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Pharmacogenetic study of anthracycline therapy in childhood acute lymphoblastic leukemia

Childhood acute lymphoblastic leukemia (ALL) is highly curable disease; most of the children recover and live for decades. However, chemotherapy has many acute and chronic side effects developing during treatment, years or even decades after treatment. There is a high interindividual variability in the occurrence and severity of the side effects; therefore, it is assumed that their formation is affected by the genetic background of the individuals. Our goal in this project was the pharmacogenetic investigation of adverse drug reactions of chemotherapeutic agents used in the treatment of ALL, particularly with regard to the long term cardiotoxicity of anthracyclines. We also analyzed acute adverse drug reactions, side effects of other drugs, survival of the patients and built our data- and biobank.

During the project we collected data and blood samples from ALL patients. The samples were taken from patients in remission at their annual checkup, or at the time of diagnosis. These patients were treated in the Hungarian pediatric hematology centers (in Budapest, Debrecen, Miskolc, Pécs, Szeged or Szombathely) or in countries participating in the recently used international ALL IC-BFM 2009 treatment protocol (Argentina, Slovakia, Slovenia and Greece). We designed and built up a Microsoft Access based database (named ALL_database) and located on a network folder accessible with a common username and password known by all members of the ALL research group. The biobank and database is aimed to be used in our future projects.

During the project the cardiotoxicity of anthracyclines, myelosuppression of cytarabine and the survival of the patients were also studied. Pharmacogenetic study of cytarabine and the survival analysis of the patients are already published therefore briefly presented here. The results regarding pharmacogenetics of anthracycline therapy are described in details in our recently submitted article. The manuscript of this paper is attached to the end of this report.

Survival of our patients in association with SNPs in *CYP3A4* and *CYP3A5* (cytochrome P450, family 3, subfamily A, polypeptide 4 and 5) genes were investigated. CYP3A4 is the most abundant CYP450 enzyme in the liver and the gut, and the main drug-metabolizing protein in humans. It plays an important role in the metabolisms of many drugs used in ALL therapy, including doxorubicin. We genotyped 8 common SNPs in the *CYP3A4* and *CYP3A5* genes in 511 children with ALL and investigated whether they influenced the survival of the patients. Significant association between the overall survival rates and the common rs2246709 SNP in the *CYP3A4* gene was observed (p=0.0028). We calculated new risk assessments involving the gender-rs2246709 interaction. If this finding is confirmed in other populations, it can have a considerable prognostic significance.

Cytarabine (cytosine arabinoside, Ara-C) is a chemotherapeutical agent used in the treatment of pediatric ALL in combination with methotrexate, cyclophosphamide and doxorubicin. The major toxicities of ara-C at standard doses are myelosuppression, mucositis and infection. Cytopenias as the result of myelosuppression can rapidly become life threatening or affect the quality of life, often leading to interruptions in chemotherapy and a subsequent increase in the risk of relapse. We studied 8 SNPs in 5 genes which are important in the metabolism and transport of Ara-C, in 144 patients with childhood ALL. *DCK* (deoxycytidine kinase) rs12648166 and *DCK* rs4694362 SNPs were associated with hematologic toxicity (OR=2.63, CI 95% = 1.37–5.04, p= 0.0036 and OR = 2.53, CI 95% = 1.34–4.80, p= 0.0044, respectively). Our results indicate that *DCK* polymorphisms might be important genetic risk factors for hematologic toxicity during ALL treatment with ara-C.

During the project we prepared a review article on pharmacogenetics of anthracyclines because we realized there was no current review in this field. The main message of this article is that there are some promising potential candidate genes which might have effect on anthracycline induced cardiotoxicity. However, the pharmacogenetics of anthracyclines is a narrow research area. This implies the difficulties to investigate cardiotoxicity of anthracyclines, the studied populations are

diverse and the associations are controversial. International cooperation is required to be able to gather patient population with appropriate statistical power. The confirmed variants should be tested in clinical trials with long-term follow-up, because of the late cardiotoxicity. Today we are only at the beginning of this process, but regarding the huge development of the last decades, we can be sure that the number of the usable pharmacogenomic tests or personal therapies will be expanded in the future and the clinical usage of these trials will be present.
We presented our data and preliminary results at several national and international conferences during the project.

The manuscript submitted (26th of July, 2017) based on the results of pharmacogenetics of anthracycline induced cardiotoxicity:

Variations in *ABCC2, NQO1* and *CYP3A5* genes might influence the risk of cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma

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Abstract

Background: The treatment of acute lymphoblastic leukemia (ALL) and osteosarcoma (OSC) is very effective: the vast majorities of patients recover and survive for decades. However, they still need to face serious adverse effects of the chemotherapy. One of these is cardiotoxicity which may lead to progressive heart failure in the long term. Cardiotoxicity is contributed mainly to the use of anthracyclines and might have genetic risk factors. Our goal was to test the association between left ventricular function and genetic polymorphisms of xenobiotic transporters and metabolizing enzymes.

Methods: Echocardiography data from medical records of 661 pediatric ALL and OSC patients were collected from the period 1989-2015. Fractional shortening (FS) and ejection fraction (EF) were determined, 67 single nucleotide polymorphisms (SNPs) in 21 genes were genotyped.

Results: Our results indicate that polymorphisms in *ABCC2, NQO1* and *CYP3A5* genes might influence the left ventricular parameters. Patients with *ABCC2* rs3740066 GG genotype had lower FS during the acute phase of therapy and 5-10 years after treatment (p = 7.38E-03, p = 7.11E-04, respectively). *NQO1* rs1043470 rare T allele was associated with lower left ventricular function in the acute phase and 5-10 years after the diagnosis (p = 4.28E-03 and 5.82E-03, respectively). *CYP3A5* rs4646450 was associated with cardiotoxicity in ALL cases with FS below 28% (p = 7.00E-03; OR = 6.56 (1.68-25.71)).

Conclusions: Genetic variants in transporters and metabolic enzymes might modulate the individual risk to cardiac toxicity after chemotherapy.

Background

Acute lymphoblastic leukemia (ALL) and osteosarcoma (OSC) occur predominantly in pediatric patients. ALL is the most common childhood hematological malignancy; about 25-30% of childhood cancers are acute leukemias, 80% of which are ALL [1]. Osteosarcoma is a rare bone disease that affects 3-4 people per million and represents 3% of pediatric tumors [2]. Nowadays, the treatment of ALL is very effective: the majority of patients are cured for the long term. The 5-year event-free survival rate (EFS) is around 80% for ALL and 60% for osteosarcoma patients [2–6].

Unfortunately, despite the use of the indeed efficacious chemotherapeutic drugs, patients have to face serious side effects. Therefore, the primary goal of the scientific research is now not only to increase the survival rate, but to identify and reduce the acute and late toxic side effects of chemotherapy and to improve the quality of life in adulthood [4,7–10]. The risk for developing health problems is increased 8-fold in pediatric cancer survivors within 30-40 years after diagnosis compared to their siblings; 50% of them experience severe, disabling, or life-threatening event, including death by the age of 50. One of the late toxic side effects of the chemotherapy in childhood ALL is cardiotoxicity [5,11–13]. A 30-year-old survivor might face treatment-related cardiac damage usually present in much older patients [14–16]. There is a need for preventing cardiac damage especially in children, because they can live for decades after treatment [17]. Several treatment regimens introduce dose reduction in some cases to decrease late side effects, but patients surviving childhood cancer still require long-term follow-up for their prevention and treatment. The constant monitoring of patients is important in order to identify subclinical anomalies before the clinical symptoms occur [18–21].

Anthracyclines are among the most essential and highly effective chemotherapeutic agents in the treatment for both hematological malignancies and solid tumors (e.g. leukemia, lymphoma, breast cancer, and sarcoma) [22–26], and belong to the backbone of childhood ALL and osteosarcoma treatment protocols all around the globe [4]. However, anthracyclines damage cardiomyocytes which can manifest during the therapy, years or even decades after the exposure to the chemotherapeutic agents [19,27–31]. The pathophysiology of the toxicity is not completely understood, but it is likely that both the drug and its metabolites are cardiotoxic [32,33]. Despite the fact that the anthracycline-induced cardiotoxicity (ACT) is well known, the toxicity is unpredictable [34–36]. Risk factors of ACT include cumulative anthracycline dose, age at treatment, length of follow-up, gender, genetic factors, rate of anthracycline administration, concomitant radiation, other chemotherapy agents, black race, and trisomy 21 [37]. Cardiotoxicity is more frequent above the cumulative dose of 500 mg/m^2 of anthracyclines; therefore, most of the treatment protocols limit the use of these drugs below this value [19,38]. However, there were patients with cardiac problems who received very low doses of anthracyclines while others were administered with high doses and escaped the side effect. The variable development of anthracycline cardiotoxicity suggests that the genetic background of the patients is important in this side effect [39–47].

Pharmacogenomics of anthracyclines is a narrow field of the research area. In the investigations both patients and treatment protocols are heterogeneous; therefore, the comparison of the results is difficult. Half of the studies were executed on former pediatric patient populations and there is an urgent pressure to translate these research evidences into clinical practice [48–52]. For this purpose one of the newest publications of the topic contains evidence-based clinical practice recommendations for pharmacogenomic testing. They emphasize *RARG* (Retinoic Acid Receptor Gamma) rs2229774, *SLC28A3* (Solute Carrier Family 28 Member 3) rs7853758 and *UGT1A6* (UDP Glucuronosyltransferase Family 1 Member A6) rs17863783 as genetic variants which have the strongest association with ACT [51]. SNPs in the genes of anthracycline transporters *ABCB1* (ATP Binding Cassette Subfamily B Member 1) and *ABCC1* (ATP Binding Cassette Subfamily C Member 1) also associated with ACT in anthracycline-treated children [39]. *SLC22A7* (Solute Carrier Family 22 Member 7) and *SLC22A17* (Solute Carrier Family 22 Member 17) were also in connection with cardiotoxicity among patients with osteosarcoma [2]. Studying adult patients with non-Hodgkin lymphoma Wojnowski et al. found associations between cardiotoxicity and SNPs in

the NAD(P)H oxidase complex, and *ABCC1* and *ABCC2* (ATP Binding Cassette Subfamily C Member 2) transporters [40]. The function of cytochrome P450 enzymes is crucial in the metabolism of several drugs, among their coding genes e.g.: *CYP3A5* (Cytochrome P450 Family 3 Subfamily A Member 5) had importance in this context. In a genome wide association study *RARG* has been identified as a promising gene [50].

These results support the hypothesis that the genetic features of the patients may influence the chemotherapy-related cardiotoxicity. However, further independent studies are needed to confirm these findings. At present the dose-limiting side effect of anthracyclines is the late-onset cardiotoxicity. The definition of genotype-based maximum cumulative doses may contribute to both further improving cure rates and limiting toxicity. Our goal was to investigate the association between SNPs of transporters, enzymes and the acute or late left ventricular damage in pediatric acute lymphoblastic leukemia and osteosarcoma patients.

Methods

Patients

In this study, patients with pediatric acute lymphoblastic leukemia (ALL) or osteosarcoma (OSC), aged 0–18 years at diagnosis, were enrolled retrospectively (n = 680). Children with ALL had undergone chemotherapy between 1989 and 2015 in 6 Hungarian pediatric oncology centers, OSC patients were treated between 1989 and 2015 at the Second Department of Pediatrics, Semmelweis University. Detailed description of the ALL (n = 622) and OSC (n = 39) patients included are shown in Table 1. Patients were excluded from the analysis because of Down syndrome (n = 7), previous cardiac problems or concomitant disease with potential cardiac complications (n = 12).

Patients with ALL were treated according to one of the following study protocols: ALL BFM (Berlin–Frankfurt–Münster) 88, ALL BFM 90, ALL BFM 95, ALL IC-BFM (ALL Intercontinental) 2002 or ALL IC-BFM 2009; Interfant 98 or Interfant 2006. The chemotherapy regimen is described in detail in our previous article [53]. Patients diagnosed with ALL and OSC are treated with anthracyclines in the first year of chemotherapy. The used chemotherapy protocols differed slightly in the number or dosage of anthracycline-administrations. In the low-risk and medium-risk groups of patients with ALL the cumulative anthracycline (i.e. doxorubicin equivalent) doses were between 180 and 240 mg/m^2; in the high risk group and in the therapy of relapsed patients it were between 240-380 mg/m^2. The anthracycline treatment of patients with osteosarcoma was based on the COSS (German-Austrian-Swiss osteosarcoma study group) -86 and COSS-96 protocols. Treatment of patients with OSC included total anthracycline doses between 180-450 mg/m^2 administered in the first and 11th weeks for both of the standard and high risk groups and at the 20th and 29th weeks for the standard risk group. For detailed description of the used COSS based protocols see Hegyi et al., 2016 [54].

The patients were followed-up by echocardiography (ECHO) routinely in the clinical practice to monitor their left ventricular function. Left ventricular end-diastolic-diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) data were collected from the patients' medical records. Left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) were determined: EF = (LVEDD^3-LVESD^3)/LVEDD^3; FS= (LVEDD-LVESD)/LVEDD. Measurements were performed before the initiation of therapy, several times during the treatment and annually after finishing treatment. FS and EF data were analyzed in follow-up categories, which are: 1) at the diagnosis (used as a control); 2) in acute phase: during intensive chemotherapy phase; 3) during oral maintenance chemotherapy; 4) at the end of the treatment, which is after the oral maintenance chemotherapy period completed 2 or 3 years after the diagnosis; 5) from the end of the treatment until 5 years after the diagnosis; 6) 5-10 years after the diagnosis; 7) 10-15 years after the diagnosis; 8) more than 15 years after the diagnosis. For detailed description of the follow-up categories see Table 2. Not all of the ECHO records were available, because of the retrospective data collection. Only the data of the latest ECHO of each patient was used in each follow-up category, the redundant echocardiography measurements were excluded.

The worst heart function of each patient was used to define patients for the case-control type study. Cases were those who had echocardiograms with FS ≤ 28% at any time point during the follow-up (n=20); patients received the same chemotherapy but never had FS ≤ 28% were regarded as controls (n=641).

The alteration of FS was computed and analyzed as dichotomous variable, which was defined as the difference between the FS value at diagnosis and at the end of the treatment. In this study, patients with decreased FS (n = 105) were regarded as cases and those with increased FS (n = 94) were considered as controls. In a similar analysis, the difference between the FS value at diagnosis and at the last follow-up time point was also computed. With this method 170 cases and 152 controls were analyzed. Informed consent was requested from legal guardians of the patients. The study was approved by the Ethics Committee of the Hungarian Medical Research Council and conducted according to the principles of the Declaration of Helsinki.

Laboratory methods

Peripheral blood samples were taken from children with ALL in remission. DNA was isolated from blood using Qiagen isolation kits according to the manufacturer's instructions (QIAmp DNA Blood Midi or Maxi Kit, Qiagen, Hilden, Germany).

Based on the scientific literature, 70 single nucleotide polymorphisms (SNPs) in 21 genes were selected and genotyped. These genes encode transporters involved in drug import or elimination as well as enzymes in the metabolism of the chemotherapeutic agents. Candidate genes were chosen from previous candidate gene and GWA (genome-wide association) studies in this field. SNPs were selected prioritized on the basis of their estimated functionality in this order: non-synonymous SNPs, SNPs in promoter and 3'-UTR (3'-untranslated region) region, synonymous SNPs and intronic SNPs. During the selection the minor allele frequency data of the SNPs were validated using HapMap database release No. 27 and the CEU population (CEPH: Utah residents with ancestry from northern and western Europe) [55]. Information on the selected SNPs is shown in Table 3. Genotyping 63 of the SNPs was conducted using TaqMan® OpenArray™ Genotyping System (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions at the Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University (Budapest, Hungary). Detailed description of this procedures can be found in the article of Banlaki et al [56]. Other 7 SNPs were genotyped using KASPar (KBioscience Competitive Allele-Specific Polymerase chain reaction)-on-Demand prevalidated assays (LGC Genomics, Berlin, Germany) on 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific Waltham, MA, USA). The genotyping was unsuccessful in the case of three SNPs; call rate for the other SNPs was higher than 87.5%.

Statistical Analysis

Allele frequencies were tested by allele counting, HWE (Hardy-Weinberg equilibrium) was studied using the on-line software (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl), significant violation of HWE was considered where $p < 0.05$. In our case-control and follow-up studies, univariate and multivariate logistic regression and multi-adjusted general linear model were performed to investigate the influence of genetic polymorphisms on the left ventricular parameters. The analysis were adjusted for potential confounders, which were age at the time of diagnosis (years), gender (male-female), chemotherapy protocols (before 2000, after 2000 and OSC protocols), risk groups (standard, intermediate, high risk) and cumulative dose of anthracycline (≤ or > 240 mg/m^2). The analyses were performed studying the genotypes separately (11 vs. 12 vs. 22), using recessive (11/12 vs. 22) or dominant (11 vs. 12/22) models, with the common homozygotes signed as 11. EF and FS is indicated in the text with the standard error (SE) of the estimate of the mean. In order to deal with multiple comparisons the Benjamini-Hochberg false discovery rate (FDR) method with type I error rate of 10% ($p \leq 8{,}90E\text{-}03$) was applied as correction [57,58]. Analyses and preparation of the figures were performed using IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA) and

RStudio Version 1.0.136 (RStudio, Boston, MA, USA) programs. Estimated haplotype frequency in cases and controls and the haplotype-specific odds ratio (OR) were calculated by the Haploview 4.1 software [59]. The power of the analyses was calculated using SPSS Statistics 23.0 program. Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA) method was applied to test for potential interaction of the studied cofactors and SNPs. The method is described in our previous article [60].

Results

Association of 66 SNPs with the left ventricular parameters of childhood acute lymphoid leukemia (ALL) and osteosarcoma (OSC) patients was investigated. The minor allele frequencies of the SNPs are presented in Table 3. Genotype distributions were in Hardy-Weinberg equilibrium except for one SNP (*AKR1A1* (Aldo-Keto Reductase Family 1 Member A1) rs2934859) which was excluded from the analysis. The genotyping was unsuccessful in case of three SNPs. Minor allele frequencies in our population were found to be more than 7% for all of the SNPs (Table 3). The analyses performed on the population had adequate power (≥75%) for all of the results.

Follow-up analysis

The potential roles of genetic polymorphisms of drug transporters and metabolizing enzymes in the changes of the left ventricular function of children with ALL or OSC after anthracycline therapy were investigated. Ejection fraction (EF) and fractional shortening (FS) were used to monitor left ventricular function. Analyses were conducted on each follow-up category.

All of the SNPs were analyzed in relation with EF an FS in the acute lymphoid leukemia population in every follow-up category with all of the three models using multi-adjusted general linear model. Summary of the results from the analyses is shown in Figure 1. These are the ones with the lowest p value, except those with less than 5 patients in one group. SNPs with p values < 0.01 (Table 4) were analyzed in the whole population including both ALL and OSC patients.

Analyzing the population of ALL and OSC patients, *ABCC2* rs3740066 common GG genotype was associated with the poorest left ventricular function during intensive chemotherapy phase (acute phase) (Figure 2). Patients with GG genotype had lower mean FS (39.5% ± 1.06) value, compared to patients with AA genotype (mean FS = 42.9% ± 1.4; p = 7.38E-03). Larger difference could be observed also in 5-10 years after the diagnosis. The *ABCC2* rs3740066 GG genotype was associated (p = 7.11E-04) with lower mean FS (39.1% ± 0.5), whereas higher mean FS rates were related to the other genotypes (40.6% ± 0.5, 42.4% ± 0.8; AG, AA, respectively).

Patients with *NQO1* (NAD(P)H Quinone Dehydrogenase 1) rs1043470 rare T allele had significantly lower mean left ventricular function rates during both phase: in the intensive chemotherapy phase (acute phase) and 5-10 years after the diagnosis. In the acute phase the T allele was associated with lower mean FS (38.1% ± 1.2; p = 4.28E-03), while patients with at least one C allele had FS = 40.7% ± 0.9. Between 5-10 years after the therapy *NQO1* rs1043470 rare T allele was associated with lower mean FS (38.5% ± 0.7, p = 5.82E-03), while the values represented with the C allele were higher (FS = 40.6% ± 0.4).

SLC22A6 (Solute Carrier Family 22 Member 6) gene rs6591722 rare AA genotype was associated with lower mean FS (37.5% ± 0.9, p=1.71E-03) 5-10 years after the diagnosis compared to higher values of TT and TA genotypes (FS: 40.6% ± 0.4).

The other SNPs could not be validated on the total cohort. Results regarding ejection fraction were correlated to those described above with fractional shortening (data not shown).

Case-control analysis

Cases with FS ≤ 28% any time during the follow-up and controls (patients who received the same chemotherapy but never had FS ≤ 28%) were compared to assess the association of the genotype

with cardiotoxicity. Multi-adjusted logistic regression analyses were used on the full cohort, while univariate logistic regression analyses on various subpopulations. Case-control analysis was performed for ALL patients in case of all SNPs. Among these the ones with at least 2 cases in one group are shown in Figure 1. SNPs with p values < 0.01 (Table 4) were analyzed in the whole population including both ALL and OSC patients.

The allele distribution of the *CYP3A5* rs4646450 differed significantly between cases and controls in the combined cohort (ALL and OSC patients) (p = 4.81E-03; OR = 7.25 (1.83–28.78)). Among cases (n=20) 15% had TT genotype vs. in controls 2.8%. Subsequently, it was investigated whether *CYP3A5* rs4646450 was associated with cardiotoxicity in various subpopulations determined by clinical characteristics of the patients (Table 5). *CYP3A5* rs4646450 genotype was associated with cardiotoxity in patients with ALL (p = 7.00E-03; OR = 6.56 (1.68-25.71)); in males (p = 4.00E-03; OR = 13.45 (2.26-80.1)) and in intermediate risk patients (p = 2.00E-04; OR = 23.34 (4.46-122.07)).

The genotype distribution of the *SLC28A3* rs7853758 was also significantly different between cases (11.7%) and controls (1.2%) in the combined cohort (p = 6.53E-03; OR = 11.56 (1.98–67.45).

Haplotype analyses were carried out to study the association of haplotype blocks of genes in cardiotoxicity, but no significant results were found.

Alteration of fractional shortening between the diagnosis and the end of the treatment or the last echo ever measured

The alteration of fractional shortening from diagnosis until the end of the treatment or the last echo ever measured was also analyzed for patients with ALL in case of all SNPs. Among these the ones with at least 2 cases in one group are shown in Figure 1 if the p value was lower better than the p value of case-control analysis. SNPs with p values < 0.01 (Table 4) were analyzed in the whole population including both ALL and OSC patients. These results were not significant in the whole population.

Bayesian network based Bayesian multilevel analysis of relevance

Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA) method was performed for SNPs in the most relevant genes previously identified related to cardiotoxicity or in this current study (*ABCB1, ABCC1, ABCC2, ABCG2, AKR1A1, AKR1C3, CYP3A4, CYP3A5, GSTP1, HAS3, NQO1, NQO2, RARG, SLC22A17, SLC22A6, SLC22A7, SLC22A8, SLC28A3*) along with cofactors. This method aims to find the most probably strongly relevant variables with respect to the case-control status of the patients. The strongly relevant variables have a direct influence on the target. Values for posterior probability of strong relevance (P) range from 0 to 1, where P=1 means that the probability of the given variable is 100% relevant with respect to the case-control status. Our analyses revealed the potential strongly relevant effect of a SNP in gene *CYP3A5* (rs776746, P = 0.42), two SNPs in gene *NQO1* (rs1043470 and rs1469908, P = 0.42 and 0.34, respectively), two SNPs in gene *SLC28A3* (rs7853758 and rs885004, P = 0.55 and 0.36, respectively), and several cofactors (age at the time of diagnosis, P = 0.72; gender, P = 0.44; risk group, P = 0.73; diagnosis (ALL vs. OSC), P = 0.8 and cumulative dose of anthracycline, P = 0.64). Besides, several interaction effects were found between the variables. Among these, the two SNPs (rs7853758 and rs885004) in gene *SLC28A3* showed the strongest interaction. However, as the number of cases was low, these interaction effects could not be reconfirmed with logistic regression models using interaction terms.

Discussion

In this study, we evaluated the association of 66 single nucleotide polymorphisms and anthracycline- induced cardiotoxicity (ACT) developed during or after the treatment in acute lymphoblastic leukemia and osteosarcoma patients. SNPs in four investigated genes (*ABCC2,*

NQO1, *SLC22A6* and *SLC28A3*) were associated with decreased FS and EF. Regarding the aforementioned four genes the acute phase and the period of 5-10 years after the diagnosis were especially important. *CYP3A5* SNP appeared to be a predictor for ACT, the association was more prominent in boys, in ALL patients and in the intermediate risk group.

It must be noted that there are some potential biases of this study. Because of the retrospective data collection not all of the ECHO records were available. Therefore, the analysis of ECHO in every year was impossible; categories of follow-up were generated. Only the data of the latest ECHO of each patient were used in each follow-up category, the redundant echocardiography measurements were excluded. Nevertheless the large patient population and long follow-up makes our study notable.

ABCC2

In our study, during the treatment and after 5-10 years of the therapy *ABCC2* rs3740066 common GG genotype was associated with decreased FS and EF values. *ABCC2 (MRP2)* (10q24.2) is a member of the ATP binding cassette subfamily. ABC-proteins transfer various molecules through membranes. ABCC2 is responsible for organic anion transmembrane transport and its substrates also include anticancer drugs, antibiotics and statins. *ABCC2*'s efflux activity is involved in multidrug resistance. Expression of *ABCC2* is at critical sites of uptake and elimination, including the hepatobiliary tract, intestine, kidney and blood-tissue barriers [61]. *ABCC2* is a frequently investigated gene for instance in drug related toxicities, in therapy-response, resistance against various drugs, in carcinogenesis and in outcomes of osteosarcoma and leukemia [62–68]. There are also several findings in the field of cardiotoxicity regarding *ABCC2*. Wojnowski et al. studied acute and chronic ACT in adult patients with Non-Hodgkin lymphoma (NHL). Acute ACT was associated with NAD(P)H oxidase subunit (*RAC2* (Ras-Related C3 Botulinum Toxin Substrate 2)) variants, with polymorphisms of *MRP1 (ABCC1)* and with one haplotype of the *MRP2 (ABCC2)* gene (rs8187694-rs8187710) [40]. We found the same gene but different *ABCC2* SNP to be associated with acute and chronic ACT. A possible explanation for this divergence might be different phenotyping method, different target SNPs and the different population: age groups, tumor types, and chemotherapies. Armenian et al. revealed that *ABCC2* rs8187710, which spans nearby rs3740066, was over-represented in survivors of hematopoietic cell transplantation patients who developed anthracycline-related congestive heart failure. [69]. This gene was evaluated in a GWA study by Aminkeng et al. and confirmed the association with ACT (cases: FS≤24%), however, their result did not show genome-wide significance [70]. A meta-analysis of twenty-eight studies found increased risk for ACT in a strong association within *ABCC2* gene, with the above mentioned rs8187710 SNP, which is near to rs3740066 [52]. There are several studies investigating *ABCC2* rs3740066. A research of Lopez-Lopez et al. studied the methotrexate (MTX) plasma levels and SNPs in pediatric ALL patients, focusing on adverse events. They suggest rs3740066 is a predictor to prevent MTX toxicity. [9]. Hegyi et al. investigated the pharmacokinetics of MTX among osteosarcoma pediatric patients. In their analysis AUC_{0-48} (area under the concentration–time curve) was significantly lower in patients with homozygous variant genotype of rs3740066 [54]. For rs3740066 Han et al. suggested different efflux activity allele-dependently [71]. Inter-individual differences of *ABCC2* in expression and activity might be important for cardiotoxicity. The potential function of rs3740066 SNP is not fully understood yet. It may modify the mRNA stability or act together with rs572344237 SNP at the transcriptional level [72]. Aminkeng et al. highlighted also the potential role of this gene in a genetic testing used in clinical practice to avoid cardiotoxicity; however, this evidence needs further validation. It is also concluded that the insufficient number of findings and the marginally significant results are still a challenge to understand the function of polymorphisms in ABC transporters. [51].

NQO1

In our study, *NQO1* (nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase 1) rs1043470 was connected with reduced cardiac function rates during the treatment and between the fifth and tenth years after the therapy. Rs1043470 is located in the 3'UTR region of both *NQO1* and *NFAT5* (Nuclear Factor Of Activated T-Cells 5) genes, as *NQO1* is transcribed form the complementary strand. Nuclear factor-activated T- cell 5 (NFAT5) plays role against hyperosmotic stress, it is also expressed in the heart. NQO1 is a cytoplasmic 2-electron reductase, it reduces quinone to hydroquinone. *NQO1* prevents oxidative stress and defends against pro-oxidant drugs like anthracyclines [73]. Some studies indicated that it could also activate drugs like mitomycin C, E09, streptonigrin and B-lapachone into more toxic forms [74]. This gene has function in the vitamin K and E metabolism; it stabilizes the p53 in stress response. In human NQO1 is localized in the cytosol, expressed in many epithelial cells as well as cardiovascular cells and adipocytes, but not in liver cells. In solid tumors it showed high expression levels [75]. The SNP which seemed to be relevant in our study has not been studied in the literature yet, although there are several SNPs in the *NQO1* gene which reported to be important of the clinical view. In a study of childhood ALL patients the outcome was worse in carriers of an *NQO1* variant [76]. Dunna et al. studied the effect of rs1800566, which is only approximately 7000 base pair distance away from the rs1043470 investigated in our study. They found that rs1800566 had decreased enzyme-activity because of instability of the protein product. In their investigation it is suggested that the SNP has importance in the development of acute leukemia and is linked with unfavorable prognosis [77]. The rs1800566 (NQO1*2) was associated with poorer outcome in patients treated with anthracycline for breast cancer [75]. Szkandera et al. in a breast cancer population failed to demonstrate the effect of rs1800566 on the therapy-response of anthracyclines [78]. Dexrazoxane is an efficacious cardioprotective agent on chronic anthracycline cardiotoxicity. In an experiment with rabbits *NQO1*, among others, was down-regulated after daunorubicin administration, however, dexrazoxane could intercept this process [79]. In a cardiomyocyte cell culture investigation *NFAT5* showed lower levels on the protein, but not on the mRNA level after doxorubicin treatment. Effects of doxorubicin were the degradation of NFAT5 protein and limitation of cardiomyocytes' viability. Ito et al. proposed NFAT5 as a new positive marker of cardiomyocyte survival [80]. Lagoa et al. studied rats treated with doxorubicin. They experienced the down-regulation of *Nqo1* and increasing ROS production during the therapy. They suggested using this molecule as an early biomarker in the doxorubicin cardiotoxicity [81]. Zhu et al. described a SNP of *NQO1* (rs1800566) as a defender against cardiovascular damage linked to inflammatory stress [82]. Blanco et al. investigated the association between congestive heart failure and rs1800566 without any significant result in childhood cancer patients [73]. The same lack of association was obtained in a study of Armenian, where hematopoietic cell transplantation survivors and the susceptibility of anthracycline-CHF (congestive heart failure) were examined [69]. Visscher et al. examined 6 SNPs in *NQO1* in ACT among pediatric patients with different tumor types, but the association was not significant [39]. Our phenotyping method was different from theirs in their work, thus the two studies are not fully comparable. Among patients with T2DM (type 2 diabetes mellitus), who experienced coronary artery disease, rs1800566 TT genotype had higher occurrence [83]. The rs1043470, studied by us, is located in a 3'UTR region. The localization of the SNPs might provide new binding sites for miRs (microRNAs) or affect the binding ability of them and these may result changes in the translation. According to the PolymiRTS Database 3.0 database, one miR binds our investigated SNP, it is called hsa-mir-6863 [84]. In a cell-line experiment two 3'UTR SNPs were tested. One of them increased, while the other one decreased the binding of different miRs and both of them modified the expression of the target gene [85].

SLC22A6 and *SLC28A3*

According to our results five to ten years after the diagnosis rs6591722 of *SLC22A6* gene was in correlation with lower cardiac function. Solute Carrier Family 22 Member 6 is involved in renal

excretion of organic anions, toxic ones are also included. Renal *Slc22a6* was down-regulated after MTX treatment in rats [86]. In an in vitro study indoxyl sulfate correlated adverse cardiac effects was inhibited by *SL22A6*, it blocked entering the toxin into cardiac cells [87]. The genotype distribution of the *SLC28A3* rs7853758 was also significantly different between cases and controls. In many studies *SLC28A3* rs7853758 is proved to be a very important protective genetic marker against ACT. Among other SNPs *SLC28A3* rs7853758 was recommended for clinical use in pharmacogenetic testing while using doxorubicin or daunorubicin in pediatric cancer patients' treatment. For more current results regarding rs7853758 see the paper of Aminkeng et al. [51].

CYP3A5

CYP3A5 rs4646450 influenced the risk of fractional shortening being lower than 28% in our joined cohort. *CYP3A5* rs776746 was associated with alteration of FS. *CYP3A5* gene (7q21.1) is a member of cytochrome P450 proteins involved in drug metabolism, synthesis of steroids and lipids. CYP3A5 is expressed in the liver and also in extrahepatic tissues e.g. in intestines. In diffuse large B-cell lymphoma the risk of grade 2–4 cardiac toxicity was improved in patients who carried rs776746 in *CYP3A5* [88]. Among breast cancer patients this association was not shown [89]. Patients with lower *CYP3A5* expression had higher risk for drug-induced torsade de pointes arrhythmia [41]. Lesche et al. discussed the role of alleles of *CYP3A5* of dose requirements and the actual concentrations in blood while using immunosuppressive drugs in cardiac transplantation patients. They found that more doses were needed of everolimus with one functional and one nonfunctional allele of *CYP3A5* than in *CYP3A5* nonfunctional carriers to reach target concentration [90]. After heart transplantation *CYP3A5*3* homozygotes (nonexpressers) required more tacrolimus compared with *CYP3A5*1* carriers (expressers) [91]. In pediatric heart transplantation population younger age and functional *CYP3A5* correlated with higher needs of doses but lower concentrations of tacrolimus [92]. The nonfunctional type of *CYP3A5* was associated with the susceptibility for developing clopidogrel resistance in coronary artery disease patients [93]. Genetic variability of *CYP3A5* is high; even it is not expressed in 20% of African and 80% of Caucasian population. SNPs in *CYP3A5* (rs776746 and rs10264272) may modify its alternative splicing and protein truncation, which can result in a less active CYP3A5 [94].

Huang et al. studied the CYP3A5 enzyme activity with presence of its different gene polymorphisms in pediatric ALL patients. They revealed that patients with the rs776746 had lower enzyme activity. Rs776746 was in association with the mRNA expression, daunorubicin plasma concentration and adverse drug reactions. In this investigation AUC of daunorubicin was higher in children with cardiotoxicity [95]. In our population an association of an intronic *CYP3A5* SNP, the rs4646450 (function is not known according to the literature) with abnormal FS (<28%, cases) was observed, predominantly in boys. In a study the rs4646450 was in correlation with reduced protein-expression and activity of *CYP3A4* in human liver [96]. However, these results require further validation.

Conclusions

We reported SNPs which might contribute to the anthracycline-cardiotoxicity. Identification of polymorphisms in ALL or OSC pediatric patients could help follow-up patients at increased risk of cardiotoxicity. Further validation and the functional analysis of these findings in prospective independent analyses are recommended. International cooperation is required to be able to gather patient population with appropriate statistical power. The confirmed variants should be tested in clinical trials with long-term follow-up, because of the late cardiotoxicity. Today we are only at the beginning of this process, but regarding the huge development of the last decades and with the help of a user-friendly decision–support system, we can be sure that the number of the usable pharmacogenomic tests or personal therapies will be expanded in the future and the clinical usage of these trials will be present.

Table 1 Characteristics of the studied populations

	Patients with ALL	Patients with OSC	Total
Number of patients	622	39	661
Gender *n* (%)			
Male	372 (60)	27 (69)	399 (60)
Female	250 (40)	12 (31)	262 (40)
Age at diagnosis (%)			
<1 yr *n*	7 (1)	0	7 (1)
1-10 yr *n*	505 (81)	9 (23)	515 (78)
>10 yr *n*	109 (18)	30 (77)	138 (21)
Mean ±SD *yr*	6.39 (±4.3)	13.1 (±3.5)	6.6 (±4.3)
Median (range) *yr*	5.2 (0-18)	13.2 (5-18)	5.3 (0-18)
Risk group *n* (%)			
SR	165 (27)	3 (7)	168 (25)
IR	355 (57)	24 (62)	379 (58)
HR	100 (16)	12 (31)	112 (17)
Chemotherapy protocol *n* (%)			
Protocols before 2000[1]	325 (52)	–	325 (52)
Protocols after 2000[2]	297 (48)	–	297 (48)
OSC protocols	–	39	39
Anthracycline dose[3] (range, mg/m^2)	60-840	135-630	60-840
Anthracycline dose *n* (%)			
≤ 240 mg/m^2	457 (74)	6 (15)	463 (70)
> 240 mg/m^2	163 (26)	33 (85)	196 (30)
Patients with pathological LVFS[4] *n*	18	2	20

Data are reported as number with percentages, unless mentioned otherwise. Abbreviations: ALL, acute lymphoid leukemia; OSC, osteosarcoma; SD, standard deviation; SR, standard risk; IR, intermediate risk; HR, high risk; LVFS, left ventricular fractional shortening. [1]ALL patients treated with ALL BFM 88, ALL BFM 90, ALL BFM 95, Interfant 98, NHL BFM 90 or NHL BFM 95 protocol. [2]ALL patients treated with ALL IC BFM 2002, ALL IC BFM 2009 or Interfant 2006 protocol. [3]Cumulative anthracycline dose in doxorubicin or daunorubicin equivalent doses during the treatment according to protocol. [4]LVFS below 28%.

Table 2 Follow-up categories with echocardiography parameters

Follow-up category			Patients with ALL	Patients with OSC	Total	Decreased | not decreased LVFS, *n*[1]	OR (95% CI) [2]
At the diagnosis	*n*		358	29	387		
	LVFS	*Mean ±SD*	41.5 ±6.1	39.6 ±4.4	41.4 ±6.0		
<1 yr from diagnosis[1]	*n*		275	5	280	104|83	1.0
	LVFS	*Mean ±SD*	40.4 ±6.1	40.2 ±5.3	40.4 ±6.1		
1-2 yr from diagnosis	*n*		46	3	49	19|10	1.5 (0.6-3.4)
	LVFS	*Mean ±SD*	41.4 ±6.0	39.9 ±2.3	41.3 ±5.9		
End of the treatment	*n*		287	28	315	105|98	0.9 (0.6-1.3)
	LVFS	*Mean ±SD*	40.0 ±5.6	38.4 ±6.3	39.9 ±5.7		
2-5 yr from diagnosis	*n*		229	35	264	77|73	0.8 (0.5-1.3)
	LVFS	*Mean ±SD*	40.4 ±5.7	38.1 ±5.2	40.1 ±5.7		
5-10 yr from diagnosis	*n*		265	36	301	70|76	0.7 (0.5-1.1)
	LVFS	*Mean ±SD*	40.1 ±5.5	40.3 ±5.6	40.1 ±5.5		
10-15 yr from diagnosis	*n*		133	19	152	24|36	0.5 (0.3-1.0)
	LVFS	*Mean ±SD*	40.4 ±5.4	39.9 ±5.2	40.3 ±5.3		
>15 yr from diagnosis	*n*		24	8	32	5|3	1.3 (0.3-5.7)
	LVFS	*Mean ±SD*	37.6 ±7.5	40.7 ±6.1	38.4 ±7.2		

The decrease of LVFS was calculated patient by patient in every category compared to the individual value at diagnosis if this data was available.
[1]Number of patients with a decreased LVFS per number of patients with a not decreased LVFS. [2]Compared to the second category Abbreviations: LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; OR, odds ratio; CI, confidence interval; SD, standard deviation.

Table 3 Information about the studied SNPs

Gene	SNP	Chromosome	Position	Function	Alleles		MAF
					Major	Minor	
ABCB1	rs3842	7q21.1	87504050	3'UTR	A	G	0.128
ABCB1	rs1045642	7q21.1	87509329	Ile1145Ile	T	C	0.450
ABCB1	rs10280101	7q21.1	87524269	intron	A	C	0.090
ABCB1	rs2032582	7q21.1	87531302	Ala893Ser/Ala893Thr	G	T/A	0.454/0.028
ABCB1	rs11760837	7q21.1	87533700	intron	T	C	0.087
ABCB1	rs1128503	7q21.1	87550285	Gly412Gly	C	T	0.468
ABCB1	rs1202179	7q21.1	87574963	intron	A	G	0.272
ABCB1	rs9282564	7q21.1	87600124	Asn21Asp	A	G	0.111
ABCC1	rs35587	16p13.1	16045857	Asn354Asn	T	C	0.329
ABCC1	rs35605	16p13.1	16068162	Leu562Leu	C	T	0.187
ABCC1	rs8187858	16p13.1	16068182	Tyr568Tyr	C	T	0.087
ABCC1	rs4148350	16p13.1	16076620	intron	G	T	0.079
ABCC1	rs35626	16p13.1	16076758	intron	G	T	0.335
ABCC1	rs212087	16p13.1	16136433	intron	C	T	0.420
ABCC1	rs3743527	16p13.1	16141824	3'UTR	C	T	0.247
ABCC1	rs212090	16p13.1	16142147	3'UTR	T	A	0.431
ABCC1	rs212091	16p13.1	16142793	3'UTR	A	G	0.135
ABCC1	rs212093	16p13.1	16143897	downstream	A	G	0.417
ABCC2	rs717620	10q24.2	99782821	5'UTR	G	A	0.163
ABCC2	rs2273697	10q24.2	99804058	Val417Ile	G	A	0.216
ABCC2	rs3740066	10q24.2	99844450	Ile1324Ile	G	A	0.328
ABCG2	rs2231142	4q22	88131171	Gln141Lys	C	A	0.097
ABCG2	rs1564481	4q22	88140113	intron	C	T	0.388
AKR1A1	rs9147	1p34.1	45551015	5'UTR	T	C	0.454
AKR1A1	rs2934859	1p34.1	45552164	intron	G	A	0.451
AKR1C3	rs3763676	10p15	5094307	5'UTR	T	C	0.282
AKR1C3	rs7741	10p15	5096415	Pro30Pro	G	A	0.292
AKR1C3	rs12387	10p15	5097493	Lys104Lys	A	G	0.170
AKR1C3	rs10508293	10p15	5098945	intron	T	C	0.195
AKR1C3	rs3209896	10p15	5107511	3'UTR	A	G	0.448
BCL2	rs4987853	18q21.3	63126422	3'UTR	A	G	0.160

BCL2	rs1564483	18q21.3	63127421	3’UTR	G	A	0.283
BCL2	rs4987845	18q21.3	63127955	3’UTR	G	A	0.081
BCL2	rs4941195	18q21.3	63302803	intron	C	A	0.440
BCL2	rs1893806	18q21.3	63317731	intron	T	G	0.433
CEP72	rs4956954	5p15.3	607273	intron	A	G	0.339
CEP72	rs924607	5p15.3	609978	intron	C	T	0.434
CEP72	rs12522955	5p15.3	639116	Pro412Thr	C	A	0.195
CEP72	rs868649	5p15.3	640590	Thr509Ala	T	C	0.230
CEP72;TPPP	rs2458815	5p15.3	653228	3’UTR	A	G	0.216
CHTF8;HAS3	rs2232228	16q22	69109674	Ala93Ala	A	G	0.442
CYP3A4	rs3735451	7q22	99758352	intron	A	G	0.139
CYP3A4	rs2246709	7q22	99768096	intron	A	G	0.274
CYP3A5	rs4646450	7q22	99668695	intron	C	T	0.155
CYP3A5	rs776746	7q22	99672916	intron	G	A	0.087
CYP3A5	rs28365067	7q22	99674687	intron	C	T	0.076
GSTP1	rs6591256	11q13.2	67582428	5’UTR	A	G	0.360
GSTP1	rs4147581	11q13.2	67584114	intron	Unsuccessful		
GSTP1	rs1695	11q13.2	67585218	Ile105Val	A	G	0.297
GSTP1	rs749174	11q13.2	67585782	intron	C	T	0.304
HTATSF1P2;NQO2	rs1143684	6p25	3010156	Phe47Leu	T	C	0.234
HTATSF1P2;NQO2	rs6955	6p25	3019693	3’UTR	G	C	0.162
KDSR;BCL2	rs1801018	18q21.3	63318646	Thr7Thr	Unsuccessful		
LINC01011;NQO2	rs2071002	6p25	3000069	5’UTR	Unsuccessful		
MTHFR	rs1801131	1p36.2	11794419	Glu470Ala	A	C	0.318
NFAT5;NQO1	rs1043470	16q22	69704005	3’UTR	C	T	0.115
NQO1	rs1800566	16q22	69711242	Pro187Ser	C	T	0.180
NQO1	rs1469908	16q22	69730509	5’UTR	A	G	0.393
NQO2	rs4149352	6p25	3002004	intron	C	T	0.226
RARG;ITGB7	rs2229774	12q13.1	53211761	Ser427Leu	C	T	0.084
SLC22A17	rs12882406	14q11.2	23343028	downstream	G	C	0.370
SLC22A17	rs4982753	14q11.2	23345360	downstream	C	T	0.258
SLC22A6	rs10897310	11q12	62973704	downstream	T	C	0.376
SLC22A6	rs6591722	11q12	62982208	intron	T	A	0.315
SLC22A7;CRIP3	rs2270860	6p21.1	43302413	Ser425Ser	G	A	0.334

SLC22A7;CRIP3	rs4149178	6p21.1	43304450	intron	A	G	0.165
SLC22A8	rs2276299	11q12	62998959	Thr241Thr	A	T	0.179
SLC28A3	rs7853758	9q21.3	84286011	Leu461Leu	G	A	0.139
SLC28A3	rs885004	9q21.3	84294635	intron	G	A	0.138
SLC28A3	rs7867504	9q21.3	84305321	Thr89Thr	T	C	0.297

Minor/major allels according to HapMap-CEU, chromosomal position according to the Variation Viewer (GRCh38.p7). Abbreviations: MAF, global minor allele frequency; SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg Equilibrium.

Table 4 Genotyped SNPs and related LVFS in different genotype groups according to accomplished analysis types

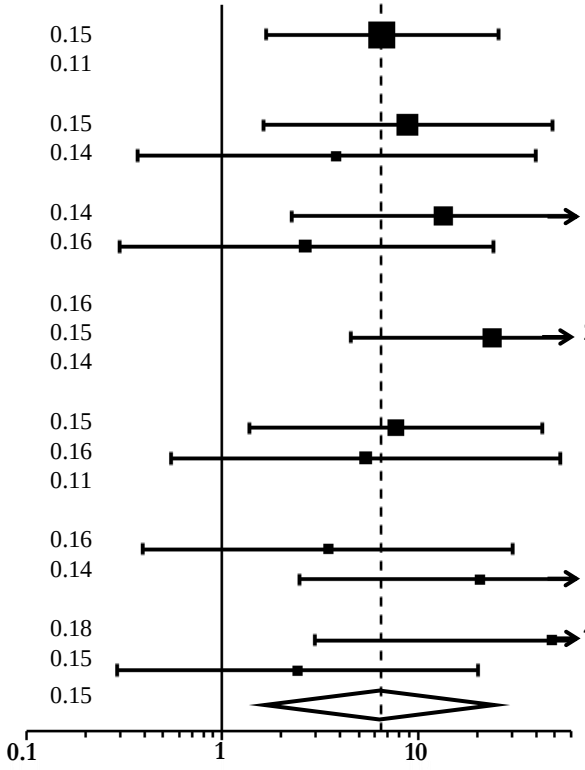
Analysis type	Gene	SNP	Model	Genotypes	Mean FS % ± SE genotype group 1 (N)	Mean FS % ± SE genotype group 2 (N)	P value	Follow-up category
Follow-up analysis	*ABCB1*	rs9282564	dom	AA / AG + GG	41.5 ± 0.7 (100)	37.9 ± 1.1 (29)	2,50E-03	10-15 years after dg
	ABCC1	rs35626	dom	GG / GT + TT	41.0 ± 0.6 (92)	39.0 ± 0.6 (127)	7,90E-03	2- 5 years after dg
	ABCC2	rs3740066	add	GG / GA / AA	39.5 ± 0.5 (112)	40.8 ± 0.5 (112) / 42.9 ± 0.9 (33)	4,50E-03	5-10 years after dg
	NQO1	rs1043470	dom	CC / CT + TT	40.9 ± 0.5 (198)	38.1 ± 0.9 (53)	2,60E-03	acute phase
	SLC22A6	rs6591722	rec	TT+TA / AA	40.7 ± 0.4 (227)	37.8 ± 1.0 (28)	5,90E-03	5-10 years after dg
	SLC28A3	rs7853758	dom	GG / GA + AA	41.3 ± 0.7 (96)	38.4 ± 1.1 (36)	4,80E-03	10-15 years after dg
	SLC28A3	rs885004	dom	GG / GA + AA	41.3 ± 0.7 (95)	38.0 ± 1.1 (33)	2,50E-03	10-15 years after dg

	Gene	SNP	Model	Genotypes	Cases in genotype groups N (%)	P value	P value	OR (CI 95%)
Case-control study	*CYP3A5*	rs4646450	rec	CC + CT / TT	15 (83) / 3 (17)	550 (97) / 17 (3)	5,60E-03	6.94 (1.76-27.39)
	SLC28A3	rs7853758	rec	GG + GA / AA	15 (88) / 2 (12)	571 (99) / 7 (1)	6,50E-03	11.56 (1.98-67.45)
Alteration of FS (dg vs. end of th)	*CYP3A4*	rs3735451	dom	AA / AG + GG	74 (82) / 16 (18)	52 (63) / 31 (37)	5,70E-03	0.36 (0.18-0.74)
	CYP3A5	rs776746	dom	GG / GA + AA	81 (91) / 8 (9)	60 (73) / 22 (27)	3,80E-03	0.26 (0.11-0.65)
Alteration of FS (dg vs. last echo)	*NQO1*	rs1043470	dom	CC / CT + TT	111 (85) / 41 (15)	112 (73) / 20 (27)	8,90E-03	0.44 (0.24-0.81)

Results are from logistic regression and multivariate general linear model performed on the ALL cohort adjusted for potential covariates. The three model used were: (add), genotypes separately (11 vs. 12 vs. 22); (rec) recessive (11/12 vs. 22); (dom) dominant (11 vs. 12/22) models, with the common homozygotes signed as 11. Abbreviations: FS, fractional shortening; dg, diagnosis; th, therapy; echo, echocardiography; N, number; OR, odds ratio; CI, confidence interval.

Table 5 Odds ratios for cardiotoxicity associated with the CYP3A5 rs4646450 genotype among subgroups of patients

Subgroup	CC|CT|TT		T allele frequency		Odds ratio (95%CI) for CC vs. TT	P value
	No. of cases	*No. of controls*	*Cases*	*Controls*		
Diagnosis						
ALL	11|4|3	409|141|17	0.28	0.15	6.56 (1.68-25.71)	**0.007**
OSC	1|1|0	29|8|0	0.25	0.11	-	-
Age at diagnosis						
≤10 yr	6|3|2	346|121|13	0.32	0.15	8.87 (1.63-48.24)	0.01
>10 yr	6|2|1	92|27|4	0.22	0.14	3.83 (0.37-39.87)	0.21
Gender						
Male	5|4|2	269|89|8	0.36	0.14	13.45 (2.26-80.1)	**0.004**
Female	7|1|1	169|60|9	0.17	0.16	2.68 (0.3-24.2)	0.38
Risk group						
SR	3|0|0	110|39|6	0.00	0.16	-	-
IR	4|4|3	249|86|8	0.45	0.15	23.81 (4.55-124.51)	**0.0002**
HR	5|1|0	69|22|2	0.08	0.14	-	-
Chemotherapy protocol						
Before 2000	7|2|2	214|72|8	0.27	0.15	7.64 (1.37-42.8)	0.02
After 2000	4|2|1	195|69|9	0.29	0.16	5.42 (0.55-53.54)	0.15
OSC protocols	1|1|0	29|8|0	0.25	0.11	-	-
Cumulative ANT dose						
≤240 mg/m^2	6|4|1	311|103|15	0.27	0.16	3.46 (0.39-30.55)	0.27
>240 mg/m^2	6|1|2	125|46|2	0.28	0.14	20.83 (2.49-174.3)	**0.005**
Recidiva occured						
yes	2|1|2	48|24|1	0.50	0.18	48.00 (2.96-778.53)	**0.006**
no	10|4|1	390|125|16	0.20	0.15	2.44 (0.29-20.22)	0.41
All	12|5|3	438|149|17	0.28	0.15	6.44 (1.66-24.97)	**0.007**



Results of the univariate logistic regression analysis performed on subpopulations of the total cohort of pateints. Black boxes represent OR, the number of cases is proportional with the width of the boxes. The lengths of the horizontal lines depict the 95% confidence intervals. Analysis of OR was not accomplished, if the number of cases was 0. Abbreviations: ALL, acute lymphoblastic leukemia; OSC, osteosarcoma; no, number; yr, year; SR, standard risk; IR, intermediate risk; HR, high risk.

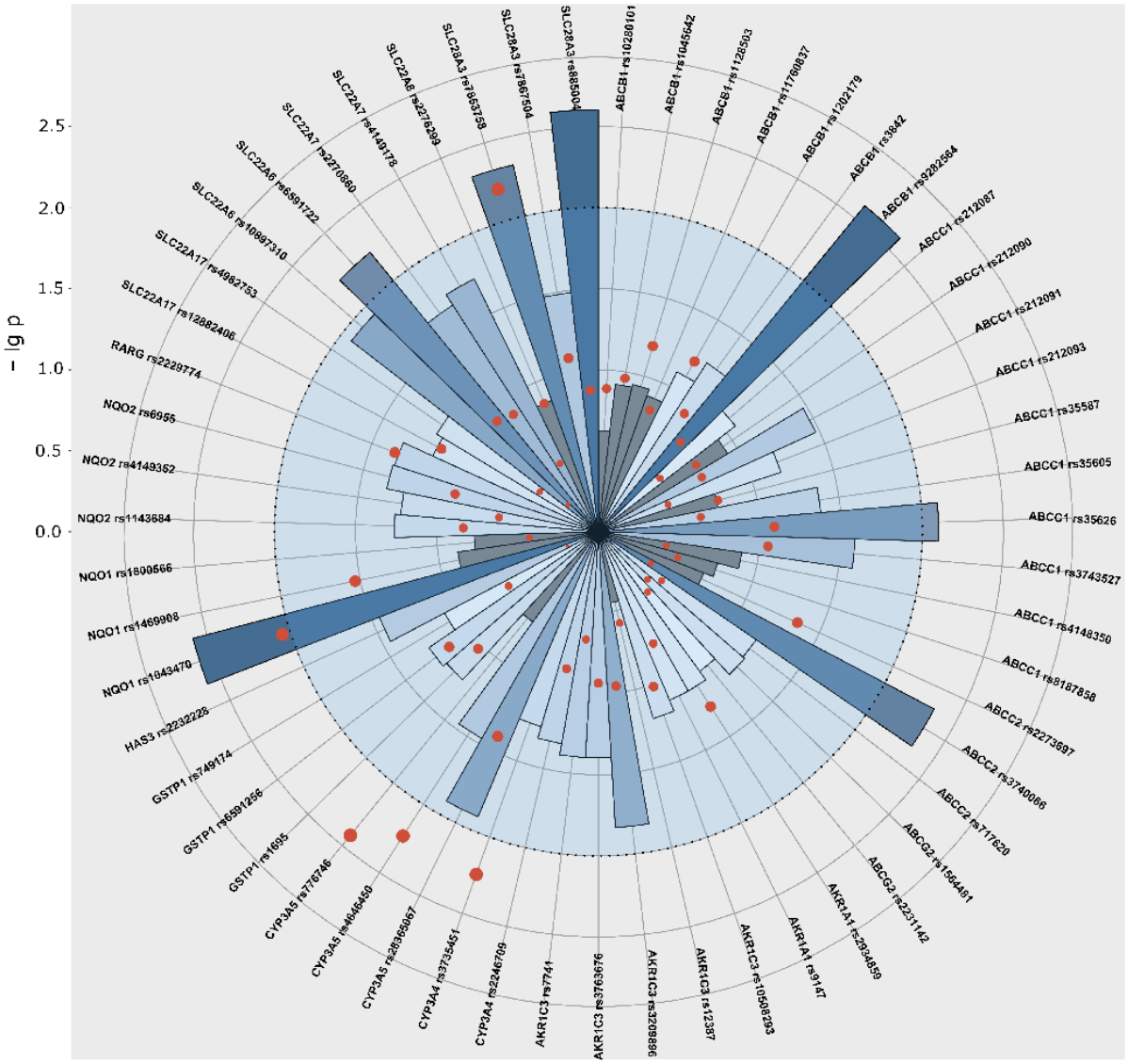


Figure 1 The p values of the follow up and case-control studies of the ALL population

Results of the analysis including data of ALL patients are presented in this Figure. The p values are illustrated in a polar coordinate system, where the circular grids represent the negative logarithm of p values (axis on the left can be projected to grids). Intermittent line indicates border at p=0.01 (- lg p = 2). Columns show the results of the follow up analysis of FS, darker shades of blue mean stronger significance. The lowest p values are chosen from every follow-up category and from every model, if the number of the cases was above 5. (The time of diagnosis was excluded.) Results of the case-control study are shown with red dots, sizes proportional with stronger significance. Most significant results of this plot were studied further on the total cohort including osteosarcoma patients as well.

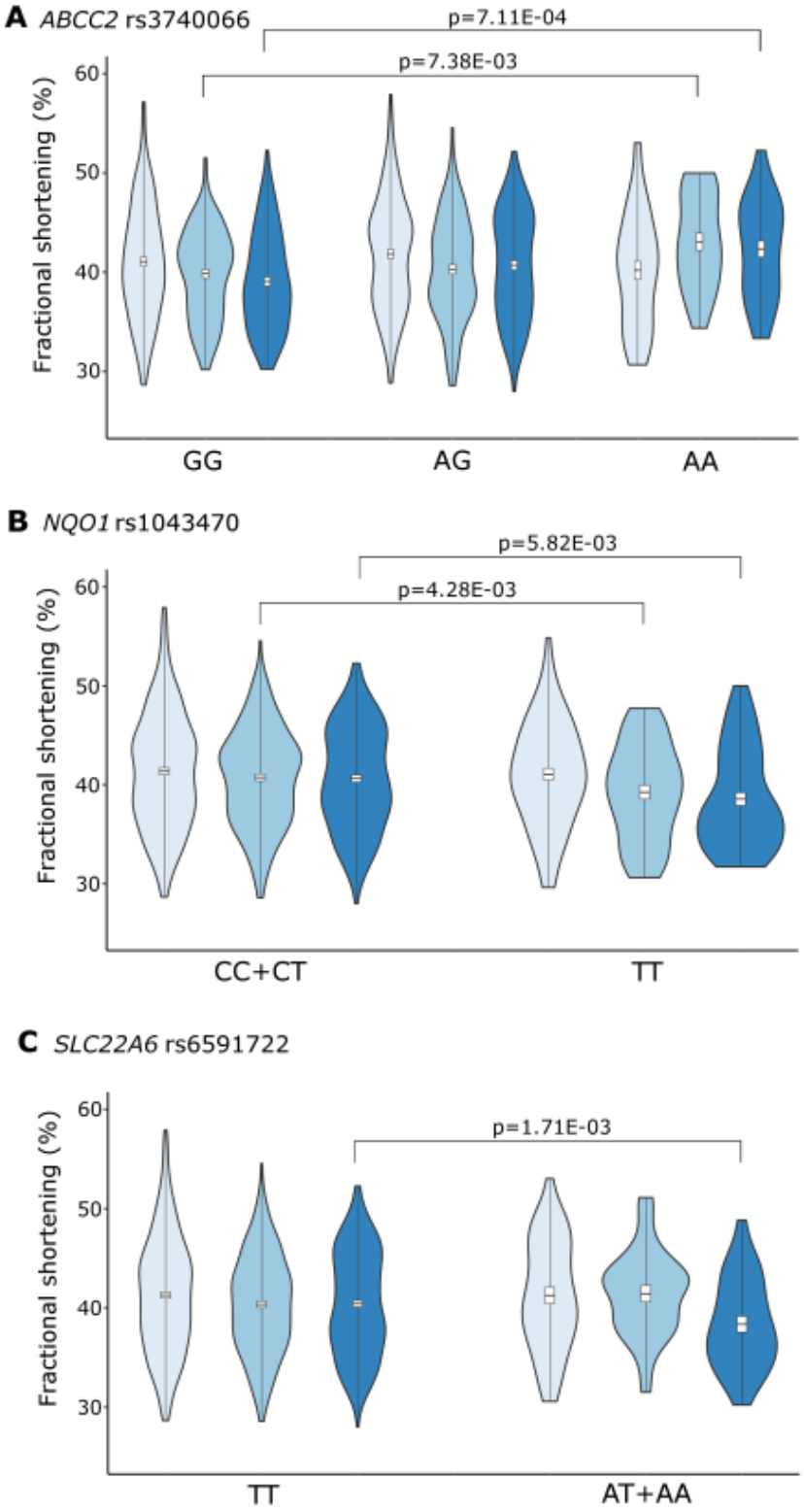


Figure 2 Violin plot of fractional shortening in total population.

FS (%) by genotypes is shown in different follow up categories. Light blue is the time of diagnosis, medium blue is the time of the anthracycline administration (acute phase), dark blue is the follow up 5-10 years after therapy. FS is indicated in box plots, box is mean ± S.D., whiskers are means ± 3 S.D. Violin plot describes the distribution of the FS data, records out of mean ± 3SD are not shown. A: *ABCC2* rs3740066; B: *NQO1* rs1043470; C: *SLC22A6* rs6591722.

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