danish Data Protection Authority (J.nr. 2005-41-4808) approved the study.

Risk grouping and therapy

According to the NOPHO protocol (ALL92 and ALL2000),1,9 the children were classified as high-risk ALL if at least one of the following parameters were present: white blood cell count > 50 × 10⁹ l⁻¹, T-lineage ALL, presence of central nervous system or testicular ALL, translocations t(9;22)(q34;q11) or t(4;11)(q21;q23) (any presence of central nervous system or testicular ALL, translocations t(1;19) or hypodiploidy (ALL2000 only), the presence of lymphomatous ALL or mediasternal lymphoma, and/or a poor treatment response (> 25% blasts in bone-marrow day 15 or > 5% blasts in bone-marrow day 29).

During the first 4 weeks of induction therapy, all patients received intrathecal MTX on days 1, 8, 15 and 29, prednisolone (60 mg m⁻² per day), weekly vincristine (2.0 mg m⁻², maximum 2.0 mg) and doxorubicin (40 mg m⁻²) on days 1 and 22. In addition, patients with high-risk ALL received an extra dose of doxorubicin on day 8 in the ALL92 protocol. In ALL92 doxorubicin was given as a 24 h infusion, but as a 4-h infusion in ALL2000.1,9

The post-remission consolidation, re-induction and maintenance therapy phases have previously been described in details.1,9

HD-MTX: Children with low-risk ALL (Supplementary Figure S1a) received HD-MTX courses (5 g m⁻² per day) three to four times during consolidation at an interval of 14–28 days and five times during maintenance therapy at an interval of 8 weeks. Leucovorin rescue (15 mg m⁻²) was given from 36 h after start of each HD-MTX course in the ALL92 protocol and from 42 h in the ALL2000 protocol, and was continued at 6-h intervals until plasma-MTX was below 200 nmol l⁻¹.20

Children with high-risk ALL received 8 mg m⁻² HD-MTX courses two to four times during the consolidation period, with an interval of at least 42 days (Supplementary Figure S1b). The initial leucovorin rescue dose at 36 h was 50 mg m⁻² (ALL2000: 15 mg m⁻²), followed by leucovorin rescue (15 mg m⁻²) at 6-h intervals until plasma-MTX was below 200 nmol l⁻¹.20

Intrathecal MTX (8–12 mg depending on age) was administered during HD-MTX courses in both low- and high-risk ALL protocols.

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Table 2. Distribution of ABCB1 haplotypes

| Haplotype       | 2677GG | 2677GA | 2677GT | 2677TT | 2677TA |
|-----------------|--------|--------|--------|--------|--------|
| 3435CC/1236CC   | 35–15%b| 4–<2%a | 0      | 0      | 0      |
| 3435CC/1236CT   | 2–<1%  | 0      | 3–<2%a | 1–<1%  | 0      |
| 3435CC/1236TT   | 0      | 0      | 1–<1%  | 0      | 0      |
| 3435CT/1236CC   | 26–11%a| 3–<2%  | 4–<2%  | 3–<2%  | 0      |
| 3435CT/1236CT   | 1–<1%  | 1–<1%  | 0      | 3–<2%  | 3–<2%  |
| 3435CT/1236TT   | 1–<1%  | 0      | 2–<1%  | 0      | 0      |
| 3435TT/1236CC   | 5–2%   | 1–<1%  | 0      | 16–7%  | 2–<1%  |
| 3435TT/1236CT   | 0      | 0      | 0      | 0      | 41–18% |
| 3435TT/1236TT   | 0      | 0      | 0      | 0      | 0      |

Two hundred and thirty patients from the induction-therapy toxicity study with all genotypes measured. The haplotype frequencies are non-random distributed, as only 5 (in boldface) out of 54 possible haplotypes have >5% patients and count for >80% of all patients. There were no patients with a 2677AA variant. The haplotypes with strong linkage disequilibrium 2677G>T/A and 1236C>T has only three haplotypes with >5% patients (GG/CC, GT/CT and TT/TT). There were 92% patients with 1199GG and 8% with 1199GA. *b6% of 1199GA (1 patient). *b75% of 1199GA (12 patients).

1236C>T, n = 518, P = 0.63; 2677G>T/A, n = 520, P = 0.51; and 3435C>T, n = 517, P = 0.22). The genotype frequencies in both donor and patient cohorts (Table 1) were in Hardy–Weinberg equilibrium.

In total, 74 patients developed relapse 0.2–8.3 years from diagnosis (median: 2.6 years). Five developed a second malignancy, 22 patients died before first HD-MTX and 5 patients died in first remission.

The 41 patients with 1199GA variant had more than twofold increased risk of relapse (HR: 2.86, 95% CI: 1.52–5.26, P = 0.0011) compared with the 477 patients with 1199GG variant. No patients had the 1199AA variant. Within the high-risk group (Figure 1a), the 15 patients with 1199GA variant had more than fourfold increased risk of relapse compared with the 149 patients with 1199GG variant in both univariate (relapse: 37, HR: 4.55, 95% CI: 2.08–10, P = 0.0001) and multivariate analysis adjusted for protocol, gender and immunophenotype (HR: 4.34, 95% CI: 2.04–9.09, P = 0.0001). In contrast, we found no statistically significant differences in risk of relapse in the low-risk group by univariate analysis (relapse: 32, HR: 1.37, 95% CI: 0.42–4.55, n = 354, P = 0.60) or multivariate analysis (HR: 1.39, 95% CI: 0.42–4.55, P = 0.59; Figure 1b), despite that data did not indicate interactions between the genotypes and risk groups. No deaths or second malignancies were registered in the 1199GA patient group compared with 16 deaths and 5 secondary malignancies in the 1199GG group. The 5-year overall probability of EFS (pEFS5y) for patients with 1199GA variant was 68% (95% CI: 50–80) and for patients with 1199GG variant was 83% (95% CI: 79–86).

The 3435C>T polymorphism was significantly associated with risk of relapse (P = 0.02). Compared with 96 patients with the 3435CC variant, 421 patients with the 3435CT or 3435CT variants had, respectively, 61% (HR: 0.39, 95% CI: 0.20–0.76, P = 0.006) and 40% (HR: 0.60, 95% CI: 0.34–1.03, P = 0.06) reduced risks of relapse in multivariate analysis adjusted for risk, immunophenotype, protocol and gender (Figure 1c).

Only one death and no second malignancy were found in the 3435CC variant group compared with eight deaths and four secondary malignancies in the 3435CT variant group, and six deaths and one secondary malignancy in the 3435TT variant group. This led to a pEFS5y of 78% (95% CI: 68–86) for the 3435CC patient group and 83% (95% CI: 79–86) for the 3435CT/TT patient group.

In risk-group-stratified analyses, no statistically significant differences in relapse risk were found for either the 1236C>T polymorphism (P = 0.37) or 2677G>A/T polymorphism (P = 0.98). Nor did we find any statistically significant difference in risk of relapse between the ABCB1 haplotypes (Table 3).

The 22 patients harboring both 1199GA and 3435CC variants had almost threefold greater risk of relapse compared with the remaining 402 patients (HR: 2.96, 95% CI: 1.36–6.49, P = 0.007).

We looked at interactions between RFC80G>A and both 1199G>A and 3435C>T with respect to risk of relapse, but no statistically significant interaction between 1199G>A and RFC80G>A was found (P = 0.14), and interaction between 3435C>T and RFC80G>A was not tested, as none of the 32 patients with RFC80AA and 3435TT had a relapse. Instead, an additive model including RFC80G>A and 3435C>T as covariates was used, and showed that both polymorphisms had effect on outcome (P = 0.024). When combining these two polymorphisms, the 78 patients with RFC80GA/GG and 3435CC had an almost fivefold higher risk of relapse (HR: 4.75, 95% CI: 1.70–13.26), and the 207 patients with RFC80GG/GA and 3435CT had almost threefold higher risk of relapse (HR: 2.87, 95% CI: 1.08–7.66) when compared with the 32 patients with both RFC80AA and 3435TT. Multivariate analysis including these polymorphisms, risk group, immunophenotype and protocol gave very similar HRs.

As we have previously demonstrated that SLC19A1 80GA and SLC19A1 80GG had similar effects on outcome, another statistical analysis was made, where SLC19A1 variants were grouped in two groups (80AA and 80GA/GG). The ABCB1 1199GA and the 3435CC combined gave the same hazard rate as each independently and were therefore grouped in one group, and the remaining variants, 1199GG, 3435CT and 3435TT, were grouped together. The multivariate Cox regression analysis showed an interaction between polymorphisms in the ABCB1 and SLC19A1 genes (P = 0.048). Patients with SLC19A1 80GG/GA and ABCB1 1199GA/3435CC had almost twofold higher risk of relapse as compared with patients with other ABCB1 variants (HR: 1.89, 95% CI: 1.10–3.27). However, patients with SLC19A1 80AA and ABCB1 1199GA/3435CC had more than 10-fold higher risk of relapse than patients with other ABCB1 variants (HR: 10.89, 95% CI: 2.10–56.38). This indicates a synergistic rather than an additive effect of the ABCB1 and SLC19A1 genes, and implies that both genes have an impact on MTX. Thus, MTX could be a substrate for P-gp. This is supported by studies showing impact of ABCB1 3435C>T variants on the toxicity or disease score in MTX monotherapy studies, which suggest that ABCB1 variants influence MTX efflux from leukemic cells.

Both univariate and multivariate analyses showed no statistical difference in end-of-therapy MTX plasma levels in relation to any of the ABCB1 variants (P > 0.20 in all univariate and multivariate analyses). No pharmacokinetic studies were carried out for doxorubicin, prednisolone and vincristine.

In both univariate and multivariate analyses, ABCB1 polymorphisms significantly influenced the risk of toxicities both after induction therapy and HD-MTX, being most pronounced for 3435C>T (Tables 4 and 5). During induction therapy, patients with the 3435CT or 3435CC variant had significantly less anemia...
(multivariate $P = 0.01$ and $P = 0.01$, respectively) and less thrombocytopenia (multivariate $P = 0.0002$ and $P < 0.0001$, respectively) when their nadir values were compared with patients with the 3435TT variant (Table 4). Similar effects were seen for neutrophil nadirs when comparing 3435TT patients with patients with the 3435CT variant.

Considering toxicity after HD-MTX, patients with the 3435CC variant had statistically significant higher alanine aminotransferase levels (univariate $P = 0.03$, multivariate $P = 0.06$), when compared with patients with 3435CT or 3435TT variants (Table 5). Including end-of-infusion plasma MTX levels and the RFC80G>A polymorphisms in the models did not significantly alter this effect of 3435C>T.

In the haplotype with strongest linkage disequilibrium 2677GG/1236CC, 2677CT/1236CT and 2677TT/1236TT, we did not see any statistically significant difference in hemoglobin values ($P = 0.11$, multivariate $P = 0.16$), but platelets and neutrophils nadir during induction therapy were statistically significantly lower in patients with 2677TT/1236TT genotype compared with patients with the 1236CC/2677GG genotype (platelets: $P = 0.01$, multivariate $P = 0.02$; neutrophils: $P = 0.006$, multivariate $P = 0.06$).

**DISCUSSION**

The study underlines the delicate balance between efficacy and toxicity when treating children with ALL with potent chemo-
therapeutic agents. 6 We have previously demonstrated that SNPs, associated with reduced relapse rates, in the reduced folate carrier 6 and thiopurine methyltransferase, 7 may lead to increased risk of toxicities or even second cancers, 23 which ultimately can precipitate in no difference on EFS in a large patient cohort. 24 Furthermore, risk of toxicities or even second cancers, 23 which ultimately can precipitate in no difference on EFS in a large patient cohort.

Table 4. Nadir of platelets, hemoglobin after induction therapy (doxorubicin, prednisolone, vincristine) and relation to ABCB1 polymorphism

| Polymorphism       | Univariate | Multivariate |
|--------------------|------------|--------------|
| Hemoglobin (mmol l⁻¹) |            |              |
| 1199GA             | 0.30       | 0.22         |
| 1199GG baseline    | —          | —            |
| 1236CC             | 0.17       | 0.14         |
| 1236CT             | 0.09       | 0.15         |
| 1236TT baseline    | —          | —            |
| 2677GG             | 0.09       | 0.09         |
| 2677GA             | 0.07       | 0.07         |
| 2677GT < 0.01*      | 0.02*      |              |
| 2677TA             | 0.12       | 0.08         |
| 2677TT baseline    | —          | —            |
| 3435CC             | 0.02*      | 0.01*        |
| 3435CT             | 0.005*     | 0.01*        |
| 3435TT baseline    | —          | —            |
| Platelets (10⁹ l⁻¹) |            |              |
| 1199GA             | 0.65       | 0.84         |
| 1199GG baseline    | —          | —            |
| 1236CC             | 0.14       | 0.06         |
| 1236CT             | 0.04*      | 0.08         |
| 1236TT baseline    | —          | —            |
| 2677GG             | 0.04*      | 0.02*        |
| 2677GA             | 0.004*     | 0.007*       |
| 2677GT             | 0.007*     | 0.01*        |
| 2677TA             | 0.10       | 0.06         |
| 2677TT baseline    | —          | —            |
| 3435CC             | 0.0002*    | < 0.001*     |
| 3435CT             | < 0.001*   | 0.0002*      |
| 3435TT baseline    | —          | —            |
| Neutrophils (10⁹ l⁻¹) |            |              |
| 1199GA             | 0.99       | 0.86         |
| 1199GG baseline    | —          | —            |
| 1236CC             | 0.22       | 0.23         |
| 1236CT             | 0.006*     | 0.002*       |
| 1236TT baseline    | —          | —            |
| 2677GG             | 0.94       | 0.94         |
| 2677GA             | 0.004*     | 0.004*       |
| 2677GT             | 0.04*      | 0.14         |
| 2677TA             | 0.53       | 0.90         |
| 2677TT baseline    | —          | —            |
| 3435CC             | 0.49       | 0.31         |
| 3435CT             | 0.01*      | 0.15         |
| 3435TT baseline    | —          | —            |

Abbreviation: ALAT, alanine aminotransferase. P-values are based on comparison between the variant and the variant below named baseline in an overall linear mixed model with repeated measurement. The variant most different in value from the other variants was chosen as baseline. Values in brackets are 95% confidence intervals. *P < 0.05 in bold. No interaction between doxorubicin treatment periods and polymorphisms could be demonstrated; thus, the percentage difference in values between the variants (that is, 1236CC, 1236CT and 1236TT) in each parameter (that is, ALAT, hemoglobin) was the same in all time periods.

HEK and LLC-PK1 cells have showed increased drug resistance to doxorubicin and/or vincristine for the 1199GA variants when compared with the 1199GG variant. 25-27 Lower efflux of vincristine in patients with the 1199GG variants compared with patients with the 1199GA variant may thus explain the effect on relapse. De Meyer et al. 28 has found better renal function in kidneys from donors with the 1199GA variant, thus another explanation could be a lower renal function in patients with the 1199GG variant allowing drugs to be retained longer in the organism. Still it remains to be determined whether patients with the unfavorable 1199GA might benefit from higher doses of vincristine or protocols with more emphasis on drugs with a lower affinity for P-gp.

The inferior outcome for ALL patients with the 3435CC genotype found in this study is in agreement with previous studies of...
polymorphisms.

MTX into the cell, and as HD-MTX is widely used in NOPHO other studies 17,18 have indicated that MTX could be a substrate for patients included in the present induction therapy study. contained at least 5% of the patients and accounted for > 80% of all polymorphisms are in strong linkage disequilibrium, and that both are ABCB1 4 1236C acid change, the C 3435C alters P-gp activity. This is consistent with our of relapse, but not with the 2677G A/T and the 1236C/T variants on the toxicity or disease score in both the present and in MTX monotherapy studies17,18 suggest that ABCB1 variants influence MTX efflux from leukemic cells.

Wang et al.35 found that in spite of the assumed linkage disequilibrium between 1236C>T, 2677G>A/T and 3435C>T, only 3435C>T accounted for altered RNA expression levels. Kimchi-Sarfaty et al.36 have hypothesized that even if the 3435C>T nucleotide substitution does not result in an amino acid change, the C>T substitution may cause an alteration in toxicity after the first HD-MTX treatment. In a previous study, we 34 found that in spite of the assumed linkage disequilibrium between 1236C>T, 2677G>A/T and 3435C>T, only 3435C>T accounted for altered RNA expression levels. Kimchi-Sarfaty et al.36 have hypothesized that even if the 3435C>T nucleotide substitution does not result in an amino acid change, the C>T substitution may cause an alteration in the more profound bone-marrow toxicity found in patients with the 3435TT variant after the doxorubicin-containing induction therapy is in accordance with their reduced risk of relapse and increased risk of death or second cancer. Among patients with a favorable prognosis due to the ABCB1 variants they harbor, future studies are needed to explore which additional genetic polymorphisms cause a small subset of these patients to die in remission or develop a second cancer. Such inherited genetic variants may involve glutathione S-transferases, cytochrome P-450 enzymes, quinine oxoreductase, or the folate pathway.38–41

Although MTX plasma levels after HD-MTX did not differ significantly between subsets of patients defined by their ABCB1 variants, the 3435CC variant was associated with more liver toxicity after the first HD-MTX treatment. In a previous study, we similarly reported higher levels of alanine aminotransferase among the patients with the highest risk of relapse.6 It remains to be clarified whether this reflects intracellular hepatic MTX exposure, a shift in the metabolism in the liver due to the genetic variant or differences in folate disposition or other pathways.

The lack of statistically significant associations between ABCB1 variants and risk of ALL is in agreement with a Hungarian study,42 although other studies with Indian43 and Polish44 patients/controls found higher incidences of ALL among patients with the 3435TT variant. Importantly, the frequency of 3435TT among patients in the Polish study, but not their controls, was very similar to the frequencies found in our and other studies with white individuals, such as the 1000genom project, HapMap studies and

### Table 5. Nadir of platelets, hemoglobin, lymphocytes and neutrophils, and maximum ALAT value after first high-dose methotrexate and relation to ABCB1 polymorphism

|                | ALAT (Ul−1) | Hemoglobin (mmol−1) | Platelets (109l−1) | Lymphocytes (109l−1) | Neutrophils (109l−1) |
|----------------|-------------|---------------------|-------------------|----------------------|----------------------|
| 1199GG         | 147 (114–188) | 5.6 (5.5–5.8)       | 84 (69–102)       | 0.7 (0.7–0.8)        | 0.8 (0.7–1.0)        |
| 1199GA         | 225 (98–518)  | 5.3 (4.8–5.9)       | 31 (16–62)        | 0.5 (0.3–0.8)        | 0.6 (0.3–1.1)        |
| P              | 0.32        | 0.18                | <0.01*            | 0.11                 | 0.25                 |
| P-values univariate | 0.99        | 0.78                | 0.06              | 0.11                 | 0.42                 |
| 1236CC         | 162 (109–239) | 5.5 (5.3–5.8)       | 64 (47–88)        | 0.6 (0.5–0.8)        | 0.6 (0.5–0.8)        |
| 1236CT         | 166 (115–240) | 5.6 (5.4–5.8)       | 87 (66–115)       | 0.8 (0.7–1.0)        | 1.0 (0.8–1.2)        |
| 1236TT         | 114 (67–194)  | 5.7 (5.4–6.1)       | 89 (57–137)       | 0.7 (0.5–0.9)        | 0.9 (0.7–1.3)        |
| P              | 0.10        | 0.51                | 0.30              | 0.15                 | 0.03*                |
| P-values univariate | 0.93        | 0.66                | 0.22              | 0.22                 | 0.03*                |
| 2677GG         | 150 (99–227)  | 5.4 (5.2–5.7)       | 58 (42–81)        | 0.5 (0.5–0.7)        | 0.6 (0.5–0.8)        |
| 2677GA         | 427 (72–2522) | 6.7 (5.7–7.8)       | 175 (53–580)      | 0.8 (0.4–1.6)        | 1.1 (0.4–2.7)        |
| 2677GT         | 155 (110–221) | 5.6 (5.4–5.9)       | 89 (68–116)       | 0.7 (0.7–1.0)        | 0.9 (0.8–1.2)        |
| 2677TT         | 132 (74–234)  | 5.6 (5.3–6.0)       | 87 (55–138)       | 0.7 (0.5–0.9)        | 0.9 (0.7–1.3)        |
| n              | 109         | 124                 | 124               | 121                   | 118                  |
| P-values univariate | 0.66        | 0.10                | 0.11              | 0.12                 | 0.07                 |
| P-values multivariate | 0.11        | 0.30                | 0.21              | 0.10                 | 0.08                 |
| 3435CC         | 280 (167–470) | 5.5 (5.2–5.9)       | 53 (34–82)        | 0.7 (0.5–0.9)        | 0.8 (0.5–1.1)        |
| 3435CT         | 142 (103–195) | 5.5 (5.3–5.7)       | 91 (70–117)       | 0.8 (0.7–0.9)        | 0.9 (0.7–1.0)        |
| 3435TT         | 111 (70–174)  | 5.9 (5.6–6.2)       | 76 (53–109)       | 0.6 (0.5–0.8)        | 0.8 (0.6–1.1)        |
| n              | 109         | 124                 | 124               | 121                   | 118                  |
| P-values univariate | 0.03*        | 0.08                | 0.11              | 0.23                 | 0.08                 |
| P-values multivariate | 0.06        | 0.35                | 0.02*             | 0.13                 | 0.55                 |

Abbreviation: ALAT, alanine aminotransferase. Values in brackets are 95% confidence intervals. *Statistically significance below 0.05 in bold. Adjustment variables: gender, risk group, protocol (NOPHO92/NOPHO2000) and immunophenotype.

These clinical observations are supported by in vitro studies of CD56+ cells and the duodenum, which have shown lower P-gp expression and/or P-gp function in 3435TT.32,33 However, as seen with the 1199G>A polymorphism, the reduced relapse rates for patients with the 3435CT and 3435TT variant were jeopardized by their higher frequency of death in remission and secondary malignancies.

In agreement with a French study,34 the present study has demonstrated that the ABCB1 1236C>T and 2677G>T/A polymorphisms are in strong linkage disequilibrium, and that both are in moderate linkage disequilibrium with the 3435C>T polymorphism. Accordingly, out of 54 possible haplotypes only 5 haplotypes contained at least 5% of the patients and accounted for > 80% of all patients included in the present induction therapy study.

The lack of statistically significant associations between ABCB1 variants and risk of ALL is in agreement with a Hungarian study,42 although other studies with Indian43 and Polish44 patients/controls found higher incidences of ALL among patients with the 3435TT variant. Importantly, the frequency of 3435TT among patients in the Polish study, but not their controls, was very similar to the frequencies found in our and other studies with white individuals, such as the 1000genom project, HapMap studies and
the Hungarian study.\textsuperscript{42} Based on the magnitude of the present study with more than 750 individuals included, the explored \textit{ABCB1} variant does not seem to be associated with the risk of ALL.

In the present study, we found that the \textit{ABCB1} genetic variants 1199G>A and 3435C>T are associated with outcome in childhood ALL. Overall, >29% of patients with 1199GA variant had relapse, in the high-risk group >60%; thus, patients with 1199G>A polymorphism should be observed more intensively. To further elucidate the role of pharmacogenetics and the biological mechanisms involved, we would recommend pharmacokinetic studies with intracellular drug-level measurements. This population-based cohort study emphasizes the need to add pharmacogenetic data to the conventional parameters routinely included in survival analyses (for example, leukemia karyotype or post-remission minimal residual disease), to improve prediction of risk in both relapse and toxicity. Further prospective studies are needed to determine whether dose adjustments according to host pharmacogenomics can significantly improve outcome in childhood ALL without unacceptable increments in toxicity.

\section*{CONFLICT OF INTEREST}
The authors declare no conflicts of interest.

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