EXPLORING PHARMACOGENETICS IN OSTEOSARCOMA

Evelien Hurkmans

Exploring pharmacogenetics in osteosarcoma

Evelien Hurkmans

The work presented in this thesis was carried out within the Radboud Institute for Health Sciences, at the department of Human Genetics of the Radboud university medical center in Nijmegen, The Netherlands.

Cover design & Lay-out: Publiss | www.publiss.nl
Print: Ridderprint | www.ridderprint.nl

Exploring pharmacogenetics in osteosarcoma

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 6 september 2022
om 12.30 uur precies

door

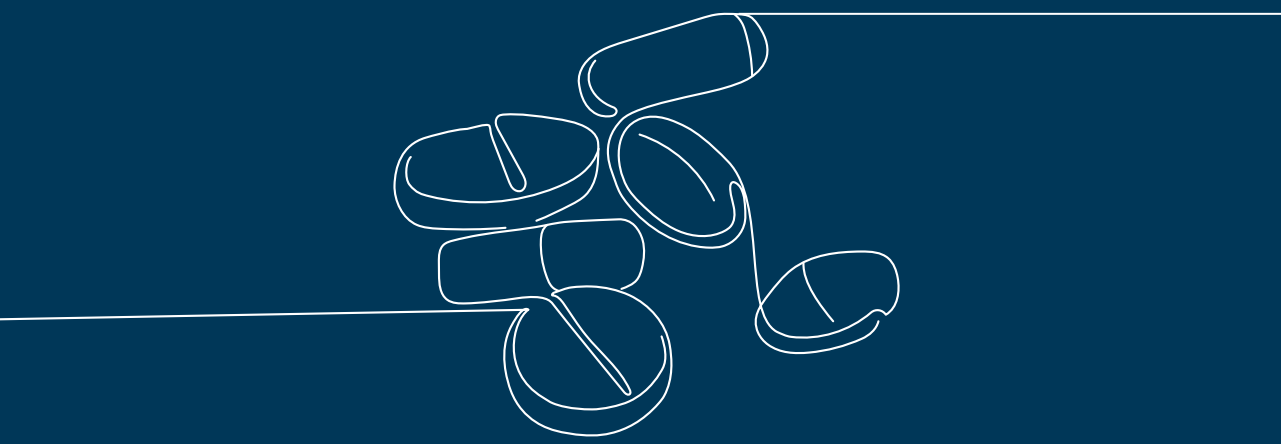
Evelien Gerarda Elisabeth Hurkmans
geboren op 12 oktober 1993
te Bakel

Promotor:
Prof. dr. H.G. Brunner

Copromotoren:
Dr. M.J.H. Coenen
Dr. D.M.W.M. te Loo

Manuscriptcommissie:
Prof. dr. C.M.L. van Herpen
Prof. dr. F.G.M. Russel
Prof. dr. R. Masereeuw (Universiteit Utrecht)

Contents



CHAPTER 1

Introduction

Introduction

Osteosarcoma is a primary bone tumor which is most prevalent in children and adolescents. In June 1986, years after the introduction of chemotherapy treatment, Link and colleagues wrote in the *New England Journal of Medicine* (1):

"Refinements in therapy for patients with osteosarcoma are needed. Although there has been a dramatic improvement in the overall outcome for children with osteosarcoma during the past 15 to 20 years, more than one third of children presenting without metastases will relapse after receiving therapy currently available. (...) The toxicity and expense of chemotherapy regimens currently in use are substantial, and the late effects of such therapy have not yet been assessed."

Today, 35 years after publication of this article, no large improvements have been made in the treatment of osteosarcoma. Survival and relapse rates have remained similar and survivors suffer from toxicities, long after they finish treatment. When compared to other pediatric malignancies, where survival rates are approaching 90%, patients with osteosarcoma have lagged behind (2). In this thesis, germline genetic variation is studied in order to gain more insight in interindividual differences in treatment response and toxicities. Eventually, these findings may lead to novel opportunities for refinements in therapy for patients with osteosarcoma.

Osteosarcoma

Osteosarcoma is the most common primary bone tumor, but it is still a rare disease. The incidence is 3-4 cases per million per year worldwide (3). Osteosarcoma can occur at any age, but it is most often diagnosed in children and young adults with a median age of 14 years at diagnosis and 59% of patients is of male sex (4). Treatment of osteosarcoma consists of chemotherapy combined with surgery. Most chemotherapeutic treatment protocols rely on a backbone of cisplatin, doxorubicin and methotrexate and this also holds true for the protocol of the European and American Osteosarcoma Study Group (EURAMOS) that is used in The Netherlands. In summary, patients are treated with two cycles of chemotherapy before surgery, consisting of one course 75 mg/m^2 doxorubicin (A) and 120 mg/m^2 cisplatin (P) in the first week, and a course of 12 g/m^2 methotrexate (M) in week 3 and in week 4. Thereafter, the primary tumor and possible metastases are resected, preferably with limb sparing procedures. Post-surgery, patients receive two additional MAP cycles and two MA cycles, supplemented with etoposide, ifosfamide or interferon-α, depending on histological response and randomization. In the latest results of the Euramos-1 trial in 2019, the 5-year overall survival (OS) was estimated 71%, along with 5-year event-free survival (EFS) of 54% (5).

The development of severe toxicities can be a major burden for patients, impairing quality of life significantly even after treatment. Cisplatin-induced hearing loss (ototoxicity) occurs in some form in approximately 60% of the patients (6). This is often irreversible and

is especially problematic when it occurs in young children, as it can lead to difficulties in learning, language development and psychosocial skills (7, 8). Whereas cisplatin-induced ototoxicity usually arises during treatment, doxorubicin-induced cardiotoxicity may also arise years after finishing treatment. The risk of cardiac dysfunction is 5-15 higher in pediatric cancer survivors treated with doxorubicin than in the general population and a diagnosis of heart failure decreases 5-year survival with 50% (9-11). Adverse events of the liver (hepatotoxicity), kidneys (nephrotoxicity) and bone marrow (bone marrow toxicity) may also develop during chemotherapy treatment of osteosarcoma. In addition to the discomfort that patients experience of these adverse events, oncologists may be forced to reduce chemotherapy doses or even discontinue treatment when toxicities are too severe. This could reduce the anti-tumor effect of chemotherapy and may affect survival. Therefore, it is of importance to know which patients have higher risk of toxicity to improve both quality-of-life and survival rates.

Many interindividual differences exist in treatment response or the development of toxicities. Identifying accurate predictors for these differences could provide new leads towards a better understanding of biological mechanisms behind this. The most important clinical parameters that can predict a poor treatment response are the presence of metastases at diagnosis or an axial skeleton tumor site (5). Clinical risk factors for toxicities are young age, male sex, high anthracycline- and cisplatin dose and poor kidney function (6, 12, 13). Despite that these predictors are used for stratification in protocols, no individualized treatment options are available yet. Germline genetic variants may influence processes regarding absorption, distribution, metabolism and excretion (pharmacokinetics or ADME) of drugs and processes at drug target sites (pharmacodynamics). Therefore, genetic variation could potentially add information to predict treatment response and toxicity and may allow us design treatment strategies that fit individual patients better.

Pharmacogenomics

Germline genetic variation can have major effects on treatment response. In the field of pharmacogenomics, we aim to use genetic variation to optimize treatment. The main goal of genetic association studies in pharmacogenomics is to identify genetic variants which may explain interpatient variability in drug response in addition to the known predictive clinical parameters, to improve drug efficacy and reduce the risk of drug-induced toxicities (14). In the early days of pharmacogenetic discovery, the focus was on candidate gene studies. These studies only study variants in genes that are known to have a role in e.g. pharmacokinetic pathways of a drug of interest. As prices for large scale genotyping have decreased and technologies advanced over the years, larger panels of genetic variation are assessed in genetic association studies. Especially panels of variants in genes involved in the ADME of drugs. This allows the identification of variants in genes that were not previously indicated in the pharmacokinetic processes of drugs. Eventually, genome-wide association studies (GWAS) gained popularity. With genome-wide coverage of common genetic variation and highly developed imputation pipelines, millions of genetic variants can be studied at once. In addition, genetic variation in genes that code for proteins

involved in pharmacodynamic processes are covered, allowing for discovery of a whole new range of genetic variants that can provide opportunities to tailor treatment towards individual patients. In this thesis, both GWAS and ADME panels will be used.

Whereas many dosing guidelines were developed to use germline genetic variation in the prescription of drugs, this is still limited for pediatric patients (15, 16). Despite that, strong evidence for the association of *NUDT15* and *TPMT* and 6-mercaptopurine-induced toxicities is present in pediatric cancer patients and genotype-guided dosing is used in some hospitals (17-21). Although, the rarity of pediatric cancer is one of the main challenges in the discovery and consistent replication of many other pharmacogenetic associations (18). When studying many genetic variants, for example in GWAS, correction for multiple testing is required to avoid false-positive findings. This means that the p-value is adjusted according to e.g. Bonferroni correction or Benjamini and Hochberg False Discovery Rate (FDR) correction, and therefore only highly powered studies can find significant associations with these more stringent p-values. Power dependents on cohort size, effect size of the association, variance in the data and minor allele frequencies of the genetic variants. In small patient cohorts it could be more sensible to study smaller numbers of variants to reduce the effect of multiple testing correction. When the power is not sufficient, only associations with very large effect sizes will be detected. Increasing patient cohort numbers is the most efficient way to increase power. As osteosarcoma is relatively rare, it is very challenging to collect large enough patient cohorts, even if it would be possible to collect all osteosarcoma patients in The Netherlands power would be low. Therefore, collaboration with international research groups that are studying osteosarcoma patient cohorts is critical to obtain the necessary power. In addition, when studying toxicities, the mechanism of action is dependent on the medication and not necessarily on the malignancy. Therefore, it is even possible to combine different patient cohorts with corresponding chemotherapeutic treatment, as will be done in chapter 5 of this thesis.

Phenotypes

In genetic association studies, the design of the study and the case definition have a great impact on study results (22). Genetic association studies often follow case-control designs with dichotomized outcomes, but in some cases it is more advantageous to study continuous, ordinal or time-dependent survival outcomes. When studying toxicities, a case control design is often preferred, because it gives clear results of genetic variants that effect risk to develop a certain clinically relevant adverse event. The U.S. National Cancer Institute Common Technology Criteria for Adverse Events (CTCAE) offers grading scales that describe many toxicities ranging from grade 0 (no toxicity) to grade 5 (death). However, in the case of cisplatin-induced ototoxicity, many additional scales exist with slightly different cutoffs for the grade designation, for example, Brock, Chang, International Society of Pediatric Oncology (SIOP) Boston and Muenster scales (23). Of these, Brock and Chang grades are most concordant (κ=0.969) and Muenster and Chang are least concordant (κ=0.665), indicating that the scales should not be used interchangeably without great caution (23). On top of that, the grade allocations are then dichotomized to

allow for a case-control study and that is not always done similarly, causing heterogeneity in results across studies. In some studies, patients with grade 0 are compared to patients with grade 1-4, and in other cases grade 1 patients are considered controls or completely excluded to allow for a clearer distinction between cases and controls. In addition to that, when dichotomizing data, information on the phenotype severity is lost. An ordinal or continuous design could be used to include all toxicity information, with ranging severity, in the statistical model, which is beneficial for the power of the statistical test.

When considering treatment efficacy, 5-year EFS or 5-year OS can be used as a survival outcome. EFS is a more relevant measure than OS because EFS only describes disease related events and OS may also describe death by other causes. An initial inadequate drug response can also be defined as progressive disease. This means that there is growth of the primary tumor or metastases or new cancerous lesions develop during or shortly after treatment, providing a very distinct group of patients with poor treatment responses. Studying progressive disease in a case-control design may identify genetic variants that are very specific for treatment response.

Objectives of this thesis

The main aim of this thesis is to identify genetic variants that are associated to treatment response or toxicities in patients with osteosarcoma. The phenotypes that are studied will be approached from different angles, including both continuous and dichotomous outcomes, with a single measure per patient or with repeated measures. At the same time, genotyping methods will differ across chapters, ranging from a genome-wide association study, an array with variants in genes coding for drug metabolizing enzymes and transporters to manual genotyping for validation cohorts. Eventually, we aim to elucidate more of the functional mechanism behind the genetic association. This may give novel opportunities for the refinement of treatment. The approaches that are used are briefly described below.

In **Chapter 2** of this thesis, a systematic literature review is presented of genetic variants that were discovered to be associated with treatment outcome or toxicities in patients with osteosarcoma. In addition to the systematic search, a structured literature search was performed to identify all studies that replicate the associations that were primarily found in osteosarcoma patient cohorts. This review thus summarizes the evidence that is available on pharmacogenetics in patients with osteosarcoma. In addition, gaps in knowledge are identified which present opportunities for future research.

Chapter 3 of this thesis is a genetic association study where methotrexate induced toxicities are the main focus. In a cohort of patients with osteosarcoma, toxicity data and methotrexate plasma levels were collected after every infusion of methotrexate during treatment. In addition, these patients were genotyped for a broad range of variants in genes in drug metabolizing enzymes and transporters. The aim of this chapter is to identify novel genetic variants associated with methotrexate toxicity using repeated measures in individual patients. Both continuous toxicity outcomes and dichotomized outcomes were analyzed to make optimal use of the information and their clinically predictive relevance.

Chemotherapy treatment is very effective for some patients, but in other patients the tumor continues to grow during treatment. In **Chapter 4** we assessed which genetic variants in genes that code for drug metabolizing enzymes and transporters are associated with disease progression. In addition, functional studies were performed to better understand the biological mechanism behind the variants that increase susceptibility to disease progression.

In **Chapter 5**, a genome-wide association study was performed to identify genetic variants that are associated to cisplatin-induced ototoxicity. The patient cohort of this study consists of pediatric patients that are treated with cisplatin, for osteosarcoma, medulloblastoma or low grade glioma. Because cohort size matters in GWASs, we collaborated with international research groups to gain as much power as possible to discover novel genetic variants that may generate opportunities to decrease hearing loss and improve patients' quality-of-life, also after treatment.

The general discussion of the thesis is given in **Chapter 6**.

References

1. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, et al. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med. 1986;314(25):1600-6.
2. Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5--a population-based study. Lancet Oncol. 2014;15(1):35-47.
3. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531-43.
4. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Ann Oncol. 2015;26(2):407-14.
5. Smeland S, Bielack SS, Whelan J, Bernstein M, Hogendoorn P, Krailo MD, et al. Survival and prognosis with osteosarcoma: outcomes in more than 2000 patients in the EURAMOS-1 (European and American Osteosarcoma Study) cohort. Eur J Cancer. 2019;109:36-50.
6. Langer T, am Zehnhoff-Dinnesen A, Radtke S, Meitert J, Zolk O. Understanding platinum-induced ototoxicity. Trends Pharmacol Sci. 2013;34(8):458-69.
7. Gurney JG, Tersak JM, Ness KK, Landier W, Matthay KK, Schmidt ML, et al. Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group. Pediatrics. 2007;120(5):e1229-36.
8. Olivier TW, Bass JK, Ashford JM, Beaulieu R, Scott SM, Schreiber JE, et al. Cognitive Implications of Ototoxicity in Pediatric Patients With Embryonal Brain Tumors. J Clin Oncol. 2019;37(18):1566-75.
9. Felker GM, Thompson RE, Hare JM, Hruban RH, Clemetson DE, Howard DL, et al. Underlying causes and long-term survival in patients with initially unexplained cardiomyopathy. N Engl J Med. 2000;342(15):1077-84.
10. Bhatia S. Genetics of Anthracycline Cardiomyopathy in Cancer Survivors: JACC: CardioOncology State-of-the-Art Review. JACC CardioOncol. 2020;2(4):539-52.
11. van Dalen EC, van der Pal HJ, Kok WE, Caron HN, Kremer LC. Clinical heart failure in a cohort of children treated with anthracyclines: a long-term follow-up study. Eur J Cancer. 2006;42(18):3191-8.
12. Armenian S, Bhatia S. Predicting and Preventing Anthracycline-Related Cardiotoxicity. Am Soc Clin Oncol Educ Book. 2018;38:3-12.
13. Cheng DH, Lu H, Liu TT, Zou XQ, Pang HM. Identification of Risk Factors in High-Dose Methotrexate-Induced Acute Kidney Injury in Childhood Acute Lymphoblastic Leukemia. Chemotherapy. 2018;63(2):101-7.
14. Roden DM, McLeod HL, Relling MV, Williams MS, Mensah GA, Peterson JF, et al. Pharmacogenomics. Lancet. 2019;394(10197):521-32.
15. van der Wouden CH, van Rhenen MH, Jama WOM, Ingelman-Sundberg M, Lauschke VM, Konta L, et al. Development of the PGx-Passport: A Panel of Actionable Germline Genetic Variants for Pre-Emptive Pharmacogenetic Testing. Clin Pharmacol Ther. 2019;106(4):866-73.
16. Relling MV, Klein TE, Gammal RS, Whirl-Carrillo M, Hoffman JM, Caudle KE. The Clinical Pharmacogenetics Implementation Consortium: 10 Years Later. Clin Pharmacol Ther. 2020;107(1):171-5.
17. Liang DC, Yang CP, Liu HC, Jaing TH, Chen SH, Hung IJ, et al. NUDT15 gene polymorphism related to mercaptopurine intolerance in Taiwan Chinese children with acute lymphoblastic leukemia. Pharmacogenomics J. 2016;16(6):536-9.
18. Bernsen EC, Hagleitner MM, Kouwenberg TW, Hanff LM. Pharmacogenomics as a Tool to Limit Acute and Long-Term Adverse Effects of Chemotherapeutics: An Update in Pediatric Oncology. Front Pharmacol. 2020;11:1184.

19. Zhu Y, Yin D, Su Y, Xia X, Moriyama T, Nishii R, et al. Combination of common and novel rare NUDT15 variants improves predictive sensitivity of thiopurine-induced leukopenia in children with acute lymphoblastic leukemia. Haematologica. 2018;103(7):e293-e5.
20. Relling MV, Gardner EE, Sandborn WJ, Schmiegelow K, Pui CH, Yee SW, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. Clin Pharmacol Ther. 2011;89(3):387-91.
21. Mlakar V, Huezo-Diaz Curtis P, Satyanarayana Uppugunduri CR, Krajinovic M, Ansari M. Pharmacogenomics in Pediatric Oncology: Review of Gene-Drug Associations for Clinical Use. Int J Mol Sci. 2016;17(9).
22. Zazuli Z, Otten LS, Drogemoller BI, Medeiros M, Monzon JG, Wright GEB, et al. Outcome Definition Influences the Relationship Between Genetic Polymorphisms of ERCC1, ERCC2, SLC22A2 and Cisplatin Nephrotoxicity in Adult Testicular Cancer Patients. Genes (Basel). 2019;10(5).
23. Clemens E, Brooks B, de Vries ACH, van Grotel M, van den Heuvel-Eibrink MM, Carleton B. A comparison of the Muenster, SIOP Boston, Brock, Chang and CTCAEv4.03 ototoxicity grading scales applied to 3,799 audiograms of childhood cancer patients treated with platinum-based chemotherapy. PLoS One. 2019;14(2):e0210646.

1

CHAPTER 2

Pharmacogenetics of chemotherapy treatment response and -toxicities in patients with osteosarcoma: a systematic review of 2010-2020

Evelien G.E. Hurkmans [1]; Annouk C. A. M. Brand [1]; Job A.J. Verdonschot [2]; D. Maroeska W.M. te Loo [3]*; Marieke J.H. Coenen [1]*

[1] Radboud university medical center, Radboud Institute for Health Sciences, Department of Human Genetics, Nijmegen, The Netherlands.

[2] Maastricht University Medical Center, Department of Clinical Genetics, Maastricht, The Netherlands

[3] Radboud university medical center, Radboud Institute for Health Sciences, Department of Pediatrics, Nijmegen, The Netherlands.

* both authors contributed equally

Under Review

Abstract

Background: Osteosarcoma is the most common bone tumor in children and adolescents. Despite multiagent chemotherapy, only 71% of patients survives and these survivors often experience long-term toxicities. The main objective of this systematic review is to provide an overview of the discovery of novel associations of germline polymorphisms with treatment response and/or chemotherapy-induced toxicities in osteosarcoma.

Methods: MEDLINE and Embase were systematically searched (2010-2020). Genetic association studies were included if they assessed >10 germline genetic variants in > 5 genes in relevant drug pathways or if they used a genotyping array or other large-scale genetic analysis. Quality was assessed using adjusted STrengthening the REporting of Genetic Association studies (STREGA)-guidelines. To find additional evidence for the identified associations, literature was searched to identify replication studies.

Results: After screening 1533 articles, fifteen articles met our inclusion criteria. These range from studies focusing on genes in relevant pharmacokinetic pathways to whole genome sequencing. Eight articles reported on doxorubicin-induced cardiomyopathy. For seven genetic variants in *CELF4, GPR35, HAS3, RARG, SLC22A17, SLC22A7* and *SLC28A3*, replication studies were performed, however without consistent results. Four small studies reported on bone marrow, nephro- and/or hepatotoxicity. Six studies included analysis for treatment efficacy. Genetic variants in *ABCC3, ABCC5, FasL, GLDC, GSTP1* were replicated in studies using heterogeneous efficacy outcomes.

Conclusions: Despite that results are promising, the majority of associations were poorly reproducible due to small patient cohorts. For the future, hypothesis-generating studies in large patient cohorts will be necessary, especially for cisplatin-induced ototoxicity as these are lacking. In order to form large patient cohorts, national and international collaboration will be essential.

Background

Osteosarcoma is the most common primary bone tumor, that occurs most often in adolescents between the age of 10 and 25 (1, 2). Treatment of osteosarcoma consist of surgical removal of the tumor combined with systematic pre- and postoperative chemotherapy protocols. High-dose methotrexate, doxorubicin and cisplatin form the backbone of this chemotherapy treatment (MAP regimen), combined with ifosfamide or etoposide in some regimens (3). The introduction of this chemotherapeutic treatment in the 1970s has drastically improved survival rates compared to surgery alone (1). However, no major improvements in the treatment protocol have been made since then, with survival rates remaining 71% in the latest publications of the Euramos-1 trial (4).

Despite the positive effect of the MAP regimen on survival rates, patients also develop toxicities that can have a major effect on patients' quality of life, during and after treatment. Moreover, severe toxicities can force oncologists to modify or even discontinue chemotherapy treatment, risking an unfavorable effect on tumor eradication. High-dose methotrexate can lead to bone marrow suppression, liver (hepato-) and renal (nephro-) toxicities (5). The most treatment-limiting side effect of doxorubicin is cardiotoxicity, which can develop during treatment, but most often manifests more than a year after treatment (6). Consequently, pediatric cancer survivors have a 5-15 times higher risk to develop congestive heart failure compared to the general population (7). Lastly, cisplatin can cause acute renal damage and long-term renal insufficiency and above all, cisplatin-induced hearing loss (ototoxicity). This ototoxicity is often irreversible and has a significant impact on quality of life. Approximately 60% of all patients treated with cisplatin will develop some form of ototoxicity, of which the risk increases significantly with exposure and the final cumulative dose (8).

Gaining more insight in the cause and the development of toxicities could lead to more personalized therapies, without compromising on survival rates. Clinical factors, such as age, sex, anthracycline- and cisplatin dose and kidney function, are known to contribute to the risk of developing these different toxicities (7, 9). Metastasis at diagnosis is the best validated predictor for treatment response and used in current protocols for stratification (4, 10, 11). However, this has not led to individualized treatment protocols so far. In addition, these clinical predictors do not explain all interpatient variation. Gaining more insight in genetic risk factors for survival and risk of toxicities (pharmacogenomics) may lead to a better understanding of biological mechanisms behind the different phenotypes and may ultimately lead to improved prediction models for these phenotypes seen in clinical practice.

The main goal of genetic association studies in the field of pharmacogenomics is to identify genetic variants which may explain interpatient variability in drug response, to improve drug efficacy and reduce the risk of drug-induced toxicities (12). In patients with osteosarcoma, pharmacogenomics may also give new opportunities to optimize treatment response and reduce toxicities. Most studies in this field have been focusing on genes that were already known to be involved in the working mechanisms of the treatments used, as described previously by our group in 2016 (13). So far, this has not resulted in the identification of genes that can be used in the clinical setting. Investigating

the genetic background of treatment outcome in a non-hypothesis driven manner has the advantage that new unexpected genes might be uncovered. An illustrative example is the association between *ACYP2* and ototoxicity in patients with pediatric brain tumors which was identified in a genome-wide association study (GWAS) (14). This association was replicated in other patients, e.g. osteosarcoma patients (15) and adult testicular cancer patients (16) and these were combined in meta-analyses (17, 18). In all studies, patients with the AA genotype have an increased risk of ototoxicity, however the evidence is not yet strong enough to implement interventions based on this association in clinical practice. With regard to hypothesis- generating studies, osteosarcoma patients are especially suitable, because treatment has been consistent for a long time and is comparable around the world allowing the formation of larger homogenous cohorts in this relatively rare disease. The size of a homogenous patient cohort is an issue for many pediatric pharmacogenetic studies, because cohorts of patients with multiple diseases treated with similar medication are often combined. Discovery of novel associations in osteosarcoma cohorts reduces variance in the data and may accelerate the path towards the implementation to clinical practice.

This current review aims to systematically summarize the findings of hypothesis-generating pharmacogenetic studies that included patients with osteosarcoma. More specifically, we studied literature about the discovery of novel associations of germline polymorphisms with treatment response and/or chemotherapy-induced toxicities (bone marrow, hepato-, nephro-, oto-, and cardiotoxicity) in osteosarcoma patients, published in the last decade. In addition to that, replication studies of these discoveries were summarized to determine the strength of the evidence at this point in time.

Methods

Systematic search

The aim of this systematic review was to assess the currently available literature about the discovery of novel genetic variants involved in treatment response or treatment toxicity in patients with osteosarcoma. MEDLINE and Embase were systematically searched for relevant publications between 2010 and 2020. The search strategy in both electronic databases consisted of three elements connected by AND, as shown in *Figure S1*. The first element describes the patient group, which are patients with osteosarcoma. To include articles with patient cohorts with multiple diagnoses, a search term is added to describe pediatric patients with cancer, studying toxicities of cisplatin, doxorubicin or methotrexate. It was assessed manually if these mixed cohorts also include patients with osteosarcoma. The second element defines that articles should investigate genetic variation or be genome-wide association studies. The third element defines all outcomes of interest. These include outcomes that describe treatment efficacy, for example overall survival, event-free survival, disease progression or relapse. In addition, search terms are added to include toxicities of interest: ototoxicity, nephrotoxicity, bone marrow toxicity, cardiotoxicity and hepatotoxicity. These toxicities were studied, because patients with these side effects are most at risk for a dose reduction or discontinuation of treatment, which may have negative

effects on treatment efficacy. In the final search strategy, all keywords with their synonyms, MeSH terms (Pubmed), emtree terms (Embase) were searched, as described in *Table S1*. Cochrane was searched manually with similar search words as the other databases. The date of the literature search was 30th of September 2020.

All articles were screened systematically by two independent reviewers (EH and AB) for eligibility of titles. Thereafter, abstracts were reviewed and before selection for full text, a secondary abstract selection was performed to filter for genetic association studies with available full text articles. During full text selection, the number of variants and genes that are genotyped for the publications were recorded to be able to filter out small candidate gene studies or replication studies. Genetic association studies are included if they assess more than 10 germline genetic variants in more than 5 genes in relevant drug pathways or if they use a genotyping array or other large-scale genetic analysis. The aim of the study should be to generate novel hypotheses as to which genes and/or genetic variants play a role in one of our outcomes of interest in patients with osteosarcoma. Exclusion criteria include: no osteosarcoma patients, replication studies, case reports, review articles, animal studies, in vitro studies, tumor DNA is studied or only a conference abstract is available. In case conflicts between reviewers occurred, a third independent reviewer evaluated documents leading to consensus (MC). The same reviewers (EH and AB) also independently collected and reported results (including study design, cohort size, genotyping methods, phenotype, associated variants, effect size with confidence intervals and p-values) of the studies that met the inclusion criteria.

Quality assessment

Quality assessment was performed to assess quality of the articles that were found in this systematic review. The quality assessment form was adapted from van Vugt and colleagues (*Table S5*), who adapted a previously published tool (19, 20). Briefly, the STrengthening the REporting of Genetic Association studies (STREGA) guidelines for reporting of genetic association studies was adjusted to be more applicable to pharmacogenetic studies (20, 21).

Follow-up research

In order to find additional evidence for the associations that were found in the systematic search, a structured literature search was performed to identify studies that aim to replicate these associations. This included a search of the 91 genetic association studies identified during selection of the articles of the systematic search. In addition, PharmGKB and Pubmed were searched manually using the gene and variant of interest, doxorubicin and/or cardiotoxicity as search words. Furthermore, Web of Science was consulted to assess all articles that cited one of our selected articles. The articles that contained relevant information on replication of the associations found in the systematic search are included in the results.

Results

Literature search

The systematic search in MEDLINE and Embase yielded a total of 1533 publications. The manual search in Cochrane yielded no additional publications. During the screening process, 229 duplicates were removed, and 1053 articles were removed because the title was not applicable to our research question (*Figure 1*). Consequently, the abstract screening was performed in 2 phases. In the first phase, 113 articles were removed because they were other studies than (pharmaco)genetic association studies, and secondly, all remaining studies were assessed again to make sure the full text was available, and the study included patients with osteosarcoma. In the full-text assessment, an inventory was made of the number of variants and genes studied in each article (*Table S2* and *S3*). Twenty articles, studying >10 variants in >5 genes, passed our inclusion criteria. Of these, two articles were excluded because they were not hypothesis-generating (22, 23), two did not have osteosarcoma patients in the discovery phase of the study (24, 25) and one article only studied methotrexate (MTX) pharmacokinetics, but not one of the outcomes of our interest (26). *Table S4* shows an overview of the articles that were excluded in the last phases of the selection as a result of the pre-defined inclusion criteria, as indicated with an asterisk in *Figure 1*. Eventually, fifteen articles were explored further in this systematic review.

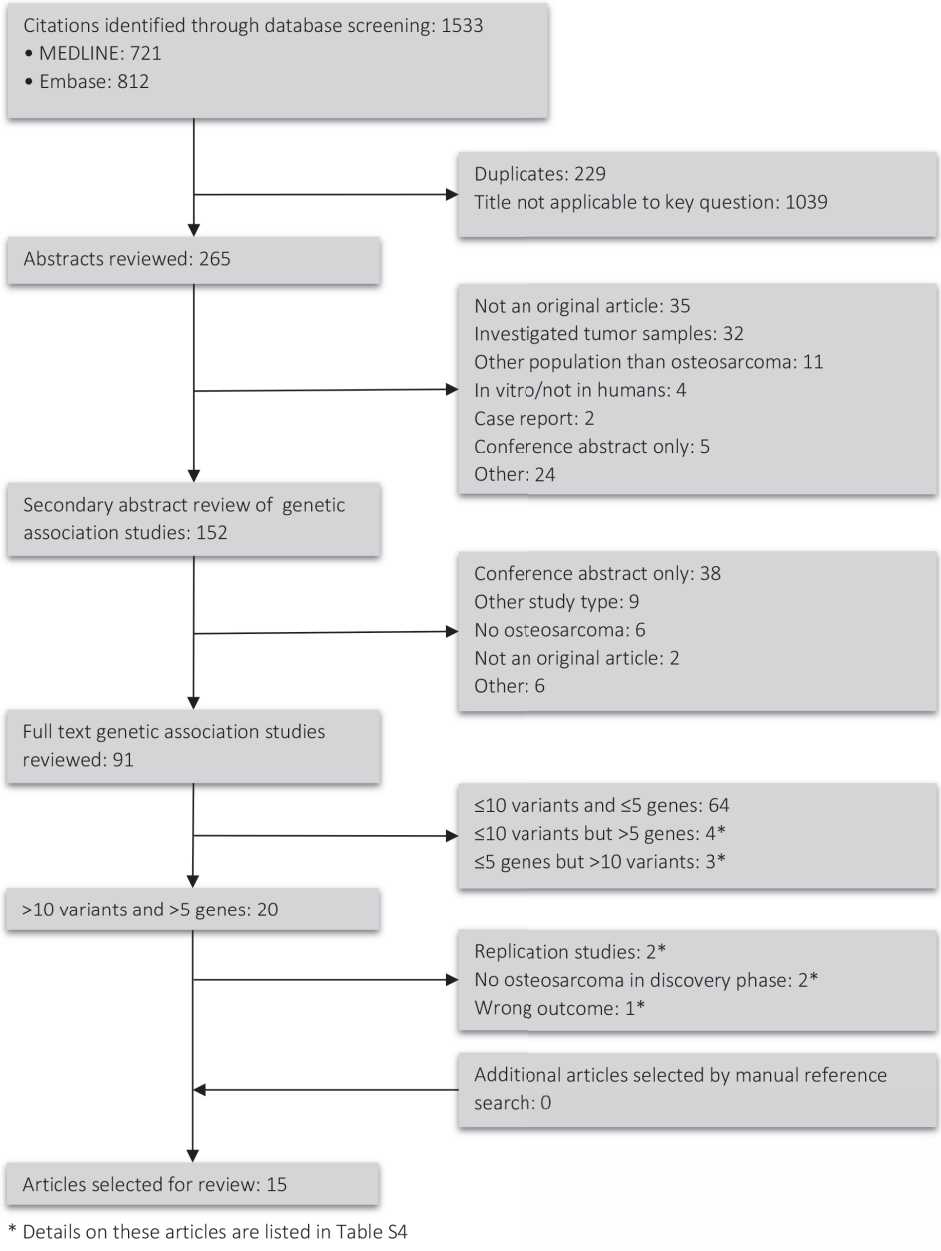


Figure 1. Flowchart for selection of articles.

Quality assessment

The 15 studies that resulted from the systematic search were assessed for their quality of reporting and the results to the questions are presented in *Table S6*. The reporting of basic characteristics of the patient cohort was sufficient in 13 of 15 studies (87%). As shown in *Table S7*, these 13 studies all reported on the sex, age and diagnosis of the patients. The reporting of additional characteristics depended on the main outcome that was studied. Of the six articles on treatment efficacy, five reported on metastasis at diagnosis, four on the location of the osteosarcoma (axial or not) and four on the histological subtype of the tumor. The most important characteristics of the eight studies focusing on cardiotoxicity were follow-up time (7/8), radiation involving chest region (6/8) and anthracycline cumulative dose (6/8). Power calculations are lacking in 12 of the 15 studies (80%). Three studies performed a retrospective power calculation to explain why variants that were previously found, were not found in their study. Seven studies contained a validation cohort to replicate their findings.

For genotyping, five studies used a genome-wide array, one study used whole genome sequencing, four studies assessed an array with variants in genes involved in absorption, distribution, metabolism and excretion (ADME) of medicines and one used a chip with genes involved in cardiovascular disease. Four studies did manual genotyping with (multiplex) allelic discrimination assays. All studies that did not analyze their data as a GWAS, had a clear rationale in their introduction or methods regarding the choice of variants (e.g. ADME or cardiovascular disease variants). Regarding quality control of the genotyping data, nine studies reported on the exclusion of variants based on call rates, with cut-offs ranging from 0.85 to 0.99. Hardy Weinberg equilibrium (HWE) calculations were performed in 13 studies and lead to exclusion of variants in 12 of these articles if a variant deviated from HWE. During statistical analyses 12 studies assessed the effects of covariates and corrected for them in their genetic association analysis accordingly. Finally, ten studies used a form of correction for multiple testing, ranging from the strict Bonferroni correction to adjusting to a stricter p-value threshold without further explanation of rationale.

Treatment response

Six of the fifteen studies identified with the systematic review focused on the role of genetic variants in treatment response or treatment efficacy of chemotherapy in patients with osteosarcoma (*Table 1*). A variation of treatment outcomes was used to describe treatment response, including progression free survival (PFS), event free survival (EFS), overall survival (OS), histological response, relapse and tumor necrosis.

Four of the six studies investigated treatment response in a broad panel of variants in genes involved in metabolism and transport of cisplatin, doxorubicin and methotrexate, as shown in *Table 1*. In the study by Caronia *et al.*, *ABCC3* rs4148416 was associated with EFS, with a hazard ratio of 6.33 (95%CI 1.79-12.7, p=0.0021)(27). This was the first evidence of the genes' clinical relevance in osteosarcoma treatment response and this was replicated successfully in two Chinese populations with consistent directions of effects, as shown in *Figure 2* and *Table S8* (28, 29). In addition, Caronia *et al*. identified

three variants in *ABCB1* to be associated with both EFS and OS (rs4148737, rs1128503, rs10276036)(27). The T-allele of the *ABCB1* rs1128503 was significantly associated with improved survival. However, replication studies of this variant yielded contradictory results, namely, one study replicated this significant association with consistent directions of effect (29), two studies found significant associations in which the T-allele was a risk allele instead of a protective allele (28, 30) and two studies found no significant associations with osteosarcoma treatment response whatsoever (31, 32). The second study using a pathway approach was the study by Hattinger *et al.*. They showed that *GGH* rs11545078, *CYP2B6**6 and *TP53* rs1642785 and *ABCC2* rs2273697 are associated with EFS (31). However, none of these four variants retained statistical significance in a multiparametric Cox proportional hazard regression analysis (31). Despite that Windsor *et al*. could not replicate the association of *ABCC2* rs2273697 with EFS, another variant in the *ABCC2* gene, namely rs717620, was identified to be associated with poor histological response (32). However, this association was not confirmed in four replications studies (28, 31, 33, 34). In addition, the presence of the G-allele of *GSTP1* rs1695 was associated with poor histological response and with PFS and a variant in *RFC1/SLC19A1* (rs1051266) was significantly associated to PFS. Three of the six studies that also assessed *GSTP1* rs1695 and one of three studies that studied *RFC/SLC19A1* rs1051266 in association to EFS or OS, also found a significant association, as shown in *Figure 2* and *Table S8* (29-31, 33, 35-38). Lastly, the study performed by Hagleitner *et al.* included a discovery cohort of 126 osteosarcoma patients and a replication cohort of 64 patients (34). Five variants were identified to be significantly associated with PFS (*Table 1*), including *FasL* rs763110, *MSH2* rs4638843, *ABCC5* rs939338, *CASP3* rs2720376, *CYP3A4* rs4646437. Genetic risk scores were generated based on these five variants, using the number of unfavorable alleles patients had for these variants. This risk score was able to distinguish between patients with good and poor outcome, both in patients with and without metastases (34). In the replication cohort by Xu *et al*., only *FasL* rs763110 and *ABCC5* rs939338 contributed to the risk score to predict treatment outcome (39). Overall, all studies that used a pathway approach found novel genetic variants that may play a role in the response to treatment of osteosarcoma, however, the study by Caronia *et al.* was the only one large enough to correct for multiple testing.

Table 1. Study design, population characteristics and outcomes of hypothesis-generating pharmacogenetic studies investigating the association between genetic variants and treatment response.

Author, year	Study design				Associated phenotype(s)	Associated variant(s)	Ref.
	Study approach	No. of osteosarcoma patients	Ethnicity; nationality	Investigated number of variants and genes			
Bhuvaneshwar *et al.*, 2019	Whole genome sequencing	INOVA: 15 TARGET: 85	INOVA: NS; American TARGET: NS; Canadian/ Brazilian	Mutation hotspot haplotypes 4,543 variants in ADME genes	Relapse Tumor necrosis and survival	Haplotypes in 26 genes 10 SNPs in 4 genes found in haplotypes in both datasets *SLC22A1* rs4646272* *SLC22A8* rs2187384* *UGT2B15* rs34073924* *CHST12* rs3735099* *CHST12* rs3735100*	(41)
Caronia *et al.*, 2011	Pathway approach	102	NS; Spanish	366 variants in 24 metabolism and transporter genes	OS EFS	*ABCC3* rs4148416 *ABCB1* rs4148737 *ABCB1* rs1128503 *ABCB1* rs10276036	(27)
Hagleitner *et al.*, 2015	Pathway approach	Disc.: 126 Repl.: 64	NS; Dutch	381 variants in 54 drug metabolism genes	PFS	*FasL* rs763110* *MSH2* rs4638843* *ABCC5* rs939338* *CASP3* rs2720376* *CYP3A4* rs4646437*	(34)
Hattinger *et al.*, 2016	Pathway approach	126	NS; Italian	47 variants in 31 drug metabolism and transport genes	EFS	*ABCC2* rs2273697* *GGH* rs11545078* *TP53* rs1642785* *CYP2B6*6*	(31)
Koster *et al.*, 2018	GWAS	GWAS: 523 Repl.: 109	GWAS: >80% European Repl.: NS; Brazilian	510,856 variants	OS	*GLDC* rs3765555 *GLDC* rs55933544	(40)
Windsor *et al.*, 2012	Pathway approach	58	Caucasian: 41 Afro-Caribbean: 8 Indian/Asian: 9	35 variants in 21 pharmacological pathway genes of MAP.	PFS Histological response	*CCND1* rs9344 * *RFC* rs1051266* *GSTT1 null** *GSTP1* rs1695* *ABCC2* rs717620* *GSTP1* rs1695* *MTHFD1* rs2236225*	(32)

GWAS: genome-wide association study; INOVA: Inova pediatric group osteosarcoma patients; TARGET: TARGET osteosarcoma dataset; Disc.: discovery cohort; Repl.: replication cohort; NS: Not specified; ADME, absorption, distribution, metabolism and excretion; MAP: Methotrexate – Adriamycin (Doxorubicin) – Cisplatin chemotherapy regimen; OS: overall survival; EFS: event-free survival; PFS; progression-free survival

* Association was not significant after multiple testing correction, but is/are the top hit(s) of the study.

A genome-wide association study (GWAS) including 523 osteosarcoma patients was performed by Koster *et al.* in 2018. They investigated 510,856 genetic variants in relation to OS (40). The variant which had the strongest association with OS, namely rs1030228, could not be replicated in their replication cohort, consisting of 109 osteosarcoma patients. However, in a combined analysis of the discovery cohort and replication cohort, another variant, rs3765555, was inversely associated with OS (HR = 1.76 per copy of the A-allele). Imputation of the region centered around this variant identified a second variant, rs55933544, significantly associated with OS (HR = 1.92 95% CI 1.53-2.41, $p=1.34\times10^{-8}$). Lin *et al*. replicated the association of rs55933544 with OS in their patient cohort with an odds ratio of 2.98 (95% CI 1.87-4.96, $p<0.001$). Furthermore, expression quantitative trait locus (eQTL) analysis showed that the T-allele of rs55933544 was significantly associated with a decreased *IL33* expression and lower *IL33* expression was independently associated with worse osteosarcoma patient survival (40). The study by Bhuvaneshwar *et al.* used whole genome sequencing data to identify haplotypes associated with relapse (41). Using both the TARGET and INOVA patient datasets, 231 haplotypes were described of which the variants could be mapped to 26 genes. From the haplotypes, only four variants in *MKI67,* one in *CACNA2D4,* three in *SLC13A2* and two in *PPP1R12C* were associated with relapse in both patient datasets independently. These variants were not previously indicated in osteosarcoma treatment response and no replication studies have been performed yet. Bhuvaneshwar *et al.* also extracted variants in ADME genes from their dataset to investigate the association with tumor necrosis and OS. A total of 281 variants were associated with tumor necrosis and five of these variants, in *SLC22A1*, *SLC22A8*, *CHST12* and *UGT2B15,* were also associated with OS, prioritizing these for future research.

Doxorubicin-induced cardiotoxicity

Eight studies reported on pharmacogenetics concerning doxorubicin-induced cardiotoxicity (*Table 2*). These range from small studies with limited numbers of patient and small numbers of variants studied to GWASs with larger patient cohorts. In an exploratory study by Windsor *et al*., 36 genetic variants in 21 genes in the pharmacokinetic pathways of cisplatin, doxorubicin and methotrexate were investigated in relation to multiple outcomes in a patient cohort of 58 osteosarcoma patients (32). With regard to doxorubicin-induced cardiotoxicity, the G-allele of *GSTP1* rs1695 was associated to both early and end-of-treatment cardiotoxicity. This variant was previously mainly indicated in treatment efficacy, as described above, however not in cardiotoxicity. Due to the exploratory nature of this study and the small patient cohort, these associations were not corrected for multiple testing. No replication studies of this association were identified in our search (*Table S9*).

In another study, Hildebrandt *et al.* assesses 12 loci that were previously indicated in hypertension by a GWAS (42). They found that the G-allele of *PLCE1* rs932764 and the G-allele of *ATP2B1* rs17249754 are protective to doxorubicin-induced cardiotoxicity in pediatric cancer survivors. In addition, they showed that doxorubicin exposure to iPSC-cardiomyocytes is associated to decreased *PLCE1* expression and increased *ATP2B1* expression in a dose-dependent manner.

In two pharmacogenetic studies by Visscher *et al.*, both studying a broad panel of ADME genes, multiple variants in the Solute Carrier (SLC) family were significantly associated with anthracycline-induced cardiotoxicity. In their patient cohorts, approximately 80% of patients was treated with doxorubicin and the rest was treated with other anthracyclines, most often daunorubicin. In the first study in 2012, the A-allele of *SLC28A3* rs7853758 was found more often in controls than in cases, and significantly associated in a protective manner (43). Another variant in the same gene (rs4877847) was also significantly associated with anthracycline-induced cardiotoxicity, even after conditioning for rs7853758 thus suggesting an independent effect. The association of the top-hit (rs7853758) was replicated in the same study in an independent patient cohort. In addition, Visscher *et al.* published a validation study in 2013 in which this effect was strengthened in a meta-analysis (22). The additional power of this meta-analysis also gained a novel top-hit association. The A-allele of *UGT1A6* rs17863783 was found to be significantly associated with anthracycline-induced cardiotoxicity (OR (95%CI) = 4.30 (1.97-9.36), $p = 2.4\times10^{-4}$). Of the additional nine patient cohorts that attempted to replicate the *SLC28A3* rs7853758 association(44-47), only two cohorts succeeded, as shown in *Figure 2* and *Table S9* (24, 48). Interestingly, the associations were only found in pediatric patient cohorts and the effect was never found in adults. In their second discovery study, Visscher *et al*. studied a more broad ADME panel, containing 4153 variants in 300 genes. For both *SLC22A17* rs4982753 and *SLC22A7* rs41491178, the minor allele was found more often in controls than in cases and had a protective effect for developing cardiotoxicity after doxorubicin treatment (49). Both variants were not statistically significantly after a strict Bonferroni correction, but finding two variants associated with cardiotoxicity within the SLC22 gene family was significantly higher than expected by chance alone. In addition, the associations were successfully replicated in an independent patient cohort in the same study. However, the associations were not found in a subsequent candidate gene study (24) and GWAS (50).

Table 2. Study design, population characteristics and outcomes of hypothesis-generating pharmacogenetic studies investigating anthracycline-induced cardiotoxicity in patients with osteosarcoma

Author (year)	Study design				Associated variant(s)	Ref.
	Study approach	No. of osteosarcoma patients	Ethnicity; nationality	Investigated number of variants and genes		
Aminkeng *et al.*, 2015	GWAS	Stage 1: 11 (of 280) Stage 2: 9 (of 96) Stage 3: 5 (of 80)	Stage 1: Caucasian; Canadian Stage 2: Caucasian; Dutch Stage 3: African, East Asian and Aboriginal	738,432 variants	*RARG* rs2229774	(54)
Hildebrandt *et al.*, 2017	Hypertension loci	41 (of 108)	White, hispanic or black; American	12 variants in *PLCE1, ATP2B1, ARHGAP42, GNAS-EDN3, C10orf107, CSK, BAG6, CACNB2, MTHFR, CACNB2, HFE, NPR3*	*PLCE1* rs932764* *ATP2B1* rs17249754*	(42)
Ruiz-Pinto *et al.*, 2017	Exome array with low frequency variants	15 (of 93)	NS; Spanish	247,870 variants	*GPR35* rs12468485	(48)
Visscher *et al.*, 2012	ADME panel	Disc.: 11 (of 156) Repl.: 10 (of 188) Repl.2: 6 (of 96)	Disc. and repl.: (non-) European; Canadian Repl.2: NS; Dutch	1,931 variants in 220 drug bio-transformation genes	*SLC28A3* rs7853758	(43)
Visscher *et al.*, 2015	ADME panel	Discover.: 21 (of 344) Repl.: 16 (of 218)	NS; Canadian and Dutch	4,153 variants in 300 pharmacokinetics and -dynamics genes	*SLC22A17* rs4982753* *SLC22A7* rs4149178*	(49)
Wang *et al.*, 2014	Cardiovascular panel: gene – environment interaction	Disc.: 54[a] (of 287) Repl.: 0 (of 76)	Disc.: Non-Hispanic white; American Repl.: Non-Hispanic white, Hispanic, other; American	34,912 variants in 2,100 genes associated with cardiovascular disease.	*HAS3* rs2232228	(51)
Wang *et al.*, 2016	GWAS: gene – environment interaction	Disc.: 96[b] (of 331) Repl.: 17 (of 54)	Disc.: Non-Hispanic white; American Repl.: Non-Hispanic white, Hispanic, Other; American	709,358 variants	*CELF4* rs1786814	(52)
Windsor *et al.*, 2012	Pathway approach	58 (of 58)	Caucasian, Afro-Caribbean, Indian, Asian; UK	35 variants in 21 pharmacological pathway genes of MAP.	*GSTP1* rs1695*	(32)

NS, not specified; GWAS, genome-wide association study; ADME, absorption, distribution, metabolism and excretion; disc., discovery cohort; repl., replication cohort; MAP: methotrexate, adriamycin (doxorubicin), cisplatin chemotherapy regimen.

[a] Number of bone tumors, osteosarcoma is not specified.

[b] Number of sarcomas, osteosarcoma is not specified.

* Association was not significant after multiple testing correction, but is/are the top hit(s) of the study.

A study by Wang *et al.* focused on genes that were previously associated with cardiovascular disease, since cardiotoxicity risk is influenced by coexistence of cardiovascular disease risk factors like hypertension and diabetes (51). In this study, cumulative anthracycline exposure was calculated by multiplying the cumulative dose of individual anthracyclines with a factor that describes the drug's cardiotoxic potential, but it was not indicated what percentage of the cohort received which anthracycline. Despite that, for this systematic review it was assumed that the majority was treated with doxorubicin. No variants were associated with anthracycline-induced cardiomyopathy, but a gene-environment interaction analysis identified the variant *HAS3* rs2232228. Among patients with the GG genotype for this variant, cardiomyopathy was infrequent and not dose related. However, the AA genotype conferred an 8.9-fold increased cardiomyopathy risk when also exposed to anthracycline doses > 250 mg/m^2, compared to the GG genotype. In the GWAS that Wang *et al*. executed two years later, they found no variants associated with anthracycline-induced cardiomyopathy (52). Again, they carried out a gene-environment analysis, which identified *CELF4* rs1786814 to have a gene-environment interaction with anthracycline dose. Patients with the *CELF4* rs1786814 GG genotype who were exposed to anthracycline levels greater than 300 mg/m^2, had a 10.2-fold increased risk of cardiomyopathy compared to patients with the GA/AA genotype and exposure to anthracycline levels of 300 mg/m^2 or lower. Among other variants, *HAS3* rs2232228 and *CELF4* rs1786814 were studied by Leger *et al*. in hematopoietic cell transplantation survivors treated with anthracyclines. For the association of *CELF4* rs1786814 with cardiomyopathy, the interaction between SNP and anthracycline dose was found, with p=0.02 (53). In addition, a significant association was found with an analysis limited to anthracycline doses >300 mg/m^2 (1-sided p=0.01; *Table S9*). The interaction of *HAS3* rs2232228 AG genotype and anthracycline dose had a 1-sided p=0.01 in this study. When restricting the analysis to anthracycline doses >250 mg/m2, a significant association was found (*Table S9*). However, no significant association was found when studying the main effects of the variants in a complete patient cohort containing all dosages with short- or long term cardiomyopathy, for neither *HAS3* rs2232228 and *CELF4* rs1786814.

In a GWAS by Aminkeng *et al.* an association was found which was also replicated in their two replication patient cohorts (54). In all cohorts, the majority of patients was treated with doxorubicin, followed by daunorubicin and epirubicin. The SNP, *RARG* rs2229774, was associated with anthracycline-induced cardiotoxicity for both patients receiving a low to moderate anthracycline dose and patients receiving a high anthracycline dose. Overall, rs2229774 carriers (AA or AG genotype) had significantly increased odds of developing cardiotoxicity in comparison to non-carriers after doxorubicin treatment (OR (95%CI) = 4.7 (2.7-8.3), p = 4.3×10^{-11}). The only other replication study that found a significant association, was the study by Schneider and colleagues in 2017 (50). However, they found that the A-allele causes a decreased risk rather than an increased risk for cardiotoxicity (*Table S9*). They argue that difference in direction of the effect may be due to the general heterogeneity between studies.

The genome-wide analysis of Ruiz-Pinto *et al.* focused on low frequency exome variants, as they used an exome array that is enriched with low frequency variants (80% of variants with minor allele frequency (MAF) ≤ 1%) (48). No variant showed a significant

association with chronic anthracycline-induced cardiotoxicity after correction for multiple testing, but a novel significant association for *GPR35* was identified by gene-based testing. The SNP rs12468485 made the greatest contribution toward the observed association. The T-allele was almost exclusively found in cases, and 67% of cases carrying the CT genotype had an extreme chronic cardiotoxicity phenotype. Up to now, no replications of this association have been attempted.

Cisplatin-induced ototoxicity

In this systematic review, no publications were identified that studied the association of genetic variants with cisplatin-induced ototoxicity in patients with osteosarcoma. Windsor *et al*. planned on including ototoxicity in the genetic association study described above (32). However, this analysis was not performed because of incomplete data.

Bone marrow- hepato- and nephrotoxicity

Four studies were identified that focused on nephrotoxicity, hepatotoxicity and/or bone marrow toxicity in the systematic search. *Table 3* shows that studies by Hattinger *et al*., Hegyi *et al*., and Windsor *et al*. are among the smallest studies in this review, as they studied 45, 29 and 36 variants in cohorts of 57, 59 and 58 osteosarcoma patients, respectively (31, 32, 55). Whereas significant associations were found, none of these studies corrected for multiple testing and that was also reflected by many replication studies with negative results. The results of the *ABCC2* gene is a clarifying example for this. rs2273697 was associated with hepatotoxicity and thrombocytopenia by Hattinger *et al*., however, as *Figure 2* indicates, six studies that also related this variant to hepatotoxicity did not find a significant association (31-33, 55-58), negative results were also found in four studies that related this variant to thrombocytopenia (31, 32, 56-58). In addition, rs2273697 was found to be associated with leukopenia according to both Hattinger *et al*. and Hegyi *et al*., but this association was not found in 4 other cohorts (31, 32, 55-58). Lastly, Hegyi *et al*. and Windsor *et al*. found that *ABCC2* variants rs3740066 and rs17222723, respectively, were associated with leukopenia. However these associations were not replicated in any of the replication studies (31, 32, 55, 58). Altogether, multiple replication studies were performed of the initial findings, but none of the replication studies could confirm these (*Table S10*).

Table 3. Study design, population characteristics and outcomes of hypothesis-generating pharmacogenetic studies investigating bone marrow- hepato- or nephrotoxicity in patients with osteosarcoma

Author, year	Study design					Associated phenotype(s)	Associated variant(s)	Ref.
	Study approach	No. of osteosarcoma patients	Ethnicity; nationality	Investigated number of variants and genes	Investigated phenotypes			
Hattinger et al., 2016	Pathway approach	57	NS; Italian	45 variants in 31 drug metabolism and transport genes	Leukopenia, thrombocytopenia, red blood cell transfusion, platelet transfusion, hepatotoxicity	Leukopenia Thrombocytopenia Hepatotoxicity	*ABCC2* rs2273697* *MTHFR* rs1801131* *ABCC2* rs2273697* *XPD* rs1799793* *ABCB1* rs1128503* *ABCC2* rs2273697* *GGH* rs1800909[HW]*	(31)
Hegyi et al., 2017	Pathway approach	59	NS; Hungarian	29 variants in *ABCB1, ABCC1, ABCC2, ABCC3, ABCC10, ABCG2, GGH, SLC19A1, NR1I2*	Leukocyte/ neutrophil granulocyte count	Myelotoxicity Hepatotoxicity	*ABCC2* rs2273697* *ABCC2* rs3740066* *NR1I2* rs3732361* *NR1I2* rs3814058 * *NR1I2* rs6785049 * *NR1I2* rs3732361* *NR1I2* rs3814058 * *NR1I2* rs6785049 *	(55)
Hurkmans et al., 2020	ADME panel	113	Caucasian; Dutch, Spanish, Australian	1936 variants in 231 ADME genes	Creatinine, ALAT, ASAT, hemoglobin, thrombocyte, leukocyte and neutrophil counts	Thrombocyte counts	*CYP2B6* rs4803418 *CYP4F8* rs4808326 *CYP2B6* rs4803419	(58)
Windsor et al., 2012	Pathway approach	58	Caucasian: 41 Afro-Caribbean: 8 Indian/ Asian: 9	36 variants in 21 pharmacological pathway genes of MAP	Anemia, leucopenia, myelo-suppression, GFR, infection	Leucopenia Anemia Infection Nephrotoxicity	*ERCC1* rs3212986* *GSTP1* rs1695* *ABCC2* rs17222723* *MTHFD1* rs2236225* *MTHFR* rs1801131* *CYBA* rs4673* *XPC* rs2228001* *ERCC2* rs13181* *MTHFR* rs1801133*	(32)

NS, Not specified; ADME, absorption, distribution, metabolism and excretion; MAP: Methotrexate – Adriamycin (Doxorubicin) – Cisplatin chemotherapy regimen; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase

* Association was not significant after multiple testing correction, but is/are the top hit(s) of the study.

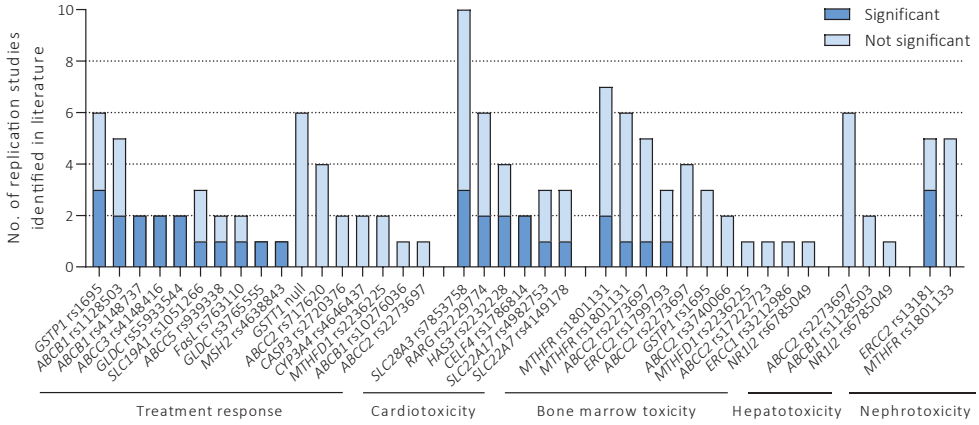


Figure 2. Replication studies of the association of genetic variants with treatment response and toxicities that were identified in literature. Associations that are indicated as significant also showed consistant direction of effect with the discovery study. *Table S8, S9* and *S10* elaborate more on phenotypes, effect sizes and significance of these replication studies.

The association of *MTHFR* variant rs1801131 with anemia that was identified by Windsor *et al*., was conformed in two cohorts of acute lymphatic leukemia patients, but the association was not significant in five other studies (32, 59-65). In addition, Hattinger *et al*. showed that this variant is also associated to leukopenia. Whereas ten studies also assessed this association, there was only one study with a significant result, however with an opposite direction of effect (31, 59-61, 64-70). The only association that shows consistent replication is the association of *ERCC2* variant rs13181 with nephrotoxicity, that was identified by Windsor *et al*.. In four of six patient cohorts treated with cisplatin-based treatment, the association of the AC or CC genotype was associated with increased risk to develop nephrotoxicity was confirmed, with odds ratios ranging from 3.16 to 4.4 (32, 71-75).

Finally, the study by Hurkmans *et al*. assessed a panel of 1936 variants in 231 genes involved in absorption, distribution, metabolism and excretion of medicines for multiple toxicities (58). Three variants in the Cytochrome P450 family were significantly associated with thrombocyte count, namely *CYP4F8* rs4808326, *CYP2B6* rs4803418 and *CYP2B6* rs4803419, and these remained significant after Bonferroni correction for multiple testing. The two variants in *CYP2B6* were in high linkage disequilibrium, and thus likely represent the same locus. Regarding *CYP4F8* rs4808326, carriers of the A-allele had higher thrombocyte counts after methotrexate infusion compared to carriers of the G-allele. The gene has not been linked to methotrexate or thrombocyte count before. The underlying mechanisms of the associations with all three variants are still unclear (58). These associations are not replicated in other patient cohorts yet, as the publication was recent.

Discussion

This systematic review provides an overview of hypothesis-generating pharmacogenetic studies in osteosarcoma patients of the last 10 years. In addition, replication studies of all top-hit associations of the studies were identified in a structured manner to give a more complete idea of the evidence that is present. Treatment response and doxorubicin-induced cardiotoxicity are the most extensively studied phenotypes. Chemotherapy-induced nephrotoxicity, hepatotoxicity and bone marrow toxicity were examined, but only in small patient cohorts. The influence of genetic variants on cisplatin-induced ototoxicity was not investigated in any of the hypothesis-generating studies.

The only GWAS that was performed in relation to treatment response to chemotherapeutic treatment in osteosarcoma patients was the GWAS by Koster *et al*. (40). In that study, two variants in in the *GLDC* gene were found to be associated to overall survival. As previously mentioned, Lin *et al*. successfully replicated the association of rs55933544 with decreased overall survival in their patient cohort (76). Noteworthy, the TT genotype of this variant was not related to *GLDC* expression, but it was associated to lower expression of interleukin-33 (*IL33*) (40, 76). On top of that, Kang and colleagues genotyped common variants in the *IL33* gene and found that the A-allele of rs1048274 was associated to survival in a osteosarcoma patient cohort of Chinese ancestry (77). The patient cohort of Koster *et al*. consisted of European and Brazilian subjects and they did not identify statistically significant associations with common variants in *IL33*. Differences in linkage disequilibrium structures between populations allowed for different variants on the same locus to be associated to survival of patients with osteosarcoma, indicating that not *GLDC*, but *IL33* is causal for decreased survival through the variant. This emphasizes that studying populations of different ethnicities helps in fine mapping the genetic background that causes a phenotype. *IL33* was previously associated to prognosis in other cancers (78-80) and it is known to have pro- and anti-tumorigenic properties mediated through immune cells (81). In osteosarcoma, *IL33* plays a role in osteosarcoma cell viability in *in vitro* experiments mediated through the PI3K/AKT pathway (82, 83). However, the exact role and the effects of genetic variants remains to be found.

A pathway approach including genes linked to osteosarcoma treatment was used by most of the included studies, so consequently members of the *ABC* transporter family were included in the pharmacogenetic investigations and sometimes found to be associated to the outcomes of interest. These genes code for membrane-bound proteins which participate in the movement of most drugs and their metabolites across cell surface and cellular organelle membranes. Defects in these genes can be important in terms of cancer therapy and pharmacokinetics (84). As indicated in the results of this review, *ABCC2* variants were repeatedly associated to toxicities in these studies, however *Figure 2* shows that these associations were scarcely replicated. Doxorubicin and methotrexate are both transport substrates for *ABCC2*, which caused these variants to be studied, but this does not necessarily explain the causative functional background of the association that is observed. On the other hand, in the pathway approach study by Caronia *et al*. it was found that per T-allele of the *ABCC3* rs4148416 variant, patients have an 8-fold higher risk of death, and 6 times lower risk on event-free survival, and this association was

consistently replicated in two other cohorts (27-29). *ABCC3* codes for multidrug resistance protein 3 (MRP3) and is an important transporter of bile salts, but is also involved in efflux of methotrexate from liver and kidney cells (85, 86).

The necessity of routine MTX plasma concentration measurement during treatment with MTX has allowed several research groups to study genetic variation involved MTX pharmacokinetics. This was not one of the clinical outcome measures of interest of this systematic review, and therefore these studies were excluded as shown in *Figure 1*, however, genetic variants that are associated to high MTX plasma levels may also give increased risk for toxicity and genetic variants that are associated to low MTX plasma levels may also predispose to a suboptimal treatment response. Lui *et al.* found three variants localized in *ABCG2* to be associated with methotrexate clearance in patients with osteosarcoma, namely rs13120400, rs13137622, rs12505410 (26). Rs13120400 was the most significant variant, and the CC genotype of this variant was previously also associated to increased response to methotrexate in psoriasis patients (87). In addition, in the study by Hegyi *et al.*, which included 59 osteosarcoma patients, *ABCG2* rs2231142 was found to be significantly associated with a longer half-life time of methotrexate (55). However, this variant is not in LD with variants identified by Lui *et al*.. *ABCG2* codes for the breast cancer resistance protein (BCRP) and has an important role in the transport of methotrexate out of the liver and kidney and knockdown of ABCG2 increased the bioavailability of methotrexate in mice (86, 88). Both *ABCC3* and *ABCG2* may be of interest for further investigation with a larger patient cohort to relate it to both pharmacokinetic parameters and clinical outcomes of treatment.

Six variants were found to be associated with doxorubicin-induced cardiotoxicity in a cohort containing osteosarcoma patients and were replicated minimally once in an independent patient cohort, namely *CELF4* rs1786814, *HAS3* rs2232228, *RARG* rs2229774, *SLC22A17* rs4982753, *SLC22A7* rs4149178, *SLC28A3* rs7853758. The associations with *HAS3* rs2232228 and *CELF4* rs1786814 identified by Wang *et al.,* consisted of gene-environment interactions, which means the variant effect is larger in patients that received a higher dose of doxorubicin. This emphasizes the importance of doxorubicin dose in the development of cardiotoxicity and in the effect size of genetic variant. CUGBP Elav-like family member 4 (*CELF4*) is involved in splicing of *TNNT2,* which codes for cardiac troponin T (cTnT). cTnT plays a role in Ca^{2+} signaling and contraction of the heart muscle and is a biomarker for myocardial damage (89). Whereas the embryonal *TNNT2* splicing variant, carrying an additional exon 5, is usually downregulated in adults, patients with *CELF4* rs1786814 GG genotype express both the adult and embryonal *TNNT2* splicing variant. This results in a temporally split myofilament response to calcium, decreasing the ventricular pumping efficiency, and thereby increasing the risk on dilated cardiomyopathy and cardiotoxicity (52, 90). In addition, pathogenic variants in *TNNT2* are an established cause of hypertrophic and dilated cardiomyopathy (91). Adult and pediatric patients with cancer who developed chemotherapy-induced cardiomyopathy have an increased prevalence of pathogenic variants in sarcomere genes compared to controls, indicating that genetics involved in susceptibility to cardiomyopathy, such as mutations in sarcomere genes, may also be of importance in doxorubicin-induced cardiotoxicity (92). *HAS3* encodes for hyaluronan which is a component of the extracellular matrix and is involved in tissue remodeling

after cardiac damage. In addition, hyaluronan reduces cardiac injury caused by ROS, which is an important element of doxorubicin-induced cardiac damage. Furthermore, the *RARG* gene codes for retinoic acid receptor gamma and binds to the topoisomerase IIβ (Top2b) promotor to repress its expression. Top2b is a target of doxorubicin mediated DNA damage, and if Top2b expression is low in cardiac tissue due to repression by *RARG*, the tissue is less susceptible to damage caused by doxorubicin (54). Despite that replication studies of the association of the *RARG* variant with cardiotoxicity were inconsistent, a functional study in iPSC-derived cardiomyocytes showed that the variant *RARG* increases sensitivity to doxorubicin-induced cardiomyopathy (93). Lastly, three variants in genes of the solute carrier transporter family were associated to doxorubicin-induced cardiomyopathy. A variant downstream of *SLC22A17* was associated to cardiotoxicity. *SLC22A17* is ubiquitously expressed, also in the heart, and plays a role in iron transport and homeostasis. Accumulation of iron in mitochondria can cause doxorubicin-induced cardiotoxicity, however the exact role of SLC22A17 in this process is not studied (94). Secondly, *SLC22A7* encodes for the organic anion transporter 2 (OAT2), which is highly expressed in liver and kidney and is known to play an important role in clearance of medicines but is not previously indicated in transport of cisplatin, doxorubicin or methotrexate. Lastly, a variant in *SLC28A3* was associated with cardiotoxicity. *SLC28A3* codes for the sodium-coupled nucleoside transporter 3 (CNT3). Only for CNT3, it was established to transport doxorubicin, indicating it may play a role in doxorubicin pharmacokinetics (95). Despite that this was not shown for OAT2, it does have considerable overlap in substrates with CNT3 and transports several nucleoside-based drugs, for example 5-fluorouracil and zidovudine (96).

While cisplatin-induced ototoxicity is one of the most prevalent adverse effects of cisplatin treatment, it was not investigated in any of the studies that were identified in this systematic review. The most recent GWAS on cisplatin-induced ototoxicity that included patients with osteosarcoma, was performed in 2009 and was therefore excluded from this review (97). In this study, Ross *et al*. identified genetic variants in the *TPMT* and *COMT* gene that are associated to cisplatin-induced hearing loss in pediatric patients with cancer. However, replication studies are very contradictive, as shown in a meta-analysis by Thiessen *et al*. in 2018 (17, 18). Whereas this was the only GWAS that included patients with osteosarcoma, work from other patient cohorts treated with cisplatin may be applicable to patients with osteosarcoma too. Xu *et al*. identified that the A-allele of *ACYP2* rs1872328 gives an increased risk to cisplatin-induced ototoxicity in a pediatric brain tumor cohort (14). In 2020, Clemens *et al*. showed in a meta-analysis of 5 studies with a total of 1418 pediatric patients that this association was also found in cohorts containing patients with osteosarcoma (OR (95%CI) = 3.94 (1.04-14.93), p = 0.04). In addition, they showed a significant association of *SLC22A2* rs316019 with cisplatin induced hearing loss in a meta-analysis of 4 studies (OR (95%CI) = 1.46 (1.07-2.00), p = 0.02). Whereas these results are significant, the heterogeneity between studies remained an obstacle (I^2=66% for *ACYP2* rs1872328 and 44% for *SLC22A2* rs316019). In conclusion, in order to find variants associated with cisplatin-induced ototoxicity, the priority would be to perform a GWAS with large patient cohorts to find reliable results. In the meantime, *ACYP2* rs1872328 and *SLC22A2* rs316019 could be studied further to find out their true potential for clinical practice.

In general, the quality of reporting data in the included studies was good, as shown with the quality assessment using STREGA guidelines. However, there was minimal reporting on follow-up and missing data. All data in the studies was collected retrospectively and there was no prospective follow-up and, therefore, it is sensible that nothing was reported on patients that were lost to follow-up. It was also not reported how missing data was handled in statistical analysis or what kind of analysis was used for the main comparison regarding the missing data. However, it is assumed that these studies did a complete case analysis, because that is most conventional in retrospective genetic association studies. Therefore, this does not compromise the quality of these articles.

The aim of this review was to identify variants that were discovered to be associated to a phenotype in a hypothesis-free manner and might form the basis for future pharmacogenomic studies in osteosarcoma. The most evident method to identify articles that describe these variants would be to limit the systematic search to GWA studies, as they are the textbook example to hypothesis-free research. However, the number of GWASs is limited in pediatric oncology cohorts and even more so in osteosarcoma cohorts. In addition, when a broad range of ADME genes is studied, it does not restrict itself to only genes that are previously indicated in the pathway of a drug. In order to include all literature with a hypothesis-free component, a boundary has been set to articles that assess more than 10 variants in more than 5 genes. The smallest studies in this review, by Hattinger *et al*., Hegyi *et al*., and Windsor *et al*. (45, 29 and 36 variants in cohorts of 57, 59 and 58 osteosarcoma patients), showed the poorest reproducibility (*Table S8, S9, S10*) (31, 32, 55). Possibly, due to the small number of patients in these studies, the power was too low to perform multiple testing correction causing the authors to report false-positive findings.

Not only in the case of small studies, but also in larger studies, replication remains laborious. Our structured search shows that many replication studies have been performed but the majority do not confirm the findings from the discovery study with significant results (see *Figure 2*). Obvious explanations for this are the general heterogeneity between cohorts, different ethnicities, phenotypes and treatment regimens. In addition, power in discovery studies is often too low to correct for multiple testing, leading to false-positive findings. Insufficient power in replication studies may also stand in the way of confirming true-positive findings. As a solution for that, meta-analysis of the discovery and replication cohorts could increase the total power, however in this review there was too much heterogeneity between studies perform reliable meta-analyses. To study treatment response different outcome parameters, such as overall survival, event-free survival, disease-free survival, progression-free survival, histological response and tumor necrosis were used, making it impossible to combine in a meta-analysis. For toxicity outcomes, there were large differences in grading systems and the exact definitions for which patients are considered cases. In pediatric patient cohorts, cardiotoxicity is defined in as fractional shortening below a limit that varies among studies. In adults, cardiotoxicity is defined as a decrease of ejection fraction below the lower limit of normal or a large absolute reduction in ejection fraction, making it impossible to combine pediatric and adult patient cohorts in meta-analysis. The introduction of more sensitive imaging tools may allow for earlier detection of cardiac dysfunction and homogenize phenotypes, for

example using global longitudinal strain (GLS), 3D volumetric echocardiographic or MRI. Lastly, confidence intervals of discovery and replication studies in this review do not always overlap, and therefore, it is already to be predicted that meta-analyses will be very heterogenous. In the future, the consistency of phenotypes would be improved if research groups with similar interests would collaborate to clearly define the phenotypes together, in order to perform powerful analyses in larger patient cohorts.

Conclusion

To conclude, in this systematic review, fifteen articles were found that aimed to identify novel genetic variants involved in treatment toxicity or treatment response in patients with osteosarcoma. Most research was done on doxorubicin-induced cardiomyopathy and for seven genetic variants *in CELF4, GPR35, HAS3, RARG, SLC22A17, SLC22A7* and *SLC28A3*, replication studies were performed without consistent results. Genetic variants *in ABCC3, ABCC5, FasL, GLDC* and *GSTP1* were repeatedly associated to osteosarcoma treatment outcome, using very heterogeneous efficacy outcomes. Studies reporting on bone marrow, nephro- and/or hepatotoxicity were small and had poor reproducibility. Moreover, none of the articles assessed cisplatin-induced ototoxicity. Despite that these results are promising and may have great potential for the future, replications often remain contradictory. Therefore, hypothesis-generating studies in large patient cohorts will be necessary to confirm these variants and to discover novel associations. Large initiatives, for example Euramos-1 or the Children's Oncology Group, could liaise with other research groups around the world with similar interests to boost the discovery of pharmacogenetic variants. Thereafter, functional studies are important to elucidate the mechanism behind the association and, ultimately, interventions should be established that make use of these associations to give patients with osteosarcoma a treatment that fits the needs of the individual best.

References

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531-43.
2. Whelan JS, Davis LE. Osteosarcoma, Chondrosarcoma, and Chordoma. J Clin Oncol. 2018;36(2):188-93.
3. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Ann Oncol. 2015;26(2):407-14.
4. Smeland S, Bielack SS, Whelan J, Bernstein M, Hogendoorn P, Krailo MD, et al. Survival and prognosis with osteosarcoma: outcomes in more than 2000 patients in the EURAMOS-1 (European and American Osteosarcoma Study) cohort. Eur J Cancer. 2019;109:36-50.
5. Howard SC, McCormick J, Pui CH, Buddington RK, Harvey RD. Preventing and Managing Toxicities of High-Dose Methotrexate. Oncologist. 2016;21(12):1471-82.
6. Lipshultz SE, Colan SD, Gelber RD, Perez-Atayde AR, Sallan SE, Sanders SP. Late cardiac effects of doxorubicin therapy for acute lymphoblastic leukemia in childhood. N Engl J Med. 1991;324(12):808-15.
7. Armenian S, Bhatia S. Predicting and Preventing Anthracycline-Related Cardiotoxicity. Am Soc Clin Oncol Educ Book. 2018;38:3-12.
8. Langer T, am Zehnhoff-Dinnesen A, Radtke S, Meitert J, Zolk O. Understanding platinum-induced ototoxicity. Trends Pharmacol Sci. 2013;34(8):458-69.
9. Cheng DH, Lu H, Liu TT, Zou XQ, Pang HM. Identification of Risk Factors in High-Dose Methotrexate-Induced Acute Kidney Injury in Childhood Acute Lymphoblastic Leukemia. Chemotherapy. 2018;63(2):101-7.
10. Hagleitner MM, de Bont ES, Te Loo DM. Survival trends and long-term toxicity in pediatric patients with osteosarcoma. Sarcoma. 2012;2012:636405.
11. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol. 2002;20(3):776-90.
12. Roden DM, McLeod HL, Relling MV, Williams MS, Mensah GA, Peterson JF, et al. Pharmacogenomics. Lancet. 2019;394(10197):521-32.
13. Vos HI, Coenen MJ, Guchelaar HJ, Te Loo DM. The role of pharmacogenetics in the treatment of osteosarcoma. Drug Discov Today. 2016;21(11):1775-86.
14. Xu H, Robinson GW, Huang J, Lim JY, Zhang H, Bass JK, et al. Common variants in ACYP2 influence susceptibility to cisplatin-induced hearing loss. Nat Genet. 2015;47(3):263-6.
15. Vos HI, Guchelaar HJ, Gelderblom H, de Bont ES, Kremer LC, Naber AM, et al. Replication of a genetic variant in ACYP2 associated with cisplatin-induced hearing loss in patients with osteosarcoma. Pharmacogenet Genomics. 2016;26(5):243-7.
16. Drogemoller BI, Brooks B, Critchley C, Monzon JG, Wright GEB, Liu G, et al. Further Investigation of the Role of ACYP2 and WFS1 Pharmacogenomic Variants in the Development of Cisplatin-Induced Ototoxicity in Testicular Cancer Patients. Clin Cancer Res. 2018;24(8):1866-71.
17. Thiesen S, Yin P, Jorgensen AL, Zhang JE, Manzo V, McEvoy L, et al. TPMT, COMT and ACYP2 genetic variants in paediatric cancer patients with cisplatin-induced ototoxicity. Pharmacogenet Genomics. 2017;27(6):213-22.
18. Tserga E, Nandwani T, Edvall NK, Bulla J, Patel P, Canlon B, et al. The genetic vulnerability to cisplatin ototoxicity: a systematic review. Sci Rep. 2019;9(1):3455.
19. van Vugt LJ, van den Reek J, Coenen MJH, de Jong E. A systematic review of pharmacogenetic studies on the response to biologics in patients with psoriasis. Br J Dermatol. 2018;178(1):86-94.

20. Maruthur NM, Gribble MO, Bennett WL, Bolen S, Wilson LM, Balakrishnan P, et al. The pharmacogenetics of type 2 diabetes: a systematic review. Diabetes Care. 2014;37(3):876-86.

21. Little J, Higgins JP, Ioannidis JP, Moher D, Gagnon F, von Elm E, et al. STrengthening the REporting of Genetic Association Studies (STREGA): an extension of the STROBE statement. PLoS Med. 2009;6(2):e22.

22. Visscher H, Ross CJ, Rassekh SR, Sandor GS, Caron HN, van Dalen EC, et al. Validation of variants in SLC28A3 and UGT1A6 as genetic markers predictive of anthracycline-induced cardiotoxicity in children. Pediatr Blood Cancer. 2013;60(8):1375-81.

23. Langer T, Clemens E, Broer L, Maier L, Uitterlinden AG, de Vries ACH, et al. Usefulness of current candidate genetic markers to identify childhood cancer patients at risk for platinum-induced ototoxicity: Results of the European PanCareLIFE cohort study. Eur J Cancer. 2020;138:212-24.

24. Sági JC, Egyed B, Kelemen A, Kutszegi N, Hegyi M, Gézsi A, et al. Possible roles of genetic variations in chemotherapy related cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma. BMC Cancer. 2018;18(1):704.

25. Ruiz-Pinto S, Pita G, Martín M, Alonso-Gordoa T, Barnes DR, Alonso MR, et al. Exome array analysis identifies ETFB as a novel susceptibility gene for anthracycline-induced cardiotoxicity in cancer patients. Breast Cancer Res Treat. 2018;167(1):249-56.

26. Lui G, Treluyer JM, Fresneau B, Piperno-Neumann S, Gaspar N, Corradini N, et al. A Pharmacokinetic and Pharmacogenetic Analysis of Osteosarcoma Patients Treated With High-Dose Methotrexate: Data From the OS2006/Sarcoma-09 Trial. J Clin Pharmacol. 2018;58(12):1541-9.

27. Caronia D, Patino-Garcia A, Perez-Martinez A, Pita G, Moreno LT, Zalacain-Diez M, et al. Effect of ABCB1 and ABCC3 polymorphisms on osteosarcoma survival after chemotherapy: a pharmacogenetic study. PLoS One. 2011;6(10):e26091.

28. Yang J, Wang ZG, Cai HQ, Li YC, Xu YL. Effect of variation of ABCB1 and ABCC3 genotypes on the survival of bone tumor cases after chemotherapy. Asian Pac J Cancer Prev. 2013;14(8):4595-8.

29. Liu S, Yi Z, Ling M, Shi J, Qiu Y, Yang S. Predictive potential of ABCB1, ABCC3, and GSTP1 gene polymorphisms on osteosarcoma survival after chemotherapy. Tumour Biol. 2014;35(10):9897-904.

30. Li JZ, Tian ZQ, Jiang SN, Feng T. Effect of variation of ABCB1 and GSTP1 on osteosarcoma survival after chemotherapy. Genet Mol Res. 2014;13(2):3186-92.

31. Hattinger CM, Biason P, Iacoboni E, Gagno S, Fanelli M, Tavanti E, et al. Candidate germline polymorphisms of genes belonging to the pathways of four drugs used in osteosarcoma standard chemotherapy associated with risk, survival and toxicity in non-metastatic high-grade osteosarcoma. Oncotarget. 2016;7(38):61970-87.

32. Windsor RE, Strauss SJ, Kallis C, Wood NE, Whelan JS. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. Cancer. 2012;118(7):1856-67.

33. Goričar K, Kovač V, Jazbec J, Zakotnik B, Lamovec J, Dolžan V. Influence of the folate pathway and transporter polymorphisms on methotrexate treatment outcome in osteosarcoma. Pharmacogenet Genomics. 2014;24(10):514-21.

34. Hagleitner MM, Coenen MJ, Gelderblom H, Makkinje RR, Vos HI, de Bont ES, et al. A First Step toward Personalized Medicine in Osteosarcoma: Pharmacogenetics as Predictive Marker of Outcome after Chemotherapy-Based Treatment. Clin Cancer Res. 2015;21(15):3436-41.

35. Yang LM, Li XH, Bao CF. Glutathione S-transferase P1 and DNA polymorphisms influence response to chemotherapy and prognosis of bone tumors. Asian Pacific journal of cancer prevention : APJCP. 2012;13(11):5883-6.

36. Zhang SL, Mao NF, Sun JY, Shi ZC, Wang B, Sun YJ. Predictive potential of glutathione S-transferase polymorphisms for prognosis of osteosarcoma patients on chemotherapy. Asian Pac J Cancer Prev. 2012;13(6):2705-9.

37. Teng JW, Yang ZM, Li J, Xu B. Predictive role of Glutathione S-transferases (GSTs) on the prognosis of osteosarcoma patients treated with chemotherapy. Pak J Med Sci. 2013;29(5):1182-6.

38. Jabeen S, Holmboe L, Alnaes GI, Andersen AM, Hall KS, Kristensen VN. Impact of genetic variants of RFC1, DHFR and MTHFR in osteosarcoma patients treated with high-dose methotrexate. Pharmacogenomics J. 2015;15(5):385-90.

39. Xu L, Xia C, Sun Q, Sheng F, Xiong J, Wang S. Variants of FasL and ABCC5 are predictive of outcome after chemotherapy-based treatment in osteosarcoma. J Bone Oncol. 2018;12:44-8.

40. Koster R, Panagiotou OA, Wheeler WA, Karlins E, Gastier-Foster JM, Caminada de Toledo SR, et al. Genome-wide association study identifies the GLDC/IL33 locus associated with survival of osteosarcoma patients. Int J Cancer. 2018;142(8):1594-601.

41. Bhuvaneshwar K, Harris M, Gusev Y, Madhavan S, Iyer R, Vilboux T, et al. Genome sequencing analysis of blood cells identifies germline haplotypes strongly associated with drug resistance in osteosarcoma patients. BMC Cancer. 2019;19(1):357.

42. Hildebrandt MAT, Reyes M, Wu X, Pu X, Thompson KA, Ma J, et al. Hypertension Susceptibility Loci are Associated with Anthracycline-related Cardiotoxicity in Long-term Childhood Cancer Survivors. Sci Rep. 2017;7(1):9698.

43. Visscher H, Ross CJ, Rassekh SR, Barhdadi A, Dubé MP, Al-Saloos H, et al. Pharmacogenomic prediction of anthracycline-induced cardiotoxicity in children. J Clin Oncol. 2012;30(13):1422-8.

44. Serie DJ, Crook JE, Necela BM, Dockter TJ, Wang X, Asmann YW, et al. Genome-wide association study of cardiotoxicity in the NCCTG N9831 (Alliance) adjuvant trastuzumab trial. Pharmacogenet Genomics. 2017;27(10):378-85.

45. Hertz DL, Caram MV, Kidwell KM, Thibert JN, Gersch C, Seewald NJ, et al. Evidence for association of SNPs in ABCB1 and CBR3, but not RAC2, NCF4, SLC28A3 or TOP2B, with chronic cardiotoxicity in a cohort of breast cancer patients treated with anthracyclines. Pharmacogenomics. 2016;17(3):231-40.

46. Reichwagen A, Ziepert M, Kreuz M, Godtel-Armbrust U, Rixecker T, Poeschel V, et al. Association of NADPH oxidase polymorphisms with anthracycline-induced cardiotoxicity in the RICOVER-60 trial of patients with aggressive CD20(+) B-cell lymphoma. Pharmacogenomics. 2015;16(4):361-72.

47. Vulsteke C, Pfeil AM, Maggen C, Schwenkglenks M, Pettengell R, Szucs TD, et al. Clinical and genetic risk factors for epirubicin-induced cardiac toxicity in early breast cancer patients. Breast Cancer Res Treat. 2015;152(1):67-76.

48. Ruiz-Pinto S, Pita G, Patiño-García A, Alonso J, Pérez-Martínez A, Cartón AJ, et al. Exome array analysis identifies GPR35 as a novel susceptibility gene for anthracycline-induced cardiotoxicity in childhood cancer. Pharmacogenet Genomics. 2017;27(12):445-53.

49. Visscher H, Rassekh SR, Sandor GS, Caron HN, van Dalen EC, Kremer LC, et al. Genetic variants in SLC22A17 and SLC22A7 are associated with anthracycline-induced cardiotoxicity in children. Pharmacogenomics. 2015;16(10):1065-76.

50. Schneider BP, Shen F, Gardner L, Radovich M, Li L, Miller KD, et al. Genome-Wide Association Study for Anthracycline-Induced Congestive Heart Failure. Clin Cancer Res. 2017;23(1):43-51.

51. Wang X, Liu W, Sun CL, Armenian SH, Hakonarson H, Hageman L, et al. Hyaluronan synthase 3 variant and anthracycline-related cardiomyopathy: a report from the children's oncology group. J Clin Oncol. 2014;32(7):647-53.

52. Wang X, Sun CL, Quiñones-Lombraña A, Singh P, Landier W, Hageman L, et al. CELF4 Variant and Anthracycline-Related Cardiomyopathy: A Children's Oncology Group Genome-Wide Association Study. J Clin Oncol. 2016;34(8):863-70.

53. Leger KJ, Cushing-Haugen K, Hansen JA, Fan W, Leisenring WM, Martin PJ, et al. Clinical and Genetic Determinants of Cardiomyopathy Risk among Hematopoietic Cell Transplantation Survivors. Biol Blood Marrow Transplant. 2016;22(6):1094-101.

54. Aminkeng F, Bhavsar AP, Visscher H, Rassekh SR, Li Y, Lee JW, et al. A coding variant in RARG confers susceptibility to anthracycline-induced cardiotoxicity in childhood cancer. Nat Genet. 2015;47(9):1079-84.

55. Hegyi M, Arany A, Semsei AF, Csordas K, Eipel O, Gezsi A, et al. Pharmacogenetic analysis of high-dose methotrexate treatment in children with osteosarcoma. Oncotarget. 2017;8(6):9388-98.

56. Sharifi MJ, Bahoush G, Zaker F, Ansari S, Rafsanjani KA, Sharafi H. Association of -24CT, 1249GA, and 3972CT ABCC2 gene polymorphisms with methotrexate serum levels and toxic side effects in children with acute lymphoblastic leukemia. Pediatr Hematol Oncol. 2014;31(2):169-77.

57. Gervasini G, de Murillo SG, Jimenez M, de la Maya MD, Vagace JM. Effect of polymorphisms in transporter genes on dosing, efficacy and toxicity of maintenance therapy in children with acute lymphoblastic leukemia. Gene. 2017;628:72-7.

58. Hurkmans EGE, Klumpers MJ, Vermeulen SH, Hagleitner MM, Flucke U, Schreuder HWB, et al. Analysis of Drug Metabolizing Gene Panel in Osteosarcoma Patients Identifies Association Between Variants in SULT1E1, CYP2B6 and CYP4F8 and Methotrexate Levels and Toxicities. Front Pharmacol. 2020;11:1241.

59. Huang L, Tissing WJ, de Jonge R, van Zelst BD, Pieters R. Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Leukemia. 2008;22(9):1798-800.

60. Kantar M, Kosova B, Cetingul N, Gumus S, Toroslu E, Zafer N, et al. Methylenetetrahydrofolate reductase C677T and A1298C gene polymorphisms and therapy-related toxicity in children treated for acute lymphoblastic leukemia and non-Hodgkin lymphoma. Leuk Lymphoma. 2009;50(6):912-7.

61. Karathanasis NV, Stiakaki E, Goulielmos GN, Kalmanti M. The role of the methylenetetrahydrofolate reductase 677 and 1298 polymorphisms in Cretan children with acute lymphoblastic leukemia. Genet Test Mol Biomarkers. 2011;15(1-2):5-10.

62. Liu SG, Li ZG, Cui L, Gao C, Li WJ, Zhao XX. Effects of methylenetetrahydrofolate reductase gene polymorphisms on toxicities during consolidation therapy in pediatric acute lymphoblastic leukemia in a Chinese population. Leuk Lymphoma. 2011;52(6):1030-40.

63. Erculj N, Kotnik BF, Debeljak M, Jazbec J, Dolzan V. Influence of folate pathway polymorphisms on high-dose methotrexate-related toxicity and survival in childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2012;53(6):1096-104.

64. Araoz HV, D'Aloi K, Foncuberta ME, Sanchez La Rosa CG, Alonso CN, Chertkoff L, et al. Pharmacogenetic studies in children with acute lymphoblastic leukemia in Argentina. Leuk Lymphoma. 2015;56(5):1370-8.

65. Yousef AM, Farhad R, Alshamaseen D, Alsheikh A, Zawiah M, Kadi T. Folate pathway genetic polymorphisms modulate methotrexate-induced toxicity in childhood acute lymphoblastic leukemia. Cancer Chemother Pharmacol. 2019;83(4):755-62.

66. van Kooten Niekerk PB, Schmiegelow K, Schroeder H. Influence of methylene tetrahydrofolate reductase polymorphisms and coadministration of antimetabolites on toxicity after high dose methotrexate. Eur J Haematol. 2008;81(5):391-8.

67. Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolzan V, Jazbec J. Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. Eur J Clin Pharmacol. 2011;67(10):993-1006.

68. Haase R, Elsner K, Merkel N, Stiefel M, Mauz-Korholz C, Kramm CM, et al. High dose methotrexate treatment in childhood ALL: pilot study on the impact of the MTHFR 677C>T and 1298A>C polymorphisms on MTX-related toxicity. Klin Padiatr. 2012;224(3):156-9.

69. Suthandiram S, Gan GG, Zain SM, Bee PC, Lian LH, Chang KM, et al. Effect of polymorphisms within methotrexate pathway genes on methotrexate toxicity and plasma levels in adults with hematological malignancies. Pharmacogenomics. 2014;15(11):1479-94.

70. Milosevic G, Kotur N, Lazic J, Krstovski N, Stankovic B, Zukic B, et al. Influence of variants in folate metabolism genes on 6-mercaptopurine induced toxicity during treatment for childhood acute lymphocytic leukemia. J BUON. 2019;24(5):2075-83.

71. Khrunin A, Ivanova F, Moisseev A, Khokhrin D, Sleptsova Y, Gorbunova V, et al. Pharmacogenomics of cisplatin-based chemotherapy in ovarian cancer patients of different ethnic origins. Pharmacogenomics. 2012;13(2):171-8.

72. Khrunin AV, Moisseev A, Gorbunova V, Limborska S. Genetic polymorphisms and the efficacy and toxicity of cisplatin-based chemotherapy in ovarian cancer patients. Pharmacogenomics J. 2010;10(1):54-61.

73. Lopes-Aguiar L, Costa EF, Nogueira GA, Lima TR, Visacri MB, Pincinato EC, et al. XPD c.934G>A polymorphism of nucleotide excision repair pathway in outcome of head and neck squamous cell carcinoma patients treated with cisplatin chemoradiation. Oncotarget. 2017;8(10):16190-201.

74. Zazuli Z, Otten LS, Drogemoller BI, Medeiros M, Monzon JG, Wright GEB, et al. Outcome Definition Influences the Relationship Between Genetic Polymorphisms of ERCC1, ERCC2, SLC22A2 and Cisplatin Nephrotoxicity in Adult Testicular Cancer Patients. Genes (Basel). 2019;10(5).

75. Garcia SL, Lauritsen J, Zhang Z, Bandak M, Dalgaard MD, Nielsen RL, et al. Prediction of Nephrotoxicity Associated With Cisplatin-Based Chemotherapy in Testicular Cancer Patients. JNCI Cancer Spectr. 2020;4(3):pkaa032.

76. Lin Q, Han J, Sun Q, Wen L, Wang S. Functional variant of IL33 is associated with survival of osteosarcoma patients. J Bone Oncol. 2020;20:100270.

77. Kang C, Zhao J, Wang Y, Yang C, Chen J, Zhi L. Relationship of common variants in Interleukin 33 gene with susceptibility and prognosis of osteosarcoma in Han Chinese population. J Cancer. 2019;10(5):1138-44.

78. Hu W, Wu C, Li X, Zheng Z, Xie Q, Deng X, et al. Serum IL-33 level is a predictor of progression-free survival after chemotherapy. Oncotarget. 2017;8(21):35116-23.

79. Rossle M, Cathomas G, Bonapace L, Sachs M, Dehler S, Storz M, et al. Interleukin-33 Expression Indicates a Favorable Prognosis in Malignant Salivary Gland Tumors. Int J Surg Pathol. 2016;24(5):394-400.

80. Musolino C, Allegra A, Profita M, Alonci A, Saitta S, Russo S, et al. Reduced IL-33 plasma levels in multiple myeloma patients are associated with more advanced stage of disease. Br J Haematol. 2013;160(5):709-10.

81. Fournie JJ, Poupot M. The Pro-tumorigenic IL-33 Involved in Antitumor Immunity: A Yin and Yang Cytokine. Front Immunol. 2018;9:2506.

82. Wang S, Zhao G, Zhao S, Qiao Y, Yang H. The Effects of Interleukin-33 (IL-33) on Osteosarcoma Cell Viability, Apoptosis, and Epithelial-Mesenchymal Transition are Mediated Through the PI3K/AKT Pathway. Med Sci Monit. 2020;26:e920766.

83. Wang JL, Liu J, Xie KG, Lan CG, Lu L, Tang YJ. Association between functional polymorphisms in IL-33/ST2 pathway and risk of osteosarcoma. J Cell Mol Med. 2018.

84. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genomics. 2009;3(3):281-90.

85. Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Res. 2001;61(19):7225-32.

86. Vlaming ML, van Esch A, van de Steeg E, Pala Z, Wagenaar E, van Tellingen O, et al. Impact of abcc2 [multidrug resistance-associated protein (MRP) 2], abcc3 (MRP3), and abcg2 (breast cancer resistance protein) on the oral pharmacokinetics of methotrexate and its main metabolite 7-hydroxymethotrexate. Drug Metab Dispos. 2011;39(8):1338-44.

87. Warren RB, Smith RL, Campalani E, Eyre S, Smith CH, Barker JN, et al. Genetic variation in efflux transporters influences outcome to methotrexate therapy in patients with psoriasis. J Invest Dermatol. 2008;128(8):1925-9.

88. Chen ZS, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, et al. Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res. 2003;63(14):4048-54.

89. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. Physiol Rev. 2000;80(2):853-924.

90. Anderson PA, Greig A, Mark TM, Malouf NN, Oakeley AE, Ungerleider RM, et al. Molecular basis of human cardiac troponin T isoforms expressed in the developing, adult, and failing heart. Circ Res. 1995;76(4):681-6.

91. Kayvanpour E, Sedaghat-Hamedani F, Amr A, Lai A, Haas J, Holzer DB, et al. Genotype-phenotype associations in dilated cardiomyopathy: meta-analysis on more than 8000 individuals. Clin Res Cardiol. 2017;106(2):127-39.

92. Garcia-Pavia P, Kim Y, Restrepo-Cordoba MA, Lunde IG, Wakimoto H, Smith AM, et al. Genetic Variants Associated With Cancer Therapy-Induced Cardiomyopathy. Circulation. 2019;140(1):31-41.

93. Christidi E, Huang H, Shafaattalab S, Maillet A, Lin E, Huang K, et al. Variation in RARG increases susceptibility to doxorubicin-induced cardiotoxicity in patient specific induced pluripotent stem cell-derived cardiomyocytes. Sci Rep. 2020;10(1):10363.

94. Ichikawa Y, Ghanefar M, Bayeva M, Wu R, Khechaduri A, Naga Prasad SV, et al. Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. J Clin Invest. 2014;124(2):617-30.

95. Nagasawa K, Nagai K, Ohnishi N, Yokoyama T, Fujimoto S. Contribution of specific transport systems to anthracycline transport in tumor and normal cells. Curr Drug Metab. 2001;2(4):355-66.

96. Errasti-Murugarren E, Pastor-Anglada M. Drug transporter pharmacogenetics in nucleoside-based therapies. Pharmacogenomics. 2010;11(6):809-41.

97. Ross CJ, Katzov-Eckert H, Dube MP, Brooks B, Rassekh SR, Barhdadi A, et al. Genetic variants in TPMT and COMT are associated with hearing loss in children receiving cisplatin chemotherapy. Nat Genet. 2009;41(12):1345-9.

CHAPTER 2

Supplementary materials

Pharmacogenetics of chemotherapy treatment response and -toxicities in patients with osteosarcoma: a systematic review of 2010-2020

Figure S1. General search strategy for electronic databases.

Table S1. Search strategy for electronic databases

Search source	Pubmed/MEDLINE and Embase
Search date	30th of September 2020
Search restrictions	None
Filters used	None
Pubmed/MEDLINE search strategy	(("Osteosarcoma"[tiab] OR "osteosarcoma"[MeSH Terms] OR "Osteosarcomas"[tiab] OR "Sarcoma, Osteogenic"[tiab] OR "Sarcomas, Osteogenic"[tiab] OR "Osteogenic Sarcomas"[tiab] OR "Osteogenic Sarcoma"[tiab] OR "Bone Neoplasms/drug therapy"[Mesh] OR "bone cancer"[tiab] OR "bone tumor"[tiab] or "bone tumour"[tiab] OR "bone tumors"[tiab] or "bone tumours"[tiab]) OR (("Child"[Mesh] OR "Adolescent"[Mesh] OR childhood cancer*[tiab] OR adolescent cancer*[tiab] OR pediatric cancer*[tiab] OR childhood tumor*[tiab] OR childhood tumour*[tiab]) AND ("Methotrexate"[Mesh] OR "Methotrexate"[tiab] OR "Cisplatin"[Mesh] OR "Cisplatin"[tiab] OR "NSC-119875"[tiab] OR "cis-Platinum"[tiab] OR "cis Platinum"[tiab] OR "CDDP"[tiab] OR "Doxorubicin"[Mesh] OR "Doxorubicin"[tiab] OR "Anthracyclines"[Mesh:NoExp] OR "anthracycline"[tiab] OR "anthracyclines"[tiab] AND ("Ototoxicity"[Mesh] OR "Hearing Loss/chemically induced"[Mesh] OR "Hearing Loss/genetics"[Mesh] OR "Hearing Loss, Sensorineural"[Mesh] OR "Ear Diseases/chemically induced"[Mesh] OR "Ear Diseases/genetics"[Mesh] OR ototoxic*[tiab] OR "hearing loss"[tiab] OR "Renal Insufficiency"[Mesh] OR "Kidney Diseases/chemically induced"[Mesh] OR "Kidney Diseases/drug effects"[Mesh] OR "Kidney Diseases/genetics"[Mesh] OR "Acute Kidney Injury/chemically induced"[Mesh] OR "Acute Kidney Injury/drug effects"[Mesh] OR "Acute Kidney Injury/genetics"[Mesh] OR nephrotoxic*[tiab] OR "renal failure"[tiab] OR "kidney failure"[tiab] OR "renal function"[tiab] OR "Heart Diseases/chemically induced"[Mesh] OR "Heart Diseases/genetics"[Mesh] OR "Cardiomyopathies/chemically induced"[Mesh] OR "Cardiomyopathies/genetics"[Mesh] OR "Cardiotoxicity"[Mesh] OR cardiac toxic*[tiab] OR cardiotoxic*[tiab] OR heart toxic*[tiab] OR "cardiomyopathy"[tiab] or "Bone Marrow/drug effects"[Mesh] OR "Hematopoiesis/drug effects"[Mesh] OR "Hematopoiesis/genetics"[Mesh] OR "Bone Marrow Cells/drug effects"[Mesh] OR "myelosuppression"[tiab] OR "myelosuppressive toxicity"[tiab] OR hematologic toxic*[tiab] OR "anemia"[tiab] OR "myelopoiesis"[tiab] OR "Chemical and Drug Induced Liver Injury"[Mesh] OR "Liver Diseases/chemically induced"[Mesh] OR "Liver Diseases/drug effects"[Mesh] OR "Liver/drug effects"[MAJR] OR "Liver Diseases/genetics"[Mesh] OR "hepatotoxicity"[tiab] OR liver toxic*[tiab] OR hepatotoxic*[tiab])))) AND (("Genome-Wide Association Study"[Mesh] OR "GWAS"[tiab] OR "Genome-wide association study"[tiab] OR "Genome-Wide Association Studies"[tiab] OR "Whole Genome Association Analysis"[tiab] OR "GWA Study"[tiab] OR "GWA Studies"[tiab] OR "Genome Wide Association Scan"[tiab] OR "Genome Wide Association Studies"[tiab] OR "Genome Wide Association Study"[tiab] OR "Genome Wide Association Analysis"[tiab] OR "Whole Genome Association Study"[tiab] OR "Genome-wide scan"[tiab] OR "Genome wide scan"[tiab] OR "Genome-wide association analysis"[tiab] OR "Genome-wide association analysis"[tiab]) OR ("Genomic Structural Variation"[Mesh] OR "Pharmacogenetics"[Mesh] OR "Polymorphism, Genetic"[Mesh] OR "Polymorphism, Single Nucleotide"[Mesh] OR "Genotype"[Mesh] OR "Germ-Line Mutation"[Mesh] OR genetic variant*[tiab] OR genetic variation*[tiab] OR gene variation*[tiab] OR gene variant*[tiab] OR polymorphism*[tiab] OR SNP[tiab] OR pharmacogenetic*[tiab] OR pharmacogenomic*[tiab] OR genotype*[tiab] OR variation*[tiab] OR variant*[tiab] OR genetic variability[tiab] OR germ-line mutation*[tiab] OR germ-line variation*[tiab])) AND ("Doxorubicin/adverse effects"[Mesh] OR "Doxorubicin/toxicity"[Mesh] OR "Doxorubicin/therapeutic use"[Mesh] OR "Doxorubicin/metabolism"[Mesh] OR "Doxorubicin/pharmacokinetics"[Mesh] OR "Doxorubicin"[tiab] OR "Anthracyclines/adverse effects"[Majr] OR "Anthracyclines/toxicity"[Majr] OR "Anthracyclines/therapeutic use"[Majr] OR "Anthracyclines/metabolism"[Majr] OR "Anthracyclines/pharmacokinetics"[Majr] OR "anthracycline"[tiab] OR "anthracyclines"[tiab] OR "Cisplatin/adverse effects"[Mesh] OR "Cisplatin/metabolism"[Mesh] OR "Cisplatin/pharmacokinetics"[Mesh] OR "Cisplatin"[tiab] OR "NSC-119875"[tiab] OR "cis-Platinum"[tiab] OR "cis Platinum"[tiab] OR "CDDP"[tiab] OR "Methotrexate/adverse effects"[Mesh] OR "Methotrexate/toxicity"[Mesh] OR "Methotrexate/therapeutic use"[Mesh]OR "Methotrexate/metabolism"[Mesh] OR "Methotrexate/pharmacokinetics"[Mesh] OR "Methotrexate"[tiab] OR "Ototoxicity"[Mesh] OR "Hearing Loss/chemically induced"[Mesh] OR "Hearing Loss/genetics"[Mesh] OR "Hearing Loss, Sensorineural"[Mesh] OR "Ear Diseases/chemically induced"[Mesh] OR

	"Ear Diseases/genetics"[Mesh] OR ototoxic*[tiab] OR "hearing loss"[tiab] OR "Renal Insufficiency"[Mesh] OR "Kidney Diseases/chemically induced"[Mesh] OR "Kidney Diseases/drug effects"[Mesh] OR "Kidney Diseases/genetics"[Mesh] OR "Acute Kidney Injury/chemically induced"[Mesh] OR "Acute Kidney Injury/drug effects"[Mesh] OR "Acute Kidney Injury/genetics"[Mesh] OR nephrotoxic*[tiab] OR "renal failure"[tiab] OR "kidney failure"[tiab] OR "renal function"[tiab] OR "Heart Diseases/chemically induced"[Mesh] OR "Heart Diseases/genetics"[Mesh] OR "Cardiomyopathies/chemically induced"[Mesh] OR "Cardiomyopathies/genetics"[Mesh] OR "Cardiotoxicity"[Mesh] OR cardiac toxic*[tiab] OR cardiotoxic*[tiab] OR heart toxic*[tiab] OR "cardiomyopathy"[tiab] or "Bone Marrow/drug effects"[Mesh] OR "Hematopoiesis/drug effects"[Mesh] OR "Hematopoiesis/genetics"[Mesh] OR "Bone Marrow Cells/drug effects"[Mesh] OR "myelosuppression"[tiab] OR "myelosuppressive toxicity"[tiab] OR hematologic toxic*[tiab] OR "anemia"[tiab] OR "myelopoiesis"[tiab] OR "Chemical and Drug Induced Liver Injury"[Mesh] OR "Liver Diseases/chemically induced"[Mesh] OR "Liver Diseases/drug effects"[Mesh] OR "Liver/drug effects"[MAJR] OR "Liver Diseases/genetics"[Mesh] OR "hepatotoxicity"[tiab] OR liver toxic*[tiab] OR hepatotoxic*[tiab] OR "Treatment Outcome"[Mesh] OR "Efficacy"[tiab] OR "Treatment efficacy"[tiab] OR "Survival Analysis"[Mesh] OR "Survival"[tiab] OR "Overall survival"[tiab] OR "Disease Progression"[Mesh] OR "Progression"[tiab] OR "Progressive disease" OR "disease, progressive"[tiab] OR "Recurrence"[Mesh] OR "Neoplasm Recurrence, Local"[Mesh] OR "Recurrence"[tiab] OR "Recurrences"[tiab] OR "Relapse"[tiab] OR "Relapses"[tiab] OR "Prognosis"[Mesh] OR "prognosis"[tiab] OR "Neoplasm Metastasis"[Mesh] OR "metastasis"[tiab])
Embase search strategy	((exp osteosarcoma/ or osteosarcoma/dt or bone tumor/dt, si or (osteosarcoma* or osteogenic sarcoma* or bone cancer* or bone tumor* or bone tumour*).ti,ab,kw.) or ((juvenile/ or adolescent/ or child/ or childhood cancer.mp. or exp childhood cancer/) and (methotrexate/ae, dt, to, pd or cisplatin/ae, dt, to, pd or doxorubicin/ae, dt, to, pd or anthracycline antibiotic agent/ae, dt, to, pd or (cisplatin or doxorubicin or methotrexate or MTX or anthracycline).ti,ab,kw.) and (exp ototoxicity/si or ototoxicity.mp. or high frequency hearing loss/si or exp blood toxicity/co, si or exp bone marrow toxicity/co, si or exp bone marrow suppression/co, si or blood toxicity.mp. or bone marrow toxicity.mp. or bone marrow suppression.mp. or anemia/si or infection/si or leukopenia/si or exp cardiotoxicity/co, si or cardiotoxicity.mp. or cardiomyopathy/co, si or cardiovascular disease/co, si or exp liver toxicity/ or liver toxicity.mp. or hepatotoxicity.mp. or liver injury/co, si or alanine aminotransferase/ or alanine aminotransferase blood level/ or exp nephrotoxicity/ or nephrotoxicity.mp. or creatinine blood level/ or creatinine/ or *"pharmacokinetic parameters"/ or *"pharmacogenetics"/ or pharmacokinetics/ or drug absorption/ or drug clearance/ or drug distribution/ or drug elimination/ or drug excretion/ or drug metabolism/ or (ototoxic* or "hearing loss" or blood toxicity or bone marrow toxic* or bone marrow suppression or cardiotoxic* or cardiomyopathy or liver toxic* or hepatotoxic* or nephrotoxic* or kidney toxic*).ti,ab,kw.))) AND (exp genetic association/ or exp genome-wide association study/ or exp single nucleotide polymorphism/ or pharmacogenetics.mp. or exp pharmacogenetics/ or exp genetic variability/ or exp genetic variation/ or exp genotype/ or genotype.mp. or exp genetic polymorphism/) AND (treatment outcome/ or clinical outcome/ or disease free interval/ or disease worsening with drug treatment/ or outcome assessment/ or outcomes research/ or partial drug response/ or treatment failure/ or exp survival/ or relapse/dr, dt, si or exp recurrent disease/dr, dt, si or (exp ototoxicity/si or ototoxicity.mp. or high frequency hearing loss/si or exp blood toxicity/co, si or exp bone marrow toxicity/co, si or exp bone marrow suppression/co, si or blood toxicity.mp. or bone marrow toxicity.mp. or bone marrow suppression.mp. or anemia/si or infection/si or leukopenia/si or exp cardiotoxicity/co, si or cardiotoxicity.mp. or cardiomyopathy/co, si or cardiovascular disease/co, si or exp liver toxicity/ or liver toxicity.mp. or hepatotoxicity.mp. or liver injury/co, si or alanine aminotransferase/ or alanine aminotransferase blood level/ or exp nephrotoxicity/ or nephrotoxicity.mp. or creatinine blood level/ or creatinine/ or (ototoxic* or "hearing loss" or blood toxicity or bone marrow toxic* or immunotoxic* or lymphocytotoxic* or bone marrow suppression or cardiotoxic* or cardiomyopathy or liver toxic* or hepatotoxic* or nephrotoxic* or kidney toxic*).ti,ab,kw.) or (methotrexate/ae, dt, to, pd or cisplatin/ae, dt, to, pd or doxorubicin/ae, dt, to, pd or anthracycline antibiotic agent/ae, dt, to, pd) or (cisplatin or doxorubicin or methotrexate or MTX or anthracycline).ti,ab,kw.)

Table S2. Inventory of number of genes studied by the 91 genetic association studies. The bold line indicates the border between studies that are excluded (above line) or included (below line)

Number of genes	Number of publications
1	31
2	10
3	12
4	7
5	7
6	1
8	2
9	2
10	1
11	1
12	1
15	1
21	1
24	1
26	1
31	1
54	1
220	1
231	1
300	1
2100	1

Table S3. Inventory of number of variants studied by the 91 genetic association studies. The bold line indicates the border between studies that are excluded (above line) or included (below line)

Number of variants	Number of publications
1-5	54
6-10	13
11-15	4
16-20	1
21-25	1
26-30	2
31-35	0
36-40	1
41-45	2
46-65	0
65-70	1
>100	2
>1000	4

Table S4. Publications that were excluded from the systematic review in the last phases of the selection as a result of the pre-defined inclusion criteria.

Author	Year	Title	Exclusion phase	Exclusion reason	Ref.
Clemens *et al.*	2020	Genetic variation of cisplatin-induced ototoxicity in non-cranial-irradiated pediatric patients using a candidate gene approach: The International PanCareLIFE Study	Inventory # variants and # genes (*Table S3 and S4*)	≤10 variants but >5 genes	(1)
Goricar *et al.*	2014	Influence of the folate pathway and transporter polymorphisms on methotrexate Treatment outcome in osteosarcoma	Inventory # variants and # genes (*Table S3 and S4*)	≤10 variants but >5 genes	(2)
Goricar *et al.*	2015	Genetic variability of DNA repair mechanisms and glutathione-S-transferase genes influences treatment outcome in osteosarcoma	Inventory # variants and # genes (*Table S3 and S4*)	≤10 variants but >5 genes	(3)
Kang *et al.*	2019	Relationship of common variants in Interleukin 33 gene with susceptibility and prognosis of osteosarcoma in Han Chinese population	Inventory # variants and # genes (*Table S3 and S4*)	≤5 genes but >10 variants	(4)
Langer *et al.*	2020	Usefulness of current candidate genetic markers to identify childhood cancer patients at risk for platinum-induced ototoxicity: Results of the European PanCareLIFE cohort study	Final full text assessment	Replication study	(5)
Lanvers-Kaminsky *et al.*	2015	Human OCT2 variant c.808G>T confers protection effect against cisplatin-induced ototoxicity	Inventory # variants and # genes (*Table S3 and S4*)	≤5 genes but >10 variants	(6)
Lui *et al.*	2018	A Pharmacokinetic and Pharmacogenetic Analysis of Osteosarcoma Patients Treated With High-Dose Methotrexate: Data From the OS2006/Sarcoma-09 Trial	Final full text assessment	Wrong outcome	(7)
Ruiz-Pinto *et al.*	2018	Exome array analysis identifies ETFB as a novel susceptibility gene for anthracycline-induced cardiotoxicity in cancer patients	Final full text assessment	No osteosarcoma in discovery phase	(8)
Ságі *et al.*	2018	Possible roles of genetic variations in chemotherapy related cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma	Final full text assessment	No osteosarcoma in discovery phase	(9)
Spracklen *et al.*	2014	Genetic variation in otos is associated with cisplatin-induced ototoxicity	Inventory # variants and # genes (*Table S3 and S4*)	≤5 genes but >10 variants	(10)
Sun *et al.*	2015	Genetic polymorphisms in nucleotide excision repair pathway influences response to chemotherapy and overall survival in osteosarcoma	Inventory # variants and # genes (*Table S3 and S4*)	≤10 variants but >5 genes	(11)
Visscher *et al.*	2013	Validation of variants in SLC28A3 and UGT1A6 as genetic markers predictive of anthracycline-induced cardiotoxicity in children	Final full text assessment	Replication study	(12)

References - Table S4.

1. Clemens E, Broer L, Langer T, Uitterlinden AG, de Vries ACH, van Grotel M, et al. Genetic variation of cisplatin-induced ototoxicity in non-cranial-irradiated pediatric patients using a candidate gene approach: The International PanCareLIFE Study. Pharmacogenomics J. 2020;20(2):294-305.
2. Goričar K, Kovač V, Jazbec J, Zakotnik B, Lamovec J, Dolžan V. Influence of the folate pathway and transporter polymorphisms on methotrexate treatment outcome in osteosarcoma. Pharmacogenet Genomics. 2014;24(10):514-21.
3. Goričar K, Kovač V, Jazbec J, Zakotnik B, Lamovec J, Dolžan V. Genetic variability of DNA repair mechanisms and glutathione-S-transferase genes influences treatment outcome in osteosarcoma. Cancer Epidemiol. 2015;39(2):182-8.
4. Kang C, Zhao J, Wang Y, Yang C, Chen J, Zhi L. Relationship of common variants in Interleukin 33 gene with susceptibility and prognosis of osteosarcoma in Han Chinese population. J Cancer. 2019;10(5):1138-44.
5. Langer T, Clemens E, Broer L, Maier L, Uitterlinden AG, de Vries ACH, et al. Usefulness of current candidate genetic markers to identify childhood cancer patients at risk for platinum-induced ototoxicity: Results of the European PanCareLIFE cohort study. Eur J Cancer. 2020;138:212-24.
6. Lanvers-Kaminsky C, Sprowl JA, Malath I, Deuster D, Eveslage M, Schlatter E, et al. Human OCT2 variant c.808G>T confers protection effect against cisplatin-induced ototoxicity. Pharmacogenomics. 2015;16(4):323-32.
7. Lui G, Treluyer JM, Fresneau B, Piperno-Neumann S, Gaspar N, Corradini N, et al. A Pharmacokinetic and Pharmacogenetic Analysis of Osteosarcoma Patients Treated With High-Dose Methotrexate: Data From the OS2006/Sarcoma-09 Trial. J Clin Pharmacol. 2018;58(12):1541-9.
8. Ruiz-Pinto S, Pita G, Martín M, Alonso-Gordoa T, Barnes DR, Alonso MR, et al. Exome array analysis identifies ETFB as a novel susceptibility gene for anthracycline-induced cardiotoxicity in cancer patients. Breast Cancer Res Treat. 2018;167(1):249-56.
9. Sági JC, Egyed B, Kelemen A, Kutszegi N, Hegyi M, Gézsi A, et al. Possible roles of genetic variations in chemotherapy related cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma. BMC Cancer. 2018;18(1):704.
10. Spracklen TF, Whitehorn H, Vorster AA, Ramma L, Dalvie S, Ramesar RS. Genetic variation in Otos is associated with cisplatin-induced ototoxicity. Pharmacogenomics. 2014;15(13):1667-76.
11. Sun Y, Wu Y, Li W, Kong Z, Zou X. Genetic polymorphisms in nucleotide excision repair pathway influences response to chemotherapy and overall survival in osteosarcoma. Int J Clin Exp Pathol. 2015;8(7):7905-12.
12. Visscher H, Ross CJ, Rassekh SR, Sandor GS, Caron HN, van Dalen EC, et al. Validation of variants in SLC28A3 and UGT1A6 as genetic markers predictive of anthracycline-induced cardiotoxicity in children. Pediatr Blood Cancer. 2013;60(8):1375-81.

Table S5. Quality assessment form.

QUALITY ASSESMENT FORM

Study rationale

1. **Was there a clear rationale for the selected candidate genes?**
   Yes; No; n/a (for GWAS)

Sample selection

2. **Was a power calculation performed?**
   Yes, before initiation of study; Yes, but retrospectively; No

3. **If a power calculation was performed (Yes to #2), were the number of inclusions required for a power of 80 met?**
   Yes; No; n/a (No to #2)

4. **Were in- and exclusion criteria clearly described?**
   Yes; No

5. **Were sufficient basic characteristics reported for the study population?***
   Yes; No, name characteristics

6. **Was a validation cohort selected (in this study) to test reproducibility of findings in the original cohort?**
   Yes; No

** The basic characteristics that were reported in 2 or more studies were considered as most relevant baseline characteristics. Based on that and depending on the outcome variable, an estimation was made if this article reported sufficient baseline characteristics.*

Treatment and outcome

7. **Were all patients treated according to label?**
   Yes; No; NR

8. **Were clinical data collected prospectively of retrospectively?**
   Prospectively; Retrospectively; NR

Follow-up and missing data

9. **Does the study describe the number of participants who withdrew and/or were lost to follow-up after the start of the observation period?**
   Yes, complete (could replicate); Yes, partial (could not replicate); No; n/a

10. **What was the overall percentage of participants who withdrew or were lost to follow-up after the start of the observation period?**
    Specify percentage; NR; n/a

11. **What was the overall percentage of missing data for the association between the pharmacogenetics interaction and the main outcome?**
    Specify percentage; NR; n/a

Genotyping

12. **Specify genotyping method(s) used.**
    PCR; Sanger sequencing; allelic discrimination assays; GWA array; other (specify); NR

13. **For studies using genome-wide association study (GWAS) data, specify the allele-calling algorithm used.**
    Specify algorithm; NR; n/a

14. **How many SNPs were excluded based on call rate, and what cut-off point for exclusion was used?**
    Specify number of excluded SNPs (specify cut-off point); NR

15. **Was duplicate genotyping performed?**
    Yes; No; NR; n/a (for GWAS)

16. **If duplicate genotyping was performed (Yes to #16), what was the duplicate genotyping concordance?**
    Specify percentage; n/a

17. **Was Hardy-Weinberg equilibrium calculated?**
    Yes; No

18. **If alleles were not in HWE, how were they handled?**
    Excluded based on *P*-value; Included (specify); Other (specify)

Statistical analyses

19. **How were missing data handled (select all that apply)?**
    Excluded (complete case analysis)
    Single imputatation (specify method)
    Multiple imputation (specify method)
    Other (specify)
    NR
    n/a

20. **What type of analysis was used for the main comparison?**
    Intention to treat analysis
    As treated/complete case analysis
    Other (specify)
    NR

21. **For studies investigating more than one genetic variant, was adjustment for multiple testing applied?**
    Yes; No; n/a

22. **Were analyses performed to assess the effect of confounders?**
    Yes (specify); No; n/a

Other

23. **Please provide any additional remarks (either positive or negative) on quality.**

Table S6. Results of quality assessment according to the STrengthening the REporting of Genetic Association studies (STREGA) guidelines for reporting of genetic association studies was adjusted to be more applicable to pharmacogenetic studies

Question	**Study rationale** **1. Was there a clear rationale for the selected candidate genes?**
Aminkeng *et al.*, 2015	n/a
Bhuvaneshwar *et al.* 2019	n/a
Caronia *et al*., 2011	n/a
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	Yes
Hegyi *et al*., 2017	Yes
Hildebrandt *et al*. 2017	Yes
Hurkmans *et al*., 2020	Yes
Koster *et al*., 2018	n/a
Ruiz-Pinto *et al.*, 2017	n/a
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	Yes
Wang *et al*., 2014	Yes
Wang *et al*., 2016	n/a
Windsor *et al*., 2012	Yes
Question	**Sample selection** **2. Was a power calculation performed?**
Aminkeng *et al.*, 2015	No
Bhuvaneshwar *et al.* 2019	No
Caronia *et al*., 2011	No
Hagleitner *et al*., 2015	Yes, but retrospectively
Hattinger *et al*. 2016	Yes, but retrospectively
Hegyi *et al*., 2017	No
Hildebrandt *et al*. 2017	No
Hurkmans *et al*., 2020	No
Koster *et al*., 2018	No
Ruiz-Pinto *et al.*, 2017	No
Visscher *et al.*, 2012	No
Visscher *et al*., 2015	No
Wang *et al*., 2014	No
Wang *et al*., 2016	Yes, but retrospectively
Windsor *et al*., 2012	No

Question	3. If a power calculation was performed (Yes to #2), were the number of inclusions required for a power of 80 met?
Aminkeng *et al.*, 2015	n/a
Bhuvaneshwar *et al.* 2019	n/a
Caronia *et al*., 2011	n/a
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	No
Hegyi *et al*., 2017	n/a
Hildebrandt *et al*. 2017	n/a
Hurkmans *et al*., 2020	n/a
Koster *et al*., 2018	n/a
Ruiz-Pinto *et al.*, 2017	n/a
Visscher *et al.*, 2012	n/a
Visscher *et al*., 2015	n/a
Wang *et al*., 2014	n/a
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	n/a
Question	**4. Were in- and exclusion criteria clearly described?**
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	Yes
Caronia *et al*., 2011	Yes
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	Yes
Hegyi *et al*., 2017	Yes
Hildebrandt *et al*. 2017	Yes
Hurkmans *et al*., 2020	Yes
Koster *et al*., 2018	No
Ruiz-Pinto *et al.*, 2017	Yes
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	Yes
Wang *et al*., 2014	Yes
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	Yes

Question	5. Were sufficient basic characteristics reported for the study population?
Aminkeng *et al.*, 2015	Yes; age, gender, cumulative anthracycline exposure, anthracycline chemotherapy, primary diagnosis, radiotherapy involving heart, use of cardioprotectants, duration follow-up
Bhuvaneshwar *et al.* 2019	No
Caronia *et al*., 2011	Yes; age, sex, tumor location, response to treatment, metastasis, survival status, relapse
Hagleitner *et al*., 2015	Yes; age, gender, primary metastasis, tumor in axial skeleton, poor histologic response, 5-year PFS
Hattinger *et al*. 2016	Yes; age, gender, tumor site, metastasis at diagnosis, histologic subtype, surgery, treatment
Hegyi *et al*., 2017	Yes; age, gender, risk group, hepatotoxicity, myelotoxicity, peak MTX, 48h MTX, AUX, T1/2
Hildebrandt *et al*. 2017	Yes; age, gender, race, chest radiation, cancer site, anthracycline cumulative dose, follow-up time, average EF, risk score, hypertension
Hurkmans *et al*., 2020	Yes; age, sex, ethnicity, metastasis, treatment protocol, MTX cumulative dose
Koster *et al*., 2018	Yes; age, sex, vital status, metastasis at diagnosis
Ruiz-Pinto *et al.*, 2017	Yes; age, sex, primary diagnosis, family history of cardiovascular disease, radiotherapy involving the heart, cumulative anthracyline dose, anthracycline type, concomitant therapy, follow-up
Visscher *et al.*, 2012	Yes; age, sex, dose, anthracycline type, tumor type, radiotherapy involving heart, follow-up
Visscher *et al*., 2015	No
Wang *et al*., 2014	Yes; race, age, sex, diagnosis, year of diagnosis, follow-up, cumulative anthracycline dose, chest radiation, age at cardiomyopathy diagnosis, ejection fraction
Wang *et al*., 2016	Yes; race, age at primary cancer, age at study participation, sex, primary diagnosis, year of primary diagnosis, follow-up, chest radiation, ejection fraction, fractional shortening
Windsor *et al*., 2012	Yes; age, follow-up, sex, ethnic group, primary tumor site, metastasis at diagnosis, histological subtype, histological response, death, relapse

Question	**6. Was a validation cohort selected (in this study) to test reproducibility of findings in the original cohort?**
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	No
Caronia *et al*., 2011	No
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	No
Hegyi *et al*., 2017	No
Hildebrandt *et al*. 2017	No
Hurkmans *et al*., 2020	No
Koster *et al*., 2018	Yes
Ruiz-Pinto *et al.*, 2017	No
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	Yes
Wang *et al*., 2014	Yes
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	No
Question	**Treatment and outcome** **7. Were all patients treated according to label?**
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	Yes
Caronia *et al*., 2011	Yes
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	Yes
Hegyi *et al*., 2017	Yes
Hildebrandt *et al*. 2017	Yes
Hurkmans *et al*., 2020	Yes
Koster *et al*., 2018	Yes
Ruiz-Pinto *et al.*, 2017	Yes
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	Yes
Wang *et al*., 2014	Yes
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	Yes

Question	**8. Were clinical data collected prospectively of retrospectively?**
Aminkeng *et al.*, 2015	Retrospectively
Bhuvaneshwar *et al.* 2019	Retrospectively
Caronia *et al*., 2011	Retrospectively
Hagleitner *et al*., 2015	Retrospectively
Hattinger *et al*. 2016	Retrospectively
Hegyi *et al*., 2017	Retrospectively
Hildebrandt *et al*. 2017	Retrospectively
Hurkmans *et al*., 2020	Retrospectively
Koster *et al*., 2018	Retrospectively
Ruiz-Pinto *et al.*, 2017	Retrospectively
Visscher *et al.*, 2012	Retrospectively
Visscher *et al*., 2015	Retrospectively
Wang *et al*., 2014	Retrospectively
Wang *et al*., 2016	Retrospectively
Windsor *et al*., 2012	Retrospectively
Question	**Follow-up and missing data** **9. Does the study describe the number of participants who withdrew and/or were lost to follow-up after the start of the observation period?**
Aminkeng *et al.*, 2015	No
Bhuvaneshwar *et al.* 2019	No
Caronia *et al*., 2011	No
Hagleitner *et al*., 2015	No
Hattinger *et al*. 2016	No
Hegyi *et al*., 2017	No
Hildebrandt *et al*. 2017	No
Hurkmans *et al*., 2020	No
Koster *et al*., 2018	No
Ruiz-Pinto *et al.*, 2017	No
Visscher *et al.*, 2012	No
Visscher *et al*., 2015	No
Wang *et al*., 2014	No
Wang *et al*., 2016	No
Windsor *et al*., 2012	Yes, three patients were excluded due to missing data

Question	**10. What was the overall percentage of participants who withdrew or were lost to follow-up after the start of the observation period?**
Aminkeng *et al.*, 2015	n/a
Bhuvaneshwar *et al.* 2019	n/a
Caronia *et al*., 2011	n/a
Hagleitner *et al*., 2015	n/a
Hattinger *et al*. 2016	n/a
Hegyi *et al*., 2017	n/a
Hildebrandt *et al*. 2017	n/a
Hurkmans *et al*., 2020	n/a
Koster *et al*., 2018	n/a
Ruiz-Pinto *et al.*, 2017	n/a
Visscher *et al.*, 2012	n/a
Visscher *et al*., 2015	n/a
Wang *et al*., 2014	n/a
Wang *et al*., 2016	n/a
Windsor *et al*., 2012	n/a
Question	**11. What was the overall percentage of missing data for the association between the pharmacogenetics interaction and the main outcome?**
Aminkeng *et al.*, 2015	NR
Bhuvaneshwar *et al.* 2019	NR
Caronia *et al*., 2011	NR
Hagleitner *et al*., 2015	NR
Hattinger *et al*. 2016	NR
Hegyi *et al*., 2017	NR
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	Yes, ranges from 8% to 66% (Table 2)
Koster *et al*., 2018	NR
Ruiz-Pinto *et al.*, 2017	NR
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	NR
Wang *et al*., 2014	NR
Wang *et al*., 2016	NR
Windsor *et al*., 2012	NR

	Genotyping
Question	**12. Specify genotyping method(s) used.**
Aminkeng *et al.*, 2015	GWA array and allelic discrimination assays
Bhuvaneshwar *et al.* 2019	Whole genome sequencing
Caronia *et al*., 2011	Multiplex allelic discrimination assays
Hagleitner *et al*., 2015	ADME array
Hattinger *et al*. 2016	Allelic discrimination assays
Hegyi *et al*., 2017	Allelic discrimination assays
Hildebrandt *et al*. 2017	Allelic discrimination assays
Hurkmans *et al*., 2020	ADME array
Koster *et al*., 2018	GWA array
Ruiz-Pinto *et al.*, 2017	GWA array
Visscher *et al.*, 2012	ADME array
Visscher *et al*., 2015	ADME array
Wang *et al*., 2014	Cardiovascular SNP array
Wang *et al*., 2016	GWA array
Windsor *et al*., 2012	GWA array
Question	**13. For studies using genome-wide association study (GWAS) data, specify the allele-calling algorithm used.**
Aminkeng *et al.*, 2015	GenomeStudio
Bhuvaneshwar *et al.* 2019	Sickle, Bowtie2, Samtools, Picard, and GATK's HaplotypeCaller.
Caronia *et al*., 2011	GenomeStudio
Hagleitner *et al*., 2015	GenomeStudio
Hattinger *et al*. 2016	n/a
Hegyi *et al*., 2017	GenomeStudio
Hildebrandt *et al*. 2017	n/a
Hurkmans *et al*., 2020	DMET console software
Koster *et al*., 2018	NR
Ruiz-Pinto *et al.*, 2017	GenomeStudio
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	GenomeStudio
Wang *et al*., 2014	NR
Wang *et al*., 2016	NR
Windsor *et al*., 2012	Beadstudio

Question	**14. How many SNPs were excluded based on call rate, and what cut-off point for exclusion was used?**
Aminkeng *et al.*, 2015	Number of SNPs NR, cut-off was 0.95
Bhuvaneshwar *et al.* 2019	NR
Caronia *et al*., 2011	20 SNPs excluded in total (call rate+HWE), cut-off at 95%
Hagleitner *et al*., 2015	7 SNPs excluded with call rate <0.85
Hattinger *et al*. 2016	NR
Hegyi *et al*., 2017	NR
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	26 SNPs excluded with of call rate <0.9
Koster *et al*., 2018	Number of SNPs NR, cut-off was at 0.9
Ruiz-Pinto *et al.*, 2017	Number of SNPs NR, cut-off was at 0.99
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	374 SNPs excluded in QC, possibly due to low call rate, cut-off was at 0.95
Wang *et al*., 2014	998 SNPs excluded with call rate < 0.95
Wang *et al*., 2016	2999 SNPs excluded with call rate < 0.95
Windsor *et al*., 2012	NR
Question	**15. Was duplicate genotyping performed?**
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	Yes
Caronia *et al*., 2011	Yes, duplicate samples and CEPH trio's
Hagleitner *et al*., 2015	NR
Hattinger *et al*. 2016	NR
Hegyi *et al*., 2017	NR
Hildebrandt *et al*. 2017	Yes
Hurkmans *et al*., 2020	No
Koster *et al*., 2018	NR
Ruiz-Pinto *et al.*, 2017	Yes, 6 duplicate samples
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	Yes, 54 duplicate SNPs
Wang *et al*., 2014	No
Wang *et al*., 2016	No
Windsor *et al*., 2012	NR

Question	16. If duplicate genotyping was performed (Yes to #16), what was the duplicate genotyping concordance?
Aminkeng *et al.*, 2015	100%
Bhuvaneshwar *et al.* 2019	9/20 (45%) in group A, 100% in group B and C
Caronia *et al*., 2011	NR
Hagleitner *et al*., 2015	n/a
Hattinger *et al*. 2016	n/a
Hegyi *et al*., 2017	n/a
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	n/a
Koster *et al*., 2018	n/a
Ruiz-Pinto *et al.*, 2017	NR
Visscher *et al.*, 2012	n/a
Visscher *et al*., 2015	Yes, 99.9% concordance
Wang *et al*., 2014	n/a
Wang *et al*., 2016	n/a
Windsor *et al*., 2012	n/a
Question	**17. Was Hardy-Weinberg equilibrium calculated?**
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	No
Caronia *et al*., 2011	Yes
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	Yes
Hegyi *et al*., 2017	Yes
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	Yes
Koster *et al*., 2018	Yes
Ruiz-Pinto *et al.*, 2017	Yes
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	Yes
Wang *et al*., 2014	Yes
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	Yes

Question	18. If alleles were not in HWE, how were they handled?
Aminkeng *et al.*, 2015	Excluded if HWE P<1.0 × 10−4
Bhuvaneshwar *et al.* 2019	n/a
Caronia *et al*., 2011	Excluded if deviated from HWE (threshold NR)
Hagleitner *et al*., 2015	31 variants with HWE p<0.05 were excluded
Hattinger *et al*. 2016	Excluded if HWE p<0.01
Hegyi *et al*., 2017	Excluded if HWE p<0.05
Hildebrandt *et al*. 2017	n/a
Hurkmans *et al*., 2020	1 variant with HWE p<0.0001 was excluded
Koster *et al*., 2018	Excluded if HWE p<10^-7
Ruiz-Pinto *et al.*, 2017	Excluded if HWE p<10^-8
Visscher *et al.*, 2012	23 SNPs with HWE p<1.5*10^-4 were excluded
Visscher *et al*., 2015	29 SNPs had HWE p<1.7 × 10-5. These SNPs were marked, but retained in the analysis.
Wang *et al*., 2014	108 SNPs with HWE p<0.000001 were excluded
Wang *et al*., 2016	3295 SNPs with HWE p<0.0001 were excluded
Windsor *et al*., 2012	Excluded if HWE p<0.001
Question	**Statistical analyses** **19. How were missing data handled (select all that apply)?**
Aminkeng *et al.*, 2015	NR
Bhuvaneshwar *et al.* 2019	NR
Caronia *et al*., 2011	NR
Hagleitner *et al*., 2015	NR
Hattinger *et al*. 2016	NR
Hegyi *et al*., 2017	NR
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	Correction for number of datapoints per patient
Koster *et al*., 2018	NR
Ruiz-Pinto *et al.*, 2017	NR
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	NR
Wang *et al*., 2014	NR
Wang *et al*., 2016	NR
Windsor *et al*., 2012	NR

Question	20. What type of analysis was used for the main comparison?
Aminkeng *et al.*, 2015	NR
Bhuvaneshwar *et al.* 2019	NR
Caronia *et al*., 2011	NR
Hagleitner *et al*., 2015	NR
Hattinger *et al*. 2016	NR
Hegyi *et al*., 2017	NR
Hildebrandt *et al*. 2017	NR
Hurkmans *et al*., 2020	NR
Koster *et al*., 2018	NR
Ruiz-Pinto *et al.*, 2017	NR
Visscher *et al.*, 2012	NR
Visscher *et al*., 2015	NR
Wang *et al*., 2014	NR
Wang *et al*., 2016	NR
Windsor *et al*., 2012	NR
Question	**21. For studies investigating more than one genetic variant, was adjustment for multiple testing applied?**
Aminkeng *et al.*, 2015	Yes stage 1: threshold of P < 1 × 10−5, stage 2: threshold of p < 0.006, stage 3 threshold p < 0.05.
Bhuvaneshwar *et al.* 2019	Yes, Benjamini Hochberg false discovery rate
Caronia *et al*., 2011	Yes, corrected for 696 tests
Hagleitner *et al*., 2015	No
Hattinger *et al*. 2016	No
Hegyi *et al*., 2017	No
Hildebrandt *et al*. 2017	No
Hurkmans *et al*., 2020	Yes, Bonferroni
Koster *et al*., 2018	Adjusted p-value threshold: SNPs with p<10^-4 were replicated in second patient cohort
Ruiz-Pinto *et al.*, 2017	Yes, FDR correction
Visscher *et al.*, 2012	Yes, simpleM correction
Visscher *et al*., 2015	Yes, a tiered analysis to identify SNPs associated at p < 0.01 in the larger discovery cohort that remained associated in the smaller replication cohort at p < 0.05. For combined cohort: Bonferroni corrected significance threshold at p<1.7*10^-5.
Wang *et al*., 2014	Yes, according to Purcell et al. (P < 5 *10^-6)
Wang *et al*., 2016	Yes, repeated sliding-window procedure
Windsor *et al*., 2012	No

Question	22. Were analyses performed to assess the effect of covariates?
Aminkeng *et al.*, 2015	Yes
Bhuvaneshwar *et al.* 2019	NR
Caronia *et al*., 2011	Yes
Hagleitner *et al*., 2015	Yes
Hattinger *et al*. 2016	No
Hegyi *et al*., 2017	Yes
Hildebrandt *et al*. 2017	Yes
Hurkmans *et al*., 2020	Yes
Koster *et al*., 2018	Yes
Ruiz-Pinto *et al.*, 2017	Yes
Visscher *et al.*, 2012	Yes
Visscher *et al*., 2015	NR
Wang *et al*., 2014	Yes
Wang *et al*., 2016	Yes
Windsor *et al*., 2012	Yes
Question	**Other** **23. Please provide any additional remarks (either positive or negative) on quality.**
Aminkeng *et al.*, 2015	-
Bhuvaneshwar *et al.* 2019	The results in the paper of the targeted DMET analysis do not correspond to the results in the supplementary material
Caronia *et al*., 2011	-
Hagleitner *et al*., 2015	-
Hattinger *et al*. 2016	-
Hegyi *et al*., 2017	-
Hildebrandt *et al*. 2017	-
Hurkmans *et al*., 2020	-
Koster *et al*., 2018	-
Ruiz-Pinto *et al.*, 2017	-
Visscher *et al.*, 2012	-
Visscher *et al*., 2015	-
Wang *et al*., 2014	-
Wang *et al*., 2016	-
Windsor *et al*., 2012	-

NR, not reported; n/a, not applicable

Table S7. Results question 5 of the quality assessment, regarding to reporting of relevant baseline characteristics. Characteristics were considered relevant if they were reported in 2 or more studies.

	Cardiotoxicity								Efficacy			Multiple outcomes		Other toxicities		
Outcome	**Amin-keng** *et al.*, **2015**	**Hilde-brandt** *et al.* **2017**	**Ruiz-Pinto** *et al.*, **2017**	**Vis-scher** *et al.*, **2012**	**Vis-scher** *et al.*, **2015**	**Wang** *et al.*, **2014**	**Wang** *et al.*, **2016**	**Bhu-vanesh-war** *et al.* **2019**	**Caronia** *et al.*, **2011**	**Hagleit-ner** *et al.*, **2015**	**Koster** *et al.*, **2018**	**Wind-sor** *et al.*, **2012**	**Hattin-ger** *et al.* **2016**	**Hegyi** *et al.*, **2017**	**Hurk-mans** *et al.*, **2020**	**Repor-ted in # of studies**
Age	x	x	x	x		x	x		x	x	x	x	x	x	x	**13**
Sex	x	x	x	x		x	x		x	x	x	x	x	x	x	**13**
Primary tumor diagnosis	x	x	x	x		x	x		x	x	x	x	x	x	x	**13**
Follow-up time	x	x	x	x		x	x					x				**7**
Metastasis at diagnosis									x	x	x	x	x		x	**6**
Radiation involving heart	x	x	x	x		x	x									**6**
Ethnicity		x				x	x					x			x	**5**
Anthracycline cumulative dose	x	x	x	x		x										**5**
Tumor in axial skeleton/ bone tumor location									x	x		x	x			**4**
Histological subtype												x	x			**2**
Survival state									x		x	x				**3**
Anthracycline type	x		x	x												**3**
Ejection fraction		x				x	x									**3**
Risk group		x												x		**2**

	Cardiotoxicity								Efficacy			Multiple outcomes		Other toxicities		
Outcome	**Amin-keng** *et al.*, **2015**	**Hilde-brandt** *et al.* **2017**	**Ruiz-Pinto** *et al.*, **2017**	**Vis-scher** *et al.*, **2012**	**Vis-scher** *et al.*, **2015**	**Wang** *et al.*, **2014**	**Wang** *et al.*, **2016**	**Bhu-vanesh-war** *et al.* **2019**	**Caronia** *et al.*, **2011**	**Hagleit-ner** *et al.*, **2015**	**Koster** *et al.*, **2018**	**Wind-sor** *et al.*, **2012**	**Hattin-ger** *et al.* **2016**	**Hegyi** *et al.*, **2017**	**Hurk-mans** *et al.*, **2020**	**Repor-ted in # of studies**
Relapse									x			x				**2**
MTX plasma levels									x						x	**2**
# characteris-tics reported in this article	**7**	**9**	**7**	**7**	**0**	**8**	**7**	**0**	**8**	**5**	**5**	**10**	**6**	**4**	**6**	

Replication studies Table S8 – S10

Table S8. Characteristics and results of independent discovery and replication cohorts, studying genetic variants associated with treatment response in patients with osteosarcoma.

Author	Outcome	No. of patients	HR (95% CI)	P value	Ref
***ABCB1* rs10276036**					
Caronia *et al.*, 2011*	EFS	102	0.42 (0.29-0.81)	**0.0021**	(1)
Liu *et al.*, 2014	PFS	186	1.24 (0.79-1.93)	0.32	(2)
***ABCB1* rs1128503**					
Caronia *et al.*, 2011*	EFS	102	0.42 (0.29-0.81)	**0.0021**	(1)
Windsor *et al.*, 2012	PFS	58	N/S	ns	(3)
Yang *et al.*, 2013	DFS	208	3.74 (1.63-7.4)	**0.003**	(4)
Li *et al.*, 2014	OS	162	3.17 (1.14-6.67)	**0.01**	(5)
Liu *et al.*, 2014	PFS	186	0.49 (0.31-0.76)	<**0.001**	(2)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)
***ABCB1* rs4148737**					
Caronia *et al.*, 2011*	EFS	102	2.6 (1.24-3.22)	**0.00051**	(1)
Liu *et al.*, 2014	PFS	186	1.47 (0.93-2.33)	0.08	(2)
***ABCC2* rs2273697**					
Hattinger *et al.*, 2016*	EFS	126	N/S	**0.049**	(6)
Windsor *et al.*, 2012	PFS	58	N/S	ns	(3)
***ABCC2* rs717620**					
Windsor *et al.*, 2012*	Histological response	58	6.3 (1.4-28.5)[a]	**0.017**	(3)
Yang *et al.*, 2013	DFS	208	N/S	ns	(4)
Goričar *et al.*, 2014	OS	44	0.5 (0.14-1.75)	0.275	(7)
Hagleitner *et al.*, 2015	PFS	126	N/S	ns	(8)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)
***ABCC3* rs4148416**					
Caronia *et al.*, 2011*	EFS	102	6.33 (1.79-12.7)	**0.00028**	(1)
Yang *et al.*, 2013	DFS	208	4.32 (1.75-15.65)	**0.006**	(4)
Liu *et al.*, 2014	PFS	186	2.73 (1.62-4.66)	<**0.001**	(2)

Author	Outcome	No. of patients	HR (95% CI)	P value	Ref
***ABCC5* rs939338**					
Hagleitner *et al.*, 2015*	PFS	126	1.86 (1.06-3.24)[a]	**0.03**	(8)
Hagleitner *et al.*, 2015	PFS	64	1.36 (0.62-2.98)[a]	0.44	(8)
Xu *et al.*, 2018	PFS	132	2.01 (1.2-3.37)[a]	**0.008**	(9)
***CASP3* rs2720376**					
Hagleitner *et al.*, 2015*	PFS	126	0.52 (0.3-0.9)[a]	**0.02**	(8)
Hagleitner *et al.*, 2015	PFS	64	0.68 (0.3-1.56)[a]	0.36	(8)
Xu *et al.*, 2018	PFS	132	1.02 (0.62-1.71)[a]	0.95	(9)
***CCND1* rs9344**					
Windsor *et al.*, 2012*	PFS	58	N/S	**0.018**	(3)
***CHST12* rs3735099**					
Bhuvaneshwar *et al.*, 2019*	OS and tumor necrosis	100	3.124 (N/S)	**0.04**	(10)
***CHST12* rs3735100**					
Bhuvaneshwar *et al.*, 2019*	OS and tumor necrosis	100	3.124 (N/S)	**0.04**	(10)
***CYP2B6*6**					
Hattinger *et al.*, 2016*	EFS	126	N/S	**0.039**	(6)
***CYP3A4* rs4646437**					
Hagleitner *et al.*, 2015*	PFS	126	0.34 (0.13-0.85)[a]	**0.02**	(8)
Hagleitner *et al.*, 2015	PFS	64	0.61 (0.19-1.94)[a]	0.4	(8)
Xu *et al.*, 2018	PFS	132	0.69 (0.39-1.26)[a]	0.24	(9)
***FasL* rs763110**					
Hagleitner *et al.*, 2015*	PFS	126	1.97 (1.04-3.73)[a]	**0.04**	(8)
Hagleitner *et al.*, 2015	PFS	64	1.5 (0.7-3.21)[a]	0.3	(8)
Xu *et al.*, 2018	PFS	132	2.26 (1.35-3.77)[a]	**0.002**	(9)
***GGH* rs11545078**					
Hattinger *et al.*, 2016*	EFS	126	N/S	**0.037**	(6)
***GLDC* rs3765555**					
Koster *et al.*, 2018*	OS	523	1.71 (1.34-2.18)	**1.60×10^{-5}**	(11)
Koster *et al.*, 2018	OS	109	2.12 (1.27-3.53)	**3.87×10^{-3}**	(11)

Author	Outcome	No. of patients	HR (95% CI)	P value	Ref
***GLDC* rs55933544**					
Koster *et al.*, 2018*	OS	523	1.91 (1.49-2.45)	**3.20×10^{-7}**	(11)
Koster *et al.*, 2018	OS	109	1.98 (1.16-3.38)	**0.012**	(11)
Lin *et al.*, 2020	OS	72	3.98 (1.87-4.96)[a]	**<0.001**	(12)
***GSTP1* rs1695**					
Windsor *et al.*, 2012*	Histological response	58	7.9 (1.5-42.5)[a]	**0.016**	(3)
Windsor *et al.*, 2012*	PFS	58	N/S	**0.025**	(3)
Yang *et al.*, 2012	OS	187	0.53 (0.24-1.16)	0.32	(13)
Zhang *et al.*, 2012	EFS	159	2.35 (1.13-4.85)	N/S	(14)
Teng *et al.*, 2013	OS	146	2.73 (1.05-7.45)	**<0.05**	(15)
Li *et al.*, 2014	OS	162	3.86 (1.41-10.2)	**0.004**	(5)
Liu *et al.*, 2014	PFS	186	2.16 (1.38-3.38)	**<0.001**	(2)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)
***GSTT1* null**					
Windsor *et al.*, 2012*	PFS	58	N/S	**0.006**	(3)
Yang *et al.*, 2012	OS	187	0.92 (0.57-1.76)	0.75	(13)
Zhang *et al.*, 2012	EFS	159	0.7 (0.39-1.33)	ns	(14)
Teng *et al.*, 2013	OS	146	1.43 (0.72-3.04)	0.31	(15)
Li *et al.*, 2014	OS	162	1.26 (0.63-2.42)	0.66	(5)
Liu *et al.*, 2014	PFS	186	1.18 (0.64-2.18)	0.58	(2)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)
***MSH2* rs4638843**					
Hagleitner *et al.*, 2015*	PFS	126	2.32 (1.02-5.27)[a]	**0.04**	(8)
Hagleitner *et al.*, 2015	PFS	64	3.86 (0.98-15.25)[a]	**0.05**	(8)
***MTHFD1* rs2236225**					
Windsor *et al.*, 2012*	Histological response	58	0.2 (0.05-0.9)[a]	**0.03**	(3)
Goričar *et al.*, 2014	OS	44	1.52 (0.54-4.27)	0.43	(7)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)

Author	Outcome	No. of patients	HR (95% CI)	P value	Ref
***RFC1/SLC19A1* rs1051266**					
Windsor *et al.,* 2012*	PFS	58	N/S	**0.02**	(3)
Goričar *et al.*, 2014	OS	44	0.77 (0.29-2.07)	0.601	(7)
Jabeen *et al.*, 2015	OS	62	N/S	**0.046**	(16)
Hattinger et al., 2016	EFS	126	N/S	ns	(6)
***SLC22A1* rs4646272**					
Bhuvaneshwar *et al.,* 2019*	OS and tumor necrosis	100	3.723 (N/S)	**0.007**	(10)
***SLC22A8* rs2187384**					
Bhuvaneshwar *et al.,* 2019*	OS and tumor necrosis	100	3.161 (N/S)	**0.019**	(10)
***TP53* rs1642785**					
Hattinger *et al.,* 2016*	EFS	126	N/S	**0.01**	(6)
***UGT2B15* rs34073924**					
Bhuvaneshwar *et al.,* 2019*	OS and tumor necrosis	100	3.462 (N/S)	**0.024**	(10)

N/S, not specified; ns, not significant; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; EFS, event-free survival; DFS, disease-free survival
* discovery study that was identified in systematic literature search
[a] Odds ratio (95% CI)

Table S9. Characteristics and results of independent discovery and replication cohorts, studying genetic variants associated with doxorubicin-induced cardiotoxicity.

	Outcome	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	P value	Ref.
***ATP2B1* rs17249754**							
Hildebrandt *et al.,* 2017*	EF 45–50% and symptoms; or FS ≤ 25% and/or EF ≤ 45%	Pediatric	Mixed cancer cohort	108	0.33 (0.12–0.92)	**0.034**	(17)
***CELF4* rs1786814**							
Wang *et al*., 2016*	FS ≤ 28% or LVEF ≤ 40% or symptoms	Pediatric	Mixed cancer cohort	331	10.16 (3.8-27.3)	**<0.001**	(18)
Wang *et al*., 2016	FS ≤ 28% or LVEF ≤ 40% or symptoms	Pediatric	Mixed cancer cohort	75	5.09 (1.03-25.23)	**0.046**	(18)
Leger *et al*., 2016	ICD codes of symptoms or self-reported through surveys	Adult	Hematopoietic cell transplantation survivors	576	22.2 (1.5-339.2)	**0.01**	(19)
***GPR35* rs12468485**							
Ruiz-Pinto *et al*., 2017*	FS ≤ 27%	Pediatric	Mixed cancer cohort	93	N/A	**7×10^{-6}**	(20)
***GSTP1* rs1695**							
Windsor *et al.,* 2012*	EF decrease with ≥1 CTCAE	Pediatric	Osteosarcoma	58	4.8 (1.4-16.4)	0.011	(3)
***HAS3* rs2232228**							
Wang *et al*., 2014*	FS ≤ 28% or LVEF ≤ 40% or symptoms	Pediatric	Mixed cancer cohort	401	8.9 (2.1-37.5)	**0.003**	(21)
Wang *et al*., 2014	FS ≤ 28% or LVEF ≤ 40% or symptoms	Pediatric	Mixed cancer cohort	76	4.5 (1.1-18.7)	**0.04**	(21)
Wang *et al*., 2016	FS ≤ 28% or LVEF ≤ 40% or symptoms	Pediatric	Mixed cancer cohort	331	N/S	0.6	(18)
Leger *et al*., 2016	ICD codes of symptoms or self-reported through surveys	Adult	Hematopoietic cell transplantation survivors	576	21.8 (1.2-386.4)	**0.02**	(19)
Sági *et al*., 2018	FS ≤ 28%	Pediatric	ALL and osteosarcoma	661	N/S	N/S, ns	(22)
***PLCE1* rs932764**							
Hildebrandt *et al.,* 2017*	EF 45–50% and symptoms; or FS ≤ 25% and/or EF ≤ 45%	Pediatric	Mixed cancer cohort	108	0.48 (0.27–0.85)	**0.012**	(17)
***RARG* rs2229774**							
Aminkeng *et al*., 2015*	FS ≤ 24% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	280	7 (2.9-17)	**5.0×10^{-6}**	(23)
Aminkeng *et al*., 2015	FS ≤ 24% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	80	N/A	**4.3×10^{-11}**	(23)
Aminkeng *et al*., 2015	FS ≤ 24% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	96	4.1 (1.5-11.5)	**0.0043**	(23)
Serie *et al*., 2017	LVEF reduction > 10% or LVEF < 53%	Adult	HER2+ breast cancer	1191	2.39 (0.91-6.23)	0.076	(24)
Schneider *et al*., 2017	LVEF reduction > 20% or LVEF < 50%	Adult	Breast cancer	102	0.11 (N/S)	**0.0000041**	(25)
Sági *et al*., 2018	FS ≤ 28%	Pediatric	ALL and osteosarcoma	661	N/S	N/S, ns	(22)
Park *et al*., 2020	LVEF reduction > 10% or LVEF < 50%	Adult	Breast cancer	257	N/S	N/S, ns	(26)

	Outcome	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	P value	Ref.
***SLC22A17* rs4982753**							
Visscher *et al*., 2015*	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	335	0.52 (0.31-0.85)	**0.0078**	(27)
Visscher *et al*., 2015	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	185	0.39 (0.19-0.81)	**0.0071**	(27)
Schneider *et al*., 2017	LVEF reduction > 20% or LVEF < 50%	Adult	Breast cancer	102	N/S	N/S, ns	(25)
Sági *et al*., 2018	FS ≤ 28%	Pediatric	ALL and osteosarcoma	661	N/S	N/S, ns	(22)
***SLC22A7* rs4149178**							
Visscher *et al*., 2015*	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	335	0.41 (0.21-0.77)	**0.0034**	(27)
Visscher *et al*., 2015	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	185	0.39 (0.14-1.05)	**0.047**	(27)
Schneider *et al*., 2017	LVEF reduction > 20% or LVEF < 50%	Adult	Breast cancer	102	N/S	N/S, ns	(25)
Sági *et al*., 2018	FS ≤ 28%	Pediatric	ALL and osteosarcoma	661	N/S	N/S, ns	(22)
***SLC28A3* rs7853758**							
Visscher *et al*., 2012*	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	156	0.29 (0.11-0.81)	**0.0071**	(28)
Visscher *et al*., 2012	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	188	0.33 (0.13-0.8)	**0.0072**	(28)
Visscher *et al*., 2012	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	96	0.69 (N/S)	0.38	(28)
Visscher *et al*., 2013	FS ≤ 26% or symptoms requiring intervention in CTCAE v3	Pediatric	Mixed cancer cohort	177	0.46 (0.2-1.08)	0.058	(29)
Vulsteke *et al*., 2015	LVEF reduction > 10% or CTCAEv4 grade 3-5	Adult	Breast cancer	877	N/S	N/S, ns	(30)
Reichwagen *et al*., 2015	CTCAE v2 > grade 0	Adult	CD20+ B-cell lymphoma	450	1.4 (0.8-2.3)	0.27	(31)
Reichwagen *et al*., 2015	CTCAE v2 > grade 0	Adult	CD20+ B-cell lymphoma	634	1.4 (0.7-3)	0.39	(31)
Hertz *et al*., 2016	EF < 55%	Adult	Breast cancer	166	0.55 (0.16-1.91)	0.43	(32)
Ruiz-Pinto *et al*., 2017	FS ≤ 27%	Pediatric	Mixed cancer cohort	93	N/S	**<0.05**	(20)
Serie *et al*., 2017	LVEF reduction > 10% or LVEF < 53%	Adult	HER2+ breast cancer	1191	0.71 (0.25-2.02)	0.52	(24)
Sági *et al*., 2018	FS ≤ 28%	Pediatric	ALL and osteosarcoma	661	9.837 (1.73-56.02)	**0.01**	(22)

N/S, not specified; N/A, not applicable; ns, not significant; FS, fractional shortening ;LVEF, left ventricular ejection fraction; EF, ejection fraction; CTCAE, Common Terminology Criteria for Adverse Events; ALL, acute lymphatic leukemia
* discovery study that was identified in systematic literature search

Table S10. Characteristics and results of independent discovery and replication cohorts, studying genetic variants associated with bone marrow- hepato- and nephrotoxicity after treatment with cisplatin, doxorubicin or methotrexate.

Author	Outcome	Induced by[a]	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	p-value	Ref.
***ABCB1* rs1128503**	**Hepatotoxicity**							
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	2.06 (1.16-3.66)	**0.014**	(6)
Hegyi et al., 2017	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	N/S	ns	(33)
Hurkmans et al., 2020	ALAT	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***ABCC2* rs17222723**	**Leukopenia**							
Windsor et al., 2012*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	58	5.2 (1.2-22.4)	**0.028**	(3)
Hurkmans et al., 2020	Leukocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***ABCC2* rs2273697**	**Hepatotoxicity**							
Windsor et al., 2012	CTCAE v3 grade 3-4	MAP	Pediatric	Osteosarcoma	58	N/S	ns	(3)
Sharifi et al., 2014	CTCAE v3 grade 1-4	MTX	Pediatric	ALL	65	0.55 (0.2-1.48)	0.32	(35)
Goričar et al., 2014	CTCAE v4 grade 3-4	MTX	Pediatric	Osteosarcoma	118	1.88 (0.1-3.49)	0.414	(7)
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	1.96 (1.2-3.2)	**0.007**	(6)
Gervasini et al., 2017	CTCAE v4	MTX	Pediatric	ALL	41	N/S	ns	(36)
Hegyi et al., 2017	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	N/S	ns	(33)
Hurkmans et al., 2020	ALAT	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***ABCC2* rs2273697**	**Leukopenia**							
Windsor et al., 2012	CTCAE v3 grade 3-4	MAP	Pediatric	Osteosarcoma	58	N/S	ns	(3)
Sharifi et al., 2014	CTCAE v3 grade 1-4	MTX	Pediatric	ALL	65	0.61 (0.13-2.97)	0.69	(35)
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	13.16 (1.56-111.12)	**0.018**	(6)
Gervasini et al., 2017	CTCAE v4	MTX	Pediatric	ALL	41	N/S	ns	(36)
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	3.3 (1.2-9.4)	**0.02**	(33)
Hurkmans et al., 2020	Leukocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***ABCC2* rs2273697**	**Thrombocytopenia**							
Windsor et al., 2012	CTCAE v3 grade 3-4	MAP	Pediatric	Osteosarcoma	58	N/S	ns	(3)
Sharifi et al., 2014	CTCAE v3 grade 1-4	MTX	Pediatric	ALL	65	2.5 (0.89-6.97)	0.12	(35)
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	4.33 (1.2-15.63)	**0.025**	(6)
Gervasini et al., 2017	CTCAE v4	MTX	Pediatric	ALL	41	N/S	ns	(36)
Hurkmans et al., 2020	Thrombocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)

Author	Outcome	Induced by[a]	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	p-value	Ref.
***ABCC2* rs3740066**	**Leukopenia**							
Hattinger et al., 2016	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	N/S	ns	(6)
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.4 (0.2-0.9)	**0.02**	(33)
Hurkmans et al., 2020	Leukocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***CYBA* rs4673**	**Anemia**							
Windsor et al., 2012*	CTCAE v3 grade 3-4	Doxorubicin, cisplatin	Pediatric	Osteosarcoma	58	0.3 (0.09-0.9)	**0.038**	(3)
***CYP2B6* rs4803418**	**Thrombocytopenia**							
Hurkmans et al., 2020*	Thrombocyte counts	MTX	Pediatric	Osteosarcoma	113	-0.19 (-0.27--0.1) [b]	**0.00003**	(34)
***CYP2B6* rs4803419**	**Thrombocytopenia**							
Hurkmans et al., 2020*	Thrombocyte counts	MTX	Pediatric	Osteosarcoma	113	0.19 (0.1-0.29) [b]	**0.00006**	(34)
***CYP4F8* rs4808326**	**Thrombocytopenia**							
Hurkmans et al., 2020*	Thrombocyte counts	MTX	Pediatric	Osteosarcoma	113	-0.19 (-0.28--0.09) [b]	**0.00009**	(34)
***ERCC1* rs3212986**	**Leukopenia**							
Windsor et al., 2012*	CTCAE v3 grade 3-4	Doxorubicin	Pediatric	Osteosarcoma	58	5.4 (1.1-26)	**0.036**	(3)
Hattinger et al., 2016	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	N/S	ns	(6)
***ERCC2/XPD* rs13181**	**Nephrotoxicity**							
Khrunin et al., 2010	CTCAE grade 1-4	Cisplatin	Adult	Ovarian cancer	104	1.385 (0.526-3.644)	0.615	(37)
Khrunin et al., 2012	CTCAE v2 grade 1-4	Cisplatin	Adult	Ovarian cancer	87	N/S	ns	(38)
Windsor et al., 2012*	CTCAE v3 grade 3-4	MAP	Pediatric	Osteosarcoma	58	4.4 (1-18.8)	**0.044**	(3)
Lopes-Aguiar et al., 2017	CTCAE v4 grade 2-5	Cisplatin	Adult	Head and neck squamous cell carcinoma	90	3.55 (1.27-9.87)	**0.01**	(39)
Zazuli et al., 2019	CTCAE grade 1-4	Cisplatin	Adult	Testicular cancer	163	3.16 (1.17-8.58)	**0.02**	(40)
Garcia et al., 2020	GFR decline	Cisplatin	Adult	Testicular cancer	433	N/S	**0.03**	(41)
***ERCC2/XPD* rs1799793**	**Thrombocytopenia**							
Tibaldi et al., 2008	CTCAE v3 grade 3-4	Cisplatin, gemcitabine	Adult	Non-Small Cell Lung Cancer Patients	65	N/S	0.35	(42)
Khrunin et al., 2010	CTCAE grade 1-4	Cisplatin	Adult	Ovarian cancer	87	4.054 (1.21-13.583)	**0.027**	(37)
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	5.59 (1.66-18.87)	**0.006**	(6)
Lopes-Aguiar et al., 2017	CTCAE v4 grade 1-4	Cisplatin	Adult	Head and neck squamous cell carcinoma	90	N/S	ns	(39)

Author	Outcome	Induced by[a]	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	p-value	Ref.
***GGH* rs1800909[HW]**	**Hepatotoxicity**							
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	2.87 (1.53-5.41)	**0.001**	(6)
***GSTP1* rs1695**	**Leukopenia**							
Windsor et al., 2012*	CTCAE v3 grade 3-4	Doxorubicin	Pediatric	Osteosarcoma	58	7.8 (1.3-47)	**0.024**	(3)
Aráoz et al., 2015	WHO grade 3-4	MTX	Pediatric	ALL	286	N/S	ns	(43)
Hattinger et al., 2016	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	N/S	ns	(6)
Hurkmans et al., 2020	Leukocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***MTHFD1* rs2236225**	**Anemia**							
Erculj et al., 2012	CTCAE grade 2-4	MTX	Pediatric	ALL	167	0.84 (0.37-1.91)	0.669	(44)
Windsor et al., 2012*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	58	5.4 (1-27.5)	**0.044**	(3)
***MTHFR* rs1801131**	**Anemia**							
Huang et al., 2008	Requirement of red blood cell transfusions	MTX	Pediatric	ALL	81	N/S	**0.043**	(45)
Kantar et al., 2009	CTCAE grade 3-4	MTX	Pediatric	ALL and NHL	37	N/S	**0.02**	(46)
Karathanasis et al., 2011	CTCAE v4 grade 1-4	MTX	Pediatric	ALL	35	N/S	0.578	(47)
Liu et al., 2011	CTCAE v1 grade 2-4	MTX	Pediatric	ALL	181	0.52 (0.25-1.09)	0.081	(48)
Erculj et al., 2012	CTCAE grade 2-4	MTX	Pediatric	ALL	167	0.85 (0.38-1.88)	0.69	(44)
Windsor et al., 2012*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	58	4.6 (1.1-19.2)	**0.038**	(3)
Aráoz et al., 2015	WHO grade 3-4	MTX	Pediatric	ALL	286	N/S	ns	(43)
Yousef et al., 2019	CTCAE v4.03 grade 3-4	MTX	Pediatric	ALL	64	N/S	0.25	(49)
***MTHFR* rs1801131**	**Leukopenia**							
Huang et al., 2008	White blood cell count	MTX	Pediatric	ALL	31	N/S	0.053	(45)
van Kooten et al., 2008	CTCAE v2 grade 3-4	MTX	Pediatric	ALL	88	N/S	ns	(50)
Kantar et al., 2009	CTCAE grade 3-4	MTX	Pediatric	ALL and NHL	37	N/S	0.09	(46)
Faganel Kotnik et al., 2011	Leukopenia ≥ grade 1	MTX	Pediatric	ALL and ML	64	0.14 (0.037-0.54)	**0.012**	(51)
Karathanasis et al., 2011	CTCAE v4 grade 1-4	MTX	Pediatric	ALL	35	N/S	0.464	(47)
Haase et al., 2012	CTCAE grade 3-4	MTX	Pediatric	ALL	34	N/S	ns	(52)
Suthandiram et al., 2014	CTCAE v2 grade 1-4	MTX	Adult	Hematological malignancies	71	0.92 (0.28-23.37)	0.9	(53)
Aráoz et al., 2015	WHO grade 3-4	MTX	Pediatric	ALL	286	N/S	ns	(43)
Hattinger et al., 2016*	CTCAE v4 grade 4	MAP	Pediatric	Osteosarcoma	57	4.15 (1.06-16.13)	**0.04**	(6)
Milosevic et al., 2019	Number of leukopenic episodes	6-MP, MTX	Pediatric	ALL	127	N/S	0.25	(54)
Yousef et al., 2019	CTCAE v4.03 grade 3-4	MTX	Pediatric	ALL	64	2.1 (0.91-5)	0.076	(49)

Author	Outcome	Induced by[a]	Patient cohort	Diagnosis	No. of patients	OR (95% CI)	p-value	Ref.
***MTHFR* rs1801133**	**Nephrotoxicity**							
Kantar et al., 2009	CTCAE grade 3-4	MTX	Pediatric	ALL and NHL	37	N/S	0.29	(46)
Karathanasis et al., 2011	CTCAE v4 grade 1-4	MTX	Pediatric	ALL	35	N/S	0.628	(47)
Windsor et al., 2012*	CTCAE v3 grade 3-4	MAP	Pediatric	Osteosarcoma	58	3.1 (0.9-11.6)	0.085 [c]	(3)
El Khodary et al., 2012	Serum alpha1-microglobulin, serum creatinin, GFR	MTX	Pediatric	ALL	40	N/S	**<0.0001**	(55)
Xu et al., 2018	CTCAE grade 3-4	MTX	Pediatric	Osteosarcoma	109	1.12 (0.61-2.01)	0.76	(56)
Chae et al., 2020	Nephrotoxicity	MTX	Pediatric	ALL	117	N/S	ns	(57)
***NR1I2/SXR/PXR* rs3732361**	**Hepatotoxicity**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.1 (0.01-0.7)	**0.014**	(33)
***NR1I2/SXR/PXR* rs3732361**	**Leukopenia**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.1 (0.01-0.7)	**0.013**	(33)
***NR1I2/SXR/PXR* rs3814058**	**Hepatotoxicity**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.3 (0.1-0.7)	**0.007**	(33)
***NR1I2/SXR/PXR* rs3814058**	**Leukopenia**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.3 (0.1-0.7)	**0.007**	(33)
***NR1I2/SXR/PXR* rs6785049**	**Hepatotoxicity**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.1 (0.01-0.7)	**0.02**	(33)
Hurkmans et al., 2020	ALAT	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***NR1I2/SXR/PXR* rs6785049**	**Leukopenia**							
Hegyi et al., 2017*	CTCAE v3 grade 3-4	MTX	Pediatric	Osteosarcoma	59	0.09 (0.01-0.7)	**0.01**	(33)
Hurkmans et al., 2020	Leukocyte counts	MTX	Pediatric	Osteosarcoma	113	N/S	ns	(34)
***XPC* rs2228001**	**Infection**							
Windsor et al., 2012*	CTCAE v3 grade 3-4	Doxorubicin	Pediatric	Osteosarcoma	58	0.2 (0.06-0.8)	**0.024**	(3)

N/S, not specified; OR, odds ratio; CTCAE, Common Terminology Criteria for Adverse Events; MAP, methotrexate, anthracycline (doxorubicin), cisplatin treatment regimen; MTX, methotrexate; ALAT, alanine aminotransferase; ALL, acute lymphatic leukemia; ML, malignant lymphoma; NHL, non-Hodgkin lymphoma

* discovery study that was identified in systematic literature search

[a] As reported by the authors

[b] Coefficient (95% CI)

[c] p=0.043 in t-test, but results from regression analysis are reported in the table for consistency

References - Table S8, S9, S10

1. Caronia D, Patino-Garcia A, Perez-Martinez A, Pita G, Moreno LT, Zalacain-Diez M, et al. Effect of ABCB1 and ABCC3 polymorphisms on osteosarcoma survival after chemotherapy: a pharmacogenetic study. PLoS One. 2011;6(10):e26091.
2. Liu S, Yi Z, Ling M, Shi J, Qiu Y, Yang S. Predictive potential of ABCB1, ABCC3, and GSTP1 gene polymorphisms on osteosarcoma survival after chemotherapy. Tumour Biol. 2014;35(10):9897-904.
3. Windsor RE, Strauss SJ, Kallis C, Wood NE, Whelan JS. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. Cancer. 2012;118(7):1856-67.
4. Yang J, Wang ZG, Cai HQ, Li YC, Xu YL. Effect of variation of ABCB1 and ABCC3 genotypes on the survival of bone tumor cases after chemotherapy. Asian Pac J Cancer Prev. 2013;14(8):4595-8.
5. Li JZ, Tian ZQ, Jiang SN, Feng T. Effect of variation of ABCB1 and GSTP1 on osteosarcoma survival after chemotherapy. Genet Mol Res. 2014;13(2):3186-92.
6. Hattinger CM, Biason P, Iacoboni E, Gagno S, Fanelli M, Tavanti E, et al. Candidate germline polymorphisms of genes belonging to the pathways of four drugs used in osteosarcoma standard chemotherapy associated with risk, survival and toxicity in non-metastatic high-grade osteosarcoma. Oncotarget. 2016;7(38):61970-87.
7. Goričar K, Kovač V, Jazbec J, Zakotnik B, Lamovec J, Dolžan V. Influence of the folate pathway and transporter polymorphisms on methotrexate treatment outcome in osteosarcoma. Pharmacogenet Genomics. 2014;24(10):514-21.
8. Hagleitner MM, Coenen MJ, Gelderblom H, Makkinje RR, Vos HI, de Bont ES, et al. A First Step toward Personalized Medicine in Osteosarcoma: Pharmacogenetics as Predictive Marker of Outcome after Chemotherapy-Based Treatment. Clin Cancer Res. 2015;21(15):3436-41.
9. Xu L, Xia C, Sun Q, Sheng F, Xiong J, Wang S. Variants of FasL and ABCC5 are predictive of outcome after chemotherapy-based treatment in osteosarcoma. J Bone Oncol. 2018;12:44-8.
10. Bhuvaneshwar K, Harris M, Gusev Y, Madhavan S, Iyer R, Vilboux T, et al. Genome sequencing analysis of blood cells identifies germline haplotypes strongly associated with drug resistance in osteosarcoma patients. BMC Cancer. 2019;19(1):357.
11. Koster R, Panagiotou OA, Wheeler WA, Karlins E, Gastier-Foster JM, Caminada de Toledo SR, et al. Genome-wide association study identifies the GLDC/IL33 locus associated with survival of osteosarcoma patients. Int J Cancer. 2018;142(8):1594-601.
12. Lin Q, Han J, Sun Q, Wen L, Wang S. Functional variant of IL33 is associated with survival of osteosarcoma patients. J Bone Oncol. 2020;20:100270.
13. Yang LM, Li XH, Bao CF. Glutathione S-transferase P1 and DNA polymorphisms influence response to chemotherapy and prognosis of bone tumors. Asian Pacific journal of cancer prevention : APJCP. 2012;13(11):5883-6.
14. Zhang SL, Mao NF, Sun JY, Shi ZC, Wang B, Sun YJ. Predictive potential of glutathione S-transferase polymorphisms for prognosis of osteosarcoma patients on chemotherapy. Asian Pac J Cancer Prev. 2012;13(6):2705-9.
15. Teng JW, Yang ZM, Li J, Xu B. Predictive role of Glutathione S-transferases (GSTs) on the prognosis of osteosarcoma patients treated with chemotherapy. Pak J Med Sci. 2013;29(5):1182-6.
16. Jabeen S, Holmboe L, Alnaes GI, Andersen AM, Hall KS, Kristensen VN. Impact of genetic variants of RFC1, DHFR and MTHFR in osteosarcoma patients treated with high-dose methotrexate. Pharmacogenomics J. 2015;15(5):385-90.

17. Hildebrandt MAT, Reyes M, Wu X, Pu X, Thompson KA, Ma J, et al. Hypertension Susceptibility Loci are Associated with Anthracycline-related Cardiotoxicity in Long-term Childhood Cancer Survivors. Sci Rep. 2017;7(1):9698.

18. Wang X, Sun CL, Quiñones-Lombraña A, Singh P, Landier W, Hageman L, et al. CELF4 Variant and Anthracycline-Related Cardiomyopathy: A Children's Oncology Group Genome-Wide Association Study. J Clin Oncol. 2016;34(8):863-70.

19. Leger KJ, Cushing-Haugen K, Hansen JA, Fan W, Leisenring WM, Martin PJ, et al. Clinical and Genetic Determinants of Cardiomyopathy Risk among Hematopoietic Cell Transplantation Survivors. Biol Blood Marrow Transplant. 2016;22(6):1094-101.

20. Ruiz-Pinto S, Pita G, Patiño-García A, Alonso J, Pérez-Martínez A, Cartón AJ, et al. Exome array analysis identifies GPR35 as a novel susceptibility gene for anthracycline-induced cardiotoxicity in childhood cancer. Pharmacogenet Genomics. 2017;27(12):445-53.

21. Wang X, Liu W, Sun CL, Armenian SH, Hakonarson H, Hageman L, et al. Hyaluronan synthase 3 variant and anthracycline-related cardiomyopathy: a report from the children's oncology group. J Clin Oncol. 2014;32(7):647-53.

22. Sági JC, Egyed B, Kelemen A, Kutszegi N, Hegyi M, Gézsi A, et al. Possible roles of genetic variations in chemotherapy related cardiotoxicity in pediatric acute lymphoblastic leukemia and osteosarcoma. BMC Cancer. 2018;18(1):704.

23. Aminkeng F, Bhavsar AP, Visscher H, Rassekh SR, Li Y, Lee JW, et al. A coding variant in RARG confers susceptibility to anthracycline-induced cardiotoxicity in childhood cancer. Nat Genet. 2015;47(9):1079-84.

24. Serie DJ, Crook JE, Necela BM, Dockter TJ, Wang X, Asmann YW, et al. Genome-wide association study of cardiotoxicity in the NCCTG N9831 (Alliance) adjuvant trastuzumab trial. Pharmacogenet Genomics. 2017;27(10):378-85.

25. Schneider BP, Shen F, Gardner L, Radovich M, Li L, Miller KD, et al. Genome-Wide Association Study for Anthracycline-Induced Congestive Heart Failure. Clin Cancer Res. 2017;23(1):43-51.

26. Park B, Sim SH, Lee KS, Kim HJ, Park IH. Genome-wide association study of genetic variants related to anthracycline-induced cardiotoxicity in early breast cancer. Cancer Sci. 2020;111(7):2579-87.

27. Visscher H, Rassekh SR, Sandor GS, Caron HN, van Dalen EC, Kremer LC, et al. Genetic variants in SLC22A17 and SLC22A7 are associated with anthracycline-induced cardiotoxicity in children. Pharmacogenomics. 2015;16(10):1065-76.

28. Visscher H, Ross CJ, Rassekh SR, Barhdadi A, Dubé MP, Al-Saloos H, et al. Pharmacogenomic prediction of anthracycline-induced cardiotoxicity in children. J Clin Oncol. 2012;30(13):1422-8.

29. Visscher H, Ross CJ, Rassekh SR, Sandor GS, Caron HN, van Dalen EC, et al. Validation of variants in SLC28A3 and UGT1A6 as genetic markers predictive of anthracycline-induced cardiotoxicity in children. Pediatr Blood Cancer. 2013;60(8):1375-81.

30. Vulsteke C, Pfeil AM, Maggen C, Schwenkglenks M, Pettengell R, Szucs TD, et al. Clinical and genetic risk factors for epirubicin-induced cardiac toxicity in early breast cancer patients. Breast Cancer Res Treat. 2015;152(1):67-76.

31. Reichwagen A, Ziepert M, Kreuz M, Godtel-Armbrust U, Rixecker T, Poeschel V, et al. Association of NADPH oxidase polymorphisms with anthracycline-induced cardiotoxicity in the RICOVER-60 trial of patients with aggressive CD20(+) B-cell lymphoma. Pharmacogenomics. 2015;16(4):361-72.

32. Hertz DL, Caram MV, Kidwell KM, Thibert JN, Gersch C, Seewald NJ, et al. Evidence for association of SNPs in ABCB1 and CBR3, but not RAC2, NCF4, SLC28A3 or TOP2B, with chronic cardiotoxicity in a cohort of breast cancer patients treated with anthracyclines. Pharmacogenomics. 2016;17(3):231-40.

33. Hegyi M, Arany A, Semsei AF, Csordas K, Eipel O, Gezsi A, et al. Pharmacogenetic analysis of high-dose methotrexate treatment in children with osteosarcoma. Oncotarget. 2017;8(6):9388-98.

34. Hurkmans EGE, Klumpers MJ, Vermeulen SH, Hagleitner MM, Flucke U, Schreuder HWB, et al. Analysis of Drug Metabolizing Gene Panel in Osteosarcoma Patients Identifies Association Between Variants in SULT1E1, CYP2B6 and CYP4F8 and Methotrexate Levels and Toxicities. Front Pharmacol. 2020;11:1241.

35. Sharifi MJ, Bahoush G, Zaker F, Ansari S, Rafsanjani KA, Sharafi H. Association of -24CT, 1249GA, and 3972CT ABCC2 gene polymorphisms with methotrexate serum levels and toxic side effects in children with acute lymphoblastic leukemia. Pediatr Hematol Oncol. 2014;31(2):169-77.

36. Gervasini G, de Murillo SG, Jimenez M, de la Maya MD, Vagace JM. Effect of polymorphisms in transporter genes on dosing, efficacy and toxicity of maintenance therapy in children with acute lymphoblastic leukemia. Gene. 2017;628:72-7.

37. Khrunin AV, Moisseev A, Gorbunova V, Limborska S. Genetic polymorphisms and the efficacy and toxicity of cisplatin-based chemotherapy in ovarian cancer patients. Pharmacogenomics J. 2010;10(1):54-61.

38. Khrunin A, Ivanova F, Moisseev A, Khokhrin D, Sleptsova Y, Gorbunova V, et al. Pharmacogenomics of cisplatin-based chemotherapy in ovarian cancer patients of different ethnic origins. Pharmacogenomics. 2012;13(2):171-8.

39. Lopes-Aguiar L, Costa EF, Nogueira GA, Lima TR, Visacri MB, Pincinato EC, et al. XPD c.934G>A polymorphism of nucleotide excision repair pathway in outcome of head and neck squamous cell carcinoma patients treated with cisplatin chemoradiation. Oncotarget. 2017;8(10):16190-201.

40. Zazuli Z, Otten LS, Drogemoller BI, Medeiros M, Monzon JG, Wright GEB, et al. Outcome Definition Influences the Relationship Between Genetic Polymorphisms of ERCC1, ERCC2, SLC22A2 and Cisplatin Nephrotoxicity in Adult Testicular Cancer Patients. Genes (Basel). 2019;10(5).

41. Garcia SL, Lauritsen J, Zhang Z, Bandak M, Dalgaard MD, Nielsen RL, et al. Prediction of Nephrotoxicity Associated With Cisplatin-Based Chemotherapy in Testicular Cancer Patients. JNCI Cancer Spectr. 2020;4(3):pkaa032.

42. Tibaldi C, Giovannetti E, Vasile E, Mey V, Laan AC, Nannizzi S, et al. Correlation of CDA, ERCC1, and XPD polymorphisms with response and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. Clin Cancer Res. 2008;14(6):1797-803.

43. Araoz HV, D'Aloi K, Foncuberta ME, Sanchez La Rosa CG, Alonso CN, Chertkoff L, et al. Pharmacogenetic studies in children with acute lymphoblastic leukemia in Argentina. Leuk Lymphoma. 2015;56(5):1370-8.

44. Erculj N, Kotnik BF, Debeljak M, Jazbec J, Dolzan V. Influence of folate pathway polymorphisms on high-dose methotrexate-related toxicity and survival in childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2012;53(6):1096-104.

45. Huang L, Tissing WJ, de Jonge R, van Zelst BD, Pieters R. Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Leukemia. 2008;22(9):1798-800.

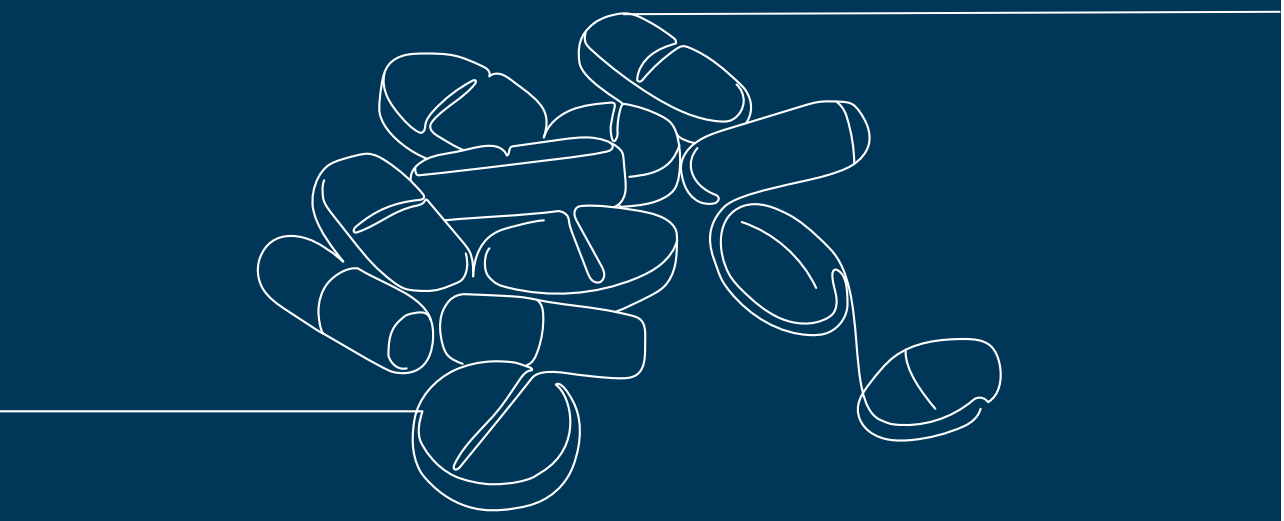
46. Kantar M, Kosova B, Cetingul N, Gumus S, Toroslu E, Zafer N, et al. Methylenetetrahydrofolate reductase C677T and A1298C gene polymorphisms and therapy-related toxicity in children treated for acute lymphoblastic leukemia and non-Hodgkin lymphoma. Leuk Lymphoma. 2009;50(6):912-7.

47. Karathanasis NV, Stiakaki E, Goulielmos GN, Kalmanti M. The role of the methylenetetrahydrofolate reductase 677 and 1298 polymorphisms in Cretan children with acute lymphoblastic leukemia. Genet Test Mol Biomarkers. 2011;15(1-2):5-10.

48. Liu SG, Li ZG, Cui L, Gao C, Li WJ, Zhao XX. Effects of methylenetetrahydrofolate reductase gene polymorphisms on toxicities during consolidation therapy in pediatric acute lymphoblastic leukemia in a Chinese population. Leuk Lymphoma. 2011;52(6):1030-40.

49. Yousef AM, Farhad R, Alshamaseen D, Alsheikh A, Zawiah M, Kadi T. Folate pathway genetic polymorphisms modulate methotrexate-induced toxicity in childhood acute lymphoblastic leukemia. Cancer Chemother Pharmacol. 2019;83(4):755-62.

50. van Kooten Niekerk PB, Schmiegelow K, Schroeder H. Influence of methylene tetrahydrofolate reductase polymorphisms and coadministration of antimetabolites on toxicity after high dose methotrexate. Eur J Haematol. 2008;81(5):391-8.
51. Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolzan V, Jazbec J. Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. Eur J Clin Pharmacol. 2011;67(10):993-1006.
52. Haase R, Elsner K, Merkel N, Stiefel M, Mauz-Korholz C, Kramm CM, et al. High dose methotrexate treatment in childhood ALL: pilot study on the impact of the MTHFR 677C>T and 1298A>C polymorphisms on MTX-related toxicity. Klin Padiatr. 2012;224(3):156-9.
53. Suthandiram S, Gan GG, Zain SM, Bee PC, Lian LH, Chang KM, et al. Effect of polymorphisms within methotrexate pathway genes on methotrexate toxicity and plasma levels in adults with hematological malignancies. Pharmacogenomics. 2014;15(11):1479-94.
54. Milosevic G, Kotur N, Lazic J, Krstovski N, Stankovic B, Zukic B, et al. Influence of variants in folate metabolism genes on 6-mercaptopurine induced toxicity during treatment for childhood acute lymphocytic leukemia. J BUON. 2019;24(5):2075-83.
55. El-Khodary NM, El-Haggar SM, Eid MA, Ebeid EN. Study of the pharmacokinetic and pharmacogenetic contribution to the toxicity of high-dose methotrexate in children with acute lymphoblastic leukemia. Med Oncol. 2012;29(3):2053-62.
56. Xu L, Wang L, Xue B, Wang S. MTHFR variant is associated with high-dose methotrexate-induced toxicity in the Chinese osteosarcoma patients. J Bone Oncol. 2018;13:143-7.
57. Chae H, Kim M, Choi SH, Kim SK, Lee JW, Chung NG, et al. Influence of plasma methotrexate level and MTHFR genotype in Korean paediatric patients with acute lymphoblastic leukaemia. J Chemother. 2020;32(5):251-9.



CHAPTER 3

Analysis of drug metabolizing gene panel in osteosarcoma patients identifies association between variants in *SULT1E1*, *CYP2B6* and *CYP4F8* and methotrexate levels and toxicities

Evelien G.E. Hurkmans[1#], Marije J. Klumpers[2#], Sita H. Vermeulen[3], Melanie M. Hagleitner[4], Uta Flucke[5], H.W. Bart Schreuder[6], Hans Gelderblom[7], Johannes Bras[8], Henk-Jan Guchelaar[9], Marieke J. H. Coenen[1]*, D. Maroeska W. M. te Loo[10]*

[1]Radboud Institute for Health Sciences, Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands

[2]Radboud Institute for Molecular Life Sciences, Department of Pediatrics, Radboud university medical center, Nijmegen, The Netherlands

[3]Radboud Institute for Health Sciences, Department for Health Evidence, Radboud university medical center, Nijmegen, The Netherlands

[4]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands

[5]Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands

[6]Department of Orthopedic Surgery, Radboud university medical center, Nijmegen, The Netherlands

[7]Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands

[8]Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

[9]Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, The Netherlands

[10]Radboud Institute for Health Sciences, Department of Pediatrics, Radboud university medical center, Nijmegen, The Netherlands

[#] both authors contributed equally

* both authors contributed equally

Published in Frontiers of Pharmacology

Abstract

High-dose methotrexate is a cornerstone agent in the chemotherapeutic treatment of patients with osteosarcoma. However, patients often develop methotrexate-induced toxicities. We aim to identify determinants of methotrexate-induced toxicities in osteosarcoma patients by investigating the relation between drug plasma levels, methotrexate-induced toxicities, and germline variants in genes related to drug absorption, distribution, metabolism and elimination.

A cohort of 114 osteosarcoma patients was genotyped for 1,931 variants in 231 genes using the Drug Metabolism Enzymes and Transporters Plus array. Methotrexate plasma levels and laboratory measurements during and after high-dose methotrexate treatment concerning renal function, liver damage and myelopoiesis to reflect toxicity outcomes were obtained. 113 patients and a subset of 545 variants in 176 genes passed quality control checks. Methotrexate plasma levels showed associations with creatinine, alanine aminotransferase, and hemoglobin.

Genetic variant rs3736599 in the 5'-untranslated region of *SULT1E1* was associated with lower 48 hour methotrexate plasma levels (coef -0.313 [95% CI -0.459 — -0.167]; $p=2.60\times10^{-5}$). Association with methotrexate-induced decreased thrombocyte counts was found for two intronic variants in *CYP2B6* (rs4803418 (coef -0.187 [95% CI -0.275 — -0.099]; $p=3.04\times10^{-5}$) and rs4803419 (coef -0.186 [95% CI -0.278 — -0.093]; $p=8.80\times10^{-5}$)). An association with increased thrombocyte counts was identified for the intronic variant rs4808326 in *CYP4F8* (coef 0.193 [95% CI 0.099 — 0.287]; $p=6.02\times10^{-5}$). Moreover, a secondary analysis with a binary approach using CTCAE toxicity criteria, resulted in a nominal significant associations ($p<0.05$) for two out of three variants (rs4803418 and rs4808326).

This is the first study to identify genetic variants in *SULT1E1, CYP2B6* and *CYP4F8* to be associated with methotrexate pharmacokinetics and toxicities. Validation of these variants in an independent cohort and further functional investigation of variants in the identified genes is needed to determine if and how they affect methotrexate plasma levels and the development of methotrexate-induced toxicities.

Introduction

Methotrexate (MTX) is an antifolate agent widely used in oncologic treatment. In high doses (≥1 g/m²), MTX is used as a cornerstone agent in the chemotherapeutic treatment of osteosarcoma, the most common primary bone tumor in children and adolescents (1, 2). Introduction of chemotherapeutic treatment with a regimen of the agents doxorubicin, cisplatin and high dose (HD-)MTX in osteosarcoma has resulted in tremendous increase of patients' survival rates compared to surgery alone (5-year overall survival up to 70% compared to <20%, respectively) (3-6). Despite its contribution to improved prognosis, HD-MTX treatment can however lead to harmful toxicities including renal toxicity, myelosuppression and liver damage (1, 7, 8). These HD-MTX-related toxicities can occur despite appropriate leucovorin rescue, intensive hydration and monitoring of drug plasma levels. Clinical factors, such as age and kidney function, are known to contribute to the risk of developing toxicities but do not sufficiently explain all interpatient variation (7, 9). Pharmacogenetic studies aim to fill the gap of unexplained interpatient variability in drug response by investigating how genetic variants affect relevant traits such as drug plasma levels and drug-related adverse events.

Studies investigating the impact of genetic variants on MTX pharmacokinetics and toxicities have already shown significant results. A genome-wide association study (GWAS) in patients with acute lymphoblastic leukemia (ALL) showed that genetic variants in *SLCO1B1* are associated with MTX clearance (10). This association has been replicated by others (11-17). Osteosarcoma patients receive a substantial higher cumulative dose of MTX compared to patients with ALL (12 g/m² compared to 2 g/m² in most treatment protocols, respectively). This is particularly relevant as important differences are described between high and lower MTX dosages concerning cellular transport pathways (18). Therefore, one needs to be precautious with direct generalization of pharmacogenetic associations found in other malignancies or other low dose MTX-treated traits such as autoimmune diseases to osteosarcoma. In osteosarcoma, only candidate gene studies have been performed to identify relevant pharmacogenetic variants, resulting in the identification of statistically significantly associations between variants in *MTHFR*, *MTR* and *ABCB1*, and MTX pharmacokinetics, toxicities and survival rates (19-23). Previously, our group investigated the role of a genetic variant in *MTHFR* (rs1801133) in MTX-induced liver toxicity in patients with osteosarcoma and ALL(24). The present study follows up on that in a larger cohort, and looking beyond candidate genes by exploring a broad panel of variants in genes involved in drug absorption, distribution, metabolism and excretion (ADME). This panel contained part of the previously identified genetic variants in MTX pathways, but also provided us with a broader and unbiased view on the contribution of ADME gene variation on variability in HD-MTX response. This study focused on MTX plasma levels as well as laboratory markers for HD-MTX-induced renal toxicity, liver damage, and bone marrow toxicity in patients with osteosarcoma.

Materials and methods

Patients and treatment

The study cohort consisted of 114 patients diagnosed with primary, high-grade osteosarcoma, who were treated between 2003 and 2014 at (pediatric) oncology departments of four Dutch hospitals (Radboud university medical center; Leiden University Medical Center; Academic Medical Center Amsterdam; University Medical Center Groningen). Inclusion criteria were: age ≤ 45 years, self-reported Caucasian ethnicity, and treatment according to the EURAMOS-1 protocol (25). The current study was approved by the institutional review board of the Radboud university medical center (Commissie Mensgebonden Onderzoek Regio Arnhem Nijmegen), and approval for inclusion of patients in other institutes was obtained from institutional ethics committees. Written informed consent was acquired from all patients and/or their parents. According to the treatment protocol, patients received a maximum of 12 courses HD-MTX (12 g/m² per course) as a 4 hour infusion, together with adequate hydration and urinary alkalinization. Leucovorin rescue (15 mg/m²) was started 24-28 hours after start of MTX infusion and was prolonged if MTX plasma levels were >0.40 μmol/L at 48 hours. In addition to HD-MTX, chemotherapeutic treatment consisted of doxorubicin (maximum cumulative dose: 450 mg/m²) and cisplatin (maximum cumulative dose: 480 mg/m²), with or without additional ifosfamide/etoposide or interferon-α depending on randomized EURAMOS-1 treatment arm. None of the patients received trimethoprim-sulfamethoxazole during treatment due to its interaction with MTX.

MTX plasma levels and toxicity data

MTX plasma levels and laboratory results of MTX-induced toxicities after each course of MTX were retrospectively collected from electronic medical records. MTX plasma levels were routinely measured at 48 hours after initiation of MTX infusion by a fluorescence polarization immunoassay (TDx/FLx, Abott Diagnostics, The Hague, The Netherlands) or enzyme immunoassay (for three patients) (Syva Emit TDM assay, Siemens Healthcare, Hoofddorp, The Netherlands), without differences in reference value. For renal toxicity, creatinine plasma levels at 48 hours and after one week (ranging from day five until day nine) after start of MTX infusion were collected, in order to assess both the acute and later effects of MTX on renal cells. To analyze liver damage, plasma levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) at 48 hours were obtained. For bone marrow toxicity, levels of hemoglobin and counts of leukocytes, thrombocytes and neutrophils were collected, approximately one week (ranging from day five until day nine) after MTX infusion.

Genotyping

Germline DNA was isolated from blood (n=54) using the QIAamp DNA Blood Midi kit (Qiagen, Venlo, The Netherlands) or from saliva (n=53) using the Oragene saliva collection kit (DNA Genotek, Kanata, Ontario, Canada) according to the manufacturer's protocols. For patients who had passed away before study inclusion (n=7), germline DNA was isolated

from normal formalin-fixed paraffin-embedded bone tissue as described previously (26). DNA samples were genotyped for 1,936 genetic variants across 231 genes involved in drug absorption, distribution, metabolism and excretion, using the DMET Plus array (Affymetrix UK Ltd, High Wycombe, UK) according to the manufacturer's instructions. Genotypes were determined with DMET console software version 1.3 (Affymetrix UK Ltd, High Wycombe, UK) using the Dynamic Genotype Boundaries algorithm version 2. After exclusion of copy number variants, X-chromosomal variants and tri-allelic variants present on the array, quality control (QC) was performed. Variants with unreliable cluster plots, i.e. plots without distinct cluster boundaries, were excluded. Further QC consisted of exclusion of variants with a call rate below 0.90, a minor allele frequency (MAF) below 0.05, and variants, which deviated from the Hardy Weinberg equilibrium (HWE $p<0.0001$), and exclusion of samples with a call rate below 0.90.

Statistical analyses with continuous outcomes

First, analyses using continuous outcomes for MTX levels and toxicities were performed. After assessing normality of the data, Spearman's rank-order correlation (r_s) was used to assess the correlation between MTX plasma levels and toxicity markers at different time points during treatment. Genetic association analyses for MTX plasma levels and toxicity markers were performed using Generalized Estimating Equation (GEE) analysis, an extended linear regression analysis in which correlations among repeated measurements obtained from individual subjects over time are taken into account. Sex, age (at diagnosis) and cumulative MTX dose were tested for association with MTX plasma levels and toxicity markers using GEE analysis. Variables that showed an association at $p<0.05$ were included as a covariate in multivariate genetic association analyses to reduce the variation explained by these covariates on the outcome variables, and thereby increase the precision of the estimate of the effects of the genetic variants. A description of included covariates in analysis of each toxicity marker is provided in the Supplementary (*Table S3-S10*). Creatinine plasma level one week after MTX infusion was included as a covariate in all association analyses (except in analyses of renal toxicity) to adjust for decreased renal clearance of MTX. Prior to the GEE analysis, MTX plasma levels, and levels of thrombocytes, leukocytes, neutrophils, ALAT and ASAT were log-transformed to obtain normal distributions. Association analyses were performed using family(gaussian), link(identity), correlation(exchangeable) and vce(robust) as options of the *xtgee* command in STATA. Regression coefficients and corresponding 95% confidence intervals were generated for each genetic variant under the assumption of an additive genetic model. Associations were considered statistically significant if they surpassed the Bonferroni-corrected p-value threshold (p=0.05 divided by the number of variants including in the analyses after QC). Genetic association analyses were performed using STATA version 11.2 (Stata corporation, College Station, TX, USA), all other analyses using IBM SPSS Statistics version 22 (SPSS Inc., Chicago, Ill, USA).

Statistical analyses with binary outcomes

To further assess the clinical relevance of genetic associations, additional analyses was performed treating the toxicity markers as binary (case-control) outcomes. Grading of laboratory results was performed according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 of the National Cancer Institute (*Table S11*) (27). Reference values for the laboratory results according to the hospital of inclusion, were used as 'upper limit of normal' and 'lower limit of normal'. The values of the CTCAE grades for each toxicity outcome were evaluated to determine a clinical relevant cut-off for case-control designation. For the CTCAE terms 'Alanine aminotransferase increased', 'Aspartate aminotransferase increased', 'White blood cell decreased' and 'Anemia', grade 0 and 1 were considered controls and grade 2, 3 and 4 (and if applicable, grade 5) were considered cases. For 'Neutrophil count decreased', grade 0, 1 and 2 were assigned as controls and grade 3 and 4 as cases. For 'Platelet count decreased', patients with grade 0 were considered controls, and patients with grade 1, 2, 3 and 4 were considered cases. Performing an association analyses with CTCAE graded outcomes was not feasible for renal damage, as over 95% of all creatinine measurements were graded as grade 0 according to CTCAE term 'Creatinine increased'. Logistic multivariate GEE analysis was performed, including covariates that showed an association ($p<0.05$) with the graded toxicity endpoint (described in *Table S3-S10*). Also, it was performed using family(binomial), link(logit), correlation(exchangeable) and vce(robust) as options of the *xtgee* command in STATA. Other than that, the same approach was used as the analyses of continuous outcomes (as descripted above).

Results

Patient characteristics

Characteristics of the included osteosarcoma patients are provided in *Table 1*. The patients received a total of 1,256 MTX courses. All patients were treated according to the EURAMOS-1 protocol. 42.0% of the patients received a chemotherapeutic regimen containing methotrexate, doxorubicin and cisplatin (MAP), 13.3% of the patients received additional ifosfamide and etoposide (MAPie), and 10.6% received additional interferon-alfa (MAPinf). 89 patients (78.8%) received all 12 courses according to protocol. Reasons for cancelled courses included ineffective treatment, poor physical conditions of the patient (whether or not caused by treatment toxicities), a patient's own request, or death.

Table 1. Characteristics of 113 osteosarcoma patients.

Age at diagnosis in years, median (range)	15.0 (5.6 – 43.0)
Sex, number of males (%)	64 (56.6)
Self-reported ethnicity, number of Caucasians (%)	113 (100)
Metastases present at diagnosis, number of patients (%)	20 (17.7)
Treatment protocol, number of patients in EURAMOS-1 (%)	113 (100)
EURAMOS-1 treatment arm, number of patients (%)	
MAP	50 (44.2)
MAPie	15 (13.3)
MAPifn	12 (10.6)
Personalized	4 (3.5)
Unknown	32 (28.3)
MTX cumulative dose (12 g/m^2 per course), number of patients	
12 – 72 g/m^2	8
84 – 132 g/m^2	16
144 g/m^2	89

MAP: regimen of methotrexate, doxorubicin, cisplatin
MAPie: regimen of methotrexate, doxorubicin, cisplatin, ifosfamide, etoposide
MAPinf: regimen of methotrexate, doxorubicin, cisplatin, interferon-alfa

Table 2. Characteristics of laboratory markers of nephrotoxicity, liver damage, and bone marrow toxicity in included osteosarcoma patients.

Measurement in plasma	**Mean (SD) or median (range)***	**Number of datapoints (of number of patients)**
MTX level (t=48 hours), µmol/L	0.25 (0.05 – 82.34)	1238 (113)
Creatinine levels (t=48 hours), µmol/L	48.2 (15.1)	551 (91)
Creatinine levels (t=week 1), µmol/L	51.7 (17.9)	857 (110)
ALAT levels (t=48 hours), U/L	260 (10 – 4,677)	475 (87)
ASAT levels (t=48 hours), U/L	106 (11 – 4,420)	461 (87)
Hemoglobin levels (t=week 1), mmol/L	6.5 (0.8)	908 (112)
Thrombocyte counts (t=week 1), x 10^3 /mm^3	184 (10 – 740)	896 (112)
Leukocyte counts (t=week 1), x 10^9 /L	4.4 (0.8 – 38.2)	905 (112)
Neutrophil counts (t=week 1), x 10^9 /L	2.3 (0.0 – 35.8)	709 (109)

* Mean and standard deviation (SD) for normally distributed data, median and range for other.

Genotyping

Copy number variants (n=5), tri-allelic variants (n=1) and X-chromosomal variants (n=46) were not included in the analysis. QC resulted in exclusion of 90 variants due to unreliable cluster plots, 26 variants because of low call rate, 1,222 variants due to MAF<0.05, and one variant which deviated from HWE. One patient was excluded because of low overall sample call rate. A total of 113 patients and 545 variants were included for genetic association analyses (see *Table S1* for the included variants). The Bonferroni corrected *p*-value threshold was $< 0.05 / 545 = 9.2\times10^{-5}$.

MTX plasma levels

Median MTX plasma level 48 hours after MTX infusion was 0.25 µmol/L, ranging from 0.05 to 82.34 µmol/L (1,238 datapoints in 113 osteosarcoma patients) (*Table 2*). Weak but statistically significant correlations were found for MTX plasma levels with creatinine levels at t=48 hours (r_s=0.187, p=1.8×10^{-5}) and t=week 1 (r_s=0.163, p=1.8×10^{-6}), hemoglobin levels (r_s=-0.190, p=0.001) and with ALAT levels (r_s=0.102, p=0.03) (*Table 4*). Neither sex, age or cumulative MTX dose were associated with MTX plasma levels (data not shown) and were not included as covariate in the genetic association analyses. 33 genetic variants in 20 genes were associated with MTX plasma levels at p<0.05 (*Table S2*). Variant rs3736599 in *SULT1E1* passed the Bonferroni corrected p-value threshold (G vs. A allele; coef -0.31, (95% CI -0.46 - -0.17), p=2.60 × 10^{-5})(*Table 5*).

Table 3. Characteristics of laboratory markers for liver damage and bone marrow toxicity, graded according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0

CTCAE term	Case-control designation	Cases, n of measurements (%)	Controls, n of measurements (%)
Alanine aminotransferase increased	CTCAE grade 0 - 1 vs. grade 2 - 4	358 (75.4)	177 (24.6)
Aspartate aminotransferase increased	CTCAE grade 0 - 1 vs. grade 2 - 4	237 (51.4)	224 (48.6)
Anemia	CTCAE grade 0 - 1 vs. grade 2 - 5	293 (32.3)	615 (67.7)
Platelet count decreased	CTCAE grade 0 vs. grade 1 - 4	351 (39.2)	545 (60.8)
White blood cell decreased	CTCAE grade 0 - 1 vs. grade 2 - 4	194 (21.4)	711 (78.6)
Neutrophil count decreased	CTCAE grade 0 - 2 vs. grade 3 - 4	87 (12.3)	622 (87.7)

Table 4. Correlation of MTX plasma levels at 48 hours with toxicity markers.

Measurement (in plasma)	Spearman's correlation coefficient	*P*-value
Creatinine level (t=48 hour)	**0.187**	**0.000018**
Creatinine level (t=week 1)	**0.163**	**0.0000018**
ALAT levels (t=48 hour)	0.102	**0.03**
ASAT levels (t=48 hour)	0.085	0.07
Hemoglobin levels (t=week 1)	**-0.109**	**0.001**
Thrombocyte counts (t=week 1)	-0.053	0.12
Leukocyte counts (t=week 1)	0.023	0.50
Neutrophil counts (t=week 1)	-0.033	0.38

Values depicted in bold are statistically significant (*P*-value<0.05)

Toxicity markers

Availability and characteristics of collected toxicity markers are shown in *Table 2* (continuous outcomes) and *Table 3* (binary outcomes). Sex, age and cumulative MTX dose were tested for association with each toxicity marker, and included as a covariate in multivariate genetic association analyses for that respective toxicity marker if associated ($p<0.05$). A description of included covariates in the analysis of each toxicity marker, and an overview of results of genetic association analyses of both continuous and binary outcomes are provided in *Tables S3-S10*.

The results of the genetic association analyses with continuous outcomes showed no genetic variants to be statistically significantly associated ($p<9.2\times10^{-5}$) with creatinine levels, ASAT and ALAT, hemoglobin levels, leukocyte counts and neutrophil counts (*Table S3-S10*). Association analysis of thrombocyte count (as a continuous variable) showed three statistically significantly associated variants in two genes (*Table 5*). These were rs4808326 in *CYP4F8* (G vs. A allele; coef 0.20, (95% CI 0.11-0.29), $p=2.91 \times 10^{-5}$), rs4803418 in *CYP2B6* (C vs. G allele; coef -0.19, (95% CI -0.27 - -0.10), $p=3.04 \times 10^{-5}$) and rs4803419 in *CYP2B6* (C vs. T allele, coef -0.19, (95% CI -0.28 - -0.09), $p=8.80 \times 10^{-5}$).

For the nominally associated genetic variants ($p<0.05$), association analyses using toxicity markers as a binary outcome (using the CTCAE graded toxicity measures) were performed to assess clinical relevance of these findings. This additional analysis was not possible for renal damage, as the vast majority of creatinine measurements were graded as grade 0. The analysis of the other binary outcomes did not yield additional variants surpassing the Bonferroni corrected p-value threshold. The statistically significant associations identified in the analysis with continuous data, being the association between thrombocyte counts and variants in *CYP2B6* and *CYP4F8*, did show a nominal significant association in binary analysis ($p<0.05$) for two of the three identified variants, as depicted in *Table 5* (rs4808326 in *CYP4F8* and rs4803418 in *CYP2B6*).

Table 5. Genetic variants with a statistically significantly associated result ($p<9.2\times10^{-5}$) in analysis with continuous outcomes, and corresponding results from analyses with binary outcomes

Outcome	Gene	Variant	Chr	Position	Minor allele	MAF	Linear multivariate GEE analyses with continuous outcome				Logistic multivariate GEE analyses with binary outcome (CTCAE)			
							Coef	95% CI		P	Coef	95% CI		P
MTX plasma levels	*SULT1E1*	rs3736599	4	70,725,821	A	0.08	-0.31	-0.46	-0.17	0.00003	n.a.	n.a.	n.a.	n.a.
Thrombocyte counts	*CYP2B6*	rs4803418	19	41,511,803	G	0.31	-0.19	-0.27	-0.10	0.00003	0.32	0.01	0.63	0.046
Thrombocyte counts	*CYP4F8*	rs4808326	19	15,726,147	A	0.10	0.19	0.10	0.29	0.00006	-0.97	-1.55	-0.39	0.001
Thrombocyte counts	*CYP2B6*	rs4803419	19	41,512,792	T	0.32	-0.19	-0.28	-0.09	0.00009	0.32	0.00	0.65	0.051

MAF, minor allele frequency; GEE, generalized estimated equations; CTCAE, common terminology criteria of adverse events; MTX, methotrexate; Coef, coefficient; CI, confidence interval; P, p-value; n.a., not applicable.

Discussion

This study identified statistically significant associations between four genetic variants in three drug metabolism genes and pharmacokinetic and toxicity markers derived from routine plasma measurements after HD-MTX infusion in osteosarcoma patients. These novel findings may add to further understanding of variation in the development of HD-MTX induced toxicities in osteosarcoma patients.

A variant in the 5' untranslated region of the *sulfotransferase family 1E member 1* gene (*SULT1E1*, rs3736599) was statistically significantly associated with MTX plasma levels. In this cohort, carriers of the A allele had significantly lower MTX plasma levels at 48 hours compared to carriers of the G allele. *SULT1E1* encodes the enzyme estrogen sulfotransferase, which is mainly responsible for sulfate conjugation of hormones and is known to be involved in metabolism of hormonal therapies (28). *SULT1E1*, or other members of the sulfotransferase family, were not linked to MTX metabolism before, as MTX is not known to undergo sulfate conjugation. Variants in this gene family were also not found to be associated with MTX clearance in the GWAS in 434 patients with ALL by Trevino *et al*. (10). Studies in rats have shown a potential link between MTX and sulfotransferases, as this drug was found to be a xenobiotic inducer of sulfotransferases expressed in the intestine and liver (29, 30). This could implicate a role for sulfotransferases in MTX elimination via the liver and intestines, tissues in which *SULT1E1* is also mainly expressed (31). However, the identified variant in this study (rs3736599) is, due to its location in the 5'UTR region, not known to have a direct impact on SULT1E1 protein function, or to be an expression quantitative trait locus (eQTL), meaning it is not associated to the expression level of *SULT1E1*, or any other gene in any tissue (31).

Three variants in two genes were statistically significantly associated with thrombocyte counts after HD-MTX using continuous outcome measures in a linear regression model, being two variants (rs4803418 and rs4803419) in *cytochrome P450 family 2 subfamily B member 6* (*CYP2B6)* and one variant (rs4808326) in *cytochrome P450 family 4 subfamily F member 8* (*CYP4F8*). Secondary analysis of these variants using a binary approach with CTCAE graded toxicity data resulted in a nominal significant associations ($p<0.05$). Although not surpassing the Bonferroni-corrected p-value threshold (most likely due to decreased power), this result does underline the potential clinical relevance of these findings. This also indicates that the primary analyses, with an approach using continues outcomes, indeed aids in the identification of genetic variants that might have clinical relevance.

The variants in *CYP2B6* (rs4803418 and rs4803419) are located in an intronic region, and are in high linkage disequilibrium ($r^2=0.88$), therefore likely represent the same locus (32). Carriers of the variant alleles (G allele in rs4803418 and T allele in rs4803419) showed lower thrombocyte counts after HD-MTX courses compared to patients carrying the reference alleles. *CYP2B6* is involved in the metabolism of many drugs, e.g. efavirenz, cyclophosphamide and bupropion (33). Both identified (intronic) variants are not known to have an direct effect on protein function, but are significant eQTLs for expression of *CYP2B6* in the transverse colon (31). Interestingly, evidence exists that a missense variant in *CYP2B6* (rs3211371) has an influence on efficacy of the thrombocyte aggregation inhibitor

clopidogrel (34). The underlying mechanism for this association was hypothesized to be a pharmacokinetic effect on clopidogrel metabolism. This association was not replicated by others, nor were associations with other variants in *CYP2B6* identified (35, 36). Both our identified variants were not investigated in relation to clopidogrel or other phenotypes related to thrombocytes. The variants in *CYP2B6* were also nominally associated (p<0.05) with leucocytes and neutrophils (*Table S9* and *S10*, respectively). As variants in *CYP2B6* were not associated to MTX pharmacokinetics in our study or previous studies, one could hypothesize there is a potential effect of the CYP2B6 protein on myelopoiesis. However, *CYP2B6* is expressed mainly in the liver, but not in the bone marrow or whole blood, leaving the underlying mechanism of these associations unclear (31).

The third statistically significant associated variant in our toxicity analyses was the intronic variant rs4808326 in *CYP4F8*. Carriers of the A allele had higher thrombocyte counts after MTX infusion compared to those carrying the G allele. *CYP4F8* codes for an enzyme known to be involved in metabolism of prostaglandins, leukotrienes and long chain fatty acids, and is mainly expressed in prostate and skin (37). This gene is not known to have a prominent role in drug metabolism, and was not linked to MTX or thrombocyte counts before. rs4808326 is a significant eQTL for expression of other CYP4F subfamily members, including *CYP4F12, CYP4F24P* and *CYP4F3*, in multiple tissues (31). However to our knowledge, none of the CYP4F subfamily members were previously implicated in MTX pharmacokinetics or MTX-induced toxicities, nor were these eQTLs present in liver, kidneys or bone marrow. A GWAS on mean platelet volume and thrombocyte count in 66,867 healthy Caucasian individuals by Gieger *et al.* showed no genome-wide significant association with genetic variants in *CYP4F8* (or our other top hits in *CYP2B6*), suggesting that our identified associations were MTX-induced effects (38).

To get more insight in the effect of the identified variants on gene function we used RegulomeDB, which annotates variants with known and predicted regulatory elements (39). Here, multiple public data sources are used to generate a predictive score, where a higher score indicates that a variant is more likely to have regulatory impact. According to this database, the two identified variants in *CYP2B6* are not likely to have a regulatory effect (RegulomeDB score of 0.18 for both variants), whereas the variants in *CYP4F8* and *SULT1E1* have higher score (RegulomeDB scores of 0.52 and 0.61, respectively), suggesting a potential regulatory effect more specifically as indicated by the database via transcription binding sides and DNase peaks.

Genetic variation associated with HD-MTX efficacy or toxicity has been studied in the past. To date, pharmacogenetic studies in this field have resulted in genetic variants annotated with level 2A evidence at highest, meaning there is moderate evidence for the variant-drug combination but no clinical implementation yet (28). These level 2A variants include rs1045642 in *ABCB1*, rs11045879 in *SLCO1B1*, rs1801133 in *MTHFR* and rs1801394 in *MTRR* and rs4673993 in *ATIC* with MTX pharmacokinetics or toxicity outcomes. *MTHFR*, *MTRR* and *ATIC* were not present on the DMET array used in our study. The previously identified variant rs1045642 in *ABCB1* was included in our genetic association analyses, showing no association (p=0.430) with MTX plasma levels, nor with any of the toxicity markers. Multiple candidate gene studies found this variant to be associated to MTX

plasma levels and different MTX-induced toxicities in patients with hematological malignancies (14, 40-43). Potential contributors to contrasting findings are differences in outcomes, different populations with different genetic backgrounds, and different HD-MTX treatment regimens (2 – 5 g/m² in hematological malignancies compared to 12 g/m² in osteosarcoma). A prospective candidate gene study investigating the relation of genetic variants and HD-MTX pharmacokinetics in osteosarcoma patients identified statistically significant associations between MTX clearance and variants in *ABCG2* and *UGT1A* (44). *ABCG2* was not included in this study but the identified variant in *UGT1A* (rs4148324) was in high LD ($r^2 > 0.90$) with three variants in the present study (rs887829, rs111741722, rs10929302), but these variants showed no association with MTX 48-hour plasma levels (using both an additive and dominant model, data not shown). The discrepancy in results is most likely due to major differences in study design and outcome measures.

The only GWAS on MTX-related phenotypes to date identified an association between variant rs11045879 in *SLCO1B1* and MTX clearance in patients with ALL, which was replicated by others (10-17). *SLCO1B1* codes for a transporter known for its function of transporting MTX into the hepatocyte (45). Unfortunately, rs11045879 was not present on the DMET array, so its association with MTX pharmacokinetic and toxicity markers was not directly investigated in our cohort. However, 18 other variants in *SLCO1B1* were present, including variant rs4149056 which is in high LD with rs11045879 (r^2=0.92) (32). We did not identify an association with this variant and MTX 48 hour plasma levels (p=0.211). A possible explanation might be a difference in outcome measure, as the original GWAS used multiple MTX plasma levels to calculate average MTX clearance, compared to MTX 48 hour plasma levels in this study. Additionally, the limited sample size could have resulted in insufficient power to detect this association with a relatively low allele frequency in this cohort (MAF=0.16). Despite the result not being significant, the direction of association is consistent with previous findings, being higher MTX 48 hour plasma levels in case of carrying the (C) allele (coef 0.112) (10-17). Our analyses did show an association with levels of liver enzyme ALAT after HD-MTX infusion (p=0.007, uncorrected for multiple testing) with the variants rs4149056 and rs11045819 in *SLCO1B1*. As this gene is a highly expressed transporter on the basolateral membrane of hepatocytes with MTX as its substrate, one could hypothesize that its activity has an influence on the intracellular accumulation of MTX in hepatocytes, and therefore could facilitate liver damage (45).

MTX induced toxicities typically develop in a dose-dependent fashion. Therefore, correlations were calculated between MTX plasma levels and the different toxicity markers in this study. The observed directions of effects were consistent with expectations, except for the positive correlation with leukocytes. Hemoglobin, thrombocyte and neutrophil counts showed a negative correlation with MTX levels, indicating more bone marrow suppression with higher MTX plasma levels. Creatinine, ALAT and ASAT showed a positive correlation with MTX plasma levels, indicating more nephrotoxicity and hepatotoxicity with higher MTX plasma levels. Some correlations were statistically significant, but all correlations were too weak for clinical relevance. The strongest significant correlation was observed for MTX plasma levels and creatinine levels, 48 hours after MTX infusion. This corresponds with the results of Tsuruwasa *et al.*, as they found significant associations of MTX plasma levels with nephrotoxicity, but not with hepatotoxicity or infections (46).

Taking into account more extensive information, for example measuring metabolite levels (e.g. 7-OH-MTX), might result in more accurate predictions of nephrotoxicity or hepatic toxicity (7, 47).

The present study aimed to gain insight in the relevance of ADME gene variation for MTX metabolism, but had its limitations. Firstly, the power of this study was limited due to a modest number of patients and independent data points. Secondly, this study focused on phenotypes that are routinely measured in clinical practice to obtain reliable repeated measures after each MTX course. These continuous measures give insight in the MTX-induced metabolism changes in a patient, but are no direct measures of toxicities. Some other clinically relevant MTX-induced adverse events, including e.g. mucositis and encephalopathy, were not included in the present study. Reason for this is that these outcomes could not be retrospectively collected in a reliable way. However, regarding the clinical relevance of these adverse events, future prospective studies to investigate these adverse events in more detail will be certainly of value. Finally, osteosarcoma patients receive a multidrug chemotherapeutic regimen, and although the other chemotherapeutic drugs are not given simultaneously with MTX, they could have an impact on laboratory results measured, especially in later stages of the treatment. Drugs with known interactions with MTX, e.g. trimethoprim-sulfamethoxazole, were not administered close to or together with MTX according to protocol. However, other drugs with unknown interactions may have influenced MTX clearance via impaired renal function or may directly influence the laboratory results that were used as outcome measures. These interactions were not mentioned in the protocols and as a consequence, these were not taken into account in this study.

In conclusion, this study identified statistically significant associations for a genetic variant in *SULT1E1* with MTX plasma levels and three genetic variants in *CYP2B6* and *CYP4F8* with thrombocyte counts after HD-MTX infusion in osteosarcoma patients. These variants were not previously described to play a role in MTX pharmacokinetics. Replication of these findings and pinpointing the exact role of these variants and genes in MTX metabolism and development of toxicities will improve understanding the interpatient variability in MTX response and may ultimately provide new opportunities to optimize treatment for osteosarcoma patients.

Acknowledgments

This work is part of the research programme *Computing Time National Computing Facilities Processing Round pilots 2018* with project number 17666, which is (partly) financed by the Dutch Research Council (NWO). This work was carried out on the Dutch national e-infrastructure with the support of SURF Cooperative.

References

1. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983;309(18):1094-104.
2. Treon SP, Chabner BA. Concepts in use of high-dose methotrexate therapy. Clin Chem. 1996;42(8 Pt 2):1322-9.
3. Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5--a population-based study. Lancet Oncol. 2014;15(1):35-47.
4. Hagleitner MM, de Bont ES, Te Loo DM. Survival trends and long-term toxicity in pediatric patients with osteosarcoma. Sarcoma. 2012;2012:636405.
5. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, et al. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med. 1986;314(25):1600-6.
6. Bacci G, Picci P, Ferrari S, Ruggieri P, Casadei R, Tienghi A, et al. Primary chemotherapy and delayed surgery for nonmetastatic osteosarcoma of the extremities. Results in 164 patients preoperatively treated with high doses of methotrexate followed by cisplatin and doxorubicin. Cancer. 1993;72(11):3227-38.
7. Csordas K, Hegyi M, Eipel OT, Muller J, Erdelyi DJ, Kovacs GT. Comparison of pharmacokinetics and toxicity after high-dose methotrexate treatments in children with acute lymphoblastic leukemia. Anticancer Drugs. 2013;24(2):189-97.
8. Rask C, Albertioni F, Bentzen SM, Schroeder H, Peterson C. Clinical and pharmacokinetic risk factors for high-dose methotrexate-induced toxicity in children with acute lymphoblastic leukemia--a logistic regression analysis. Acta Oncol. 1998;37(3):277-84.
9. Cheng DH, Lu H, Liu TT, Zou XQ, Pang HM. Identification of Risk Factors in High-Dose Methotrexate-Induced Acute Kidney Injury in Childhood Acute Lymphoblastic Leukemia. Chemotherapy. 2018;63(2):101-7.
10. Trevino LR, Shimasaki N, Yang W, Panetta JC, Cheng C, Pei D, et al. Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27(35):5972-8.
11. Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, Pinan MA, Garcia-Miguel P, Navajas A, et al. Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. Pediatric blood & cancer. 2011;57(4):612-9.
12. Radtke S, Zolk O, Renner B, Paulides M, Zimmermann M, Moricke A, et al. Germline genetic variations in methotrexate candidate genes are associated with pharmacokinetics, toxicity, and outcome in childhood acute lymphoblastic leukemia. Blood. 2013;121(26):5145-53.
13. Ramsey LB, Panetta JC, Smith C, Yang W, Fan Y, Winick NJ, et al. Genome-wide study of methotrexate clearance replicates SLCO1B1. Blood. 2013;121(6):898-904.
14. Liu SG, Gao C, Zhang RD, Zhao XX, Cui L, Li WJ, et al. Polymorphisms in methotrexate transporters and their relationship to plasma methotrexate levels, toxicity of high-dose methotrexate, and outcome of pediatric acute lymphoblastic leukemia. Oncotarget. 2017;8(23):37761-72.
15. Csordas K, Lautner-Csorba O, Semsei AF, Harnos A, Hegyi M, Erdelyi DJ, et al. 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. British journal of haematology. 2014;166(3):410-20.

16. Zhang HN, He XL, Wang C, Wang Y, Chen YJ, Li JX, et al. Impact of SLCO1B1 521T > C variant on leucovorin rescue and risk of relapse in childhood acute lymphoblastic leukemia treated with high-dose methotrexate. Pediatric blood & cancer. 2014;61(12):2203-7.
17. Goricar K, Kovac V, Jazbec J, Zakotnik B, Lamovec J, Dolzan V. Influence of the folate pathway and transporter polymorphisms on methotrexate treatment outcome in osteosarcoma. Pharmacogenetics and genomics. 2014;24(10):514-21.
18. Schmiegelow K. Advances in individual prediction of methotrexate toxicity: a review. British journal of haematology. 2009;146(5):489-503.
19. Patino-Garcia A, Zalacain M, Marrodan L, San-Julian M, Sierrasesumaga L. Methotrexate in pediatric osteosarcoma: response and toxicity in relation to genetic polymorphisms and dihydrofolate reductase and reduced folate carrier 1 expression. The Journal of pediatrics. 2009;154(5):688-93.
20. Windsor RE, Strauss SJ, Kallis C, Wood NE, Whelan JS. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. Cancer. 2012;118(7):1856-67.
21. Jabeen S, Holmboe L, Alnaes GI, Andersen AM, Hall KS, Kristensen VN. Impact of genetic variants of RFC1, DHFR and MTHFR in osteosarcoma patients treated with high-dose methotrexate. The pharmacogenomics journal. 2015;15(5):385-90.
22. Park JA, Shin HY. Influence of genetic polymorphisms in the folate pathway on toxicity after high-dose methotrexate treatment in pediatric osteosarcoma. Blood research. 2016;51(1):50-7.
23. Lambrecht L, Sleurs C, Labarque V, Dhooge C, Laenen A, Sinnaeve F, et al. The role of the MTHFR C677T polymorphism in methotrexate-induced toxicity in pediatric osteosarcoma patients. Pharmacogenomics. 2017;18(8):787-95.
24. Hagleitner MM, Coenen MJ, Aplenc R, Patino-Garcia A, Chiusolo P, Gemmati D, et al. The role of the MTHFR 677C>T polymorphism in methotrexate-induced liver toxicity: a meta-analysis in patients with cancer. The pharmacogenomics journal. 2014;14(2):115-9.
25. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Ann Oncol. 2015;26(2):407-14.
26. Vos HI, van der Straaten T, Coenen MJ, Flucke U, Te Loo DM, Guchelaar HJ. High-quality genotyping data from formalin-fixed, paraffin-embedded tissue on the drug metabolizing enzymes and transporters plus array. The Journal of molecular diagnostics : JMD. 2015;17(1):4-9.
27. National Cancer Institute PROCSG. Common Terminology Criteria for Adverse Events (CTCAE) 2017 [Version 5.0:[Available from: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_5x7.pdf.
28. Whirl-Carrillo M, McDonagh EM, Hebert JM, Gong L, Sangkuhl K, Thorn CF, et al. Pharmacogenomics knowledge for personalized medicine. Clinical pharmacology and therapeutics. 2012;92(4):414-7.
29. Maiti S, Chen G. Methotrexate is a novel inducer of rat liver and intestinal sulfotransferases. Arch Biochem Biophys. 2003;418(2):161-8.
30. Chen X, Maiti S, Zhang J, Chen G. Nuclear receptor interactions in methotrexate induction of human dehydroepiandrosterone sulfotransferase (hSULT2A1). J Biochem Mol Toxicol. 2006;20(6):309-17.
31. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45(6):580-5.
32. Machiela MJ, Chanock SJ. LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. Bioinformatics. 2015;31(21):3555-7.
33. Zanger UM, Klein K. Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. Front Genet. 2013;4:24.

34. Kassimis G, Davlouros P, Xanthopoulou I, Stavrou EF, Athanassiadou A, Alexopoulos D. CYP2C19*2 and other genetic variants affecting platelet response to clopidogrel in patients undergoing percutaneous coronary intervention. Thromb Res. 2012;129(4):441-6.

35. Cresci S, Depta JP, Lenzini PA, Li AY, Lanfear DE, Province MA, et al. Cytochrome p450 gene variants, race, and mortality among clopidogrel-treated patients after acute myocardial infarction. Circ Cardiovasc Genet. 2014;7(3):277-86.

36. Gonzalez A, Moniche F, Cayuela A, Garcia-Lozano JR, Torrecillas F, Escudero-Martinez I, et al. Effect of CYP2C19 Polymorphisms on the Platelet Response to Clopidogrel and Influence on the Effect of High Versus Standard Dose Clopidogrel in Carotid Artery Stenting. Eur J Vasc Endovasc Surg. 2016;51(2):175-86.

37. Kirischian NL, Wilson JY. Phylogenetic and functional analyses of the cytochrome P450 family 4. Mol Phylogenet Evol. 2012;62(1):458-71.

38. Gieger C, Radhakrishnan A, Cvejic A, Tang W, Porcu E, Pistis G, et al. New gene functions in megakaryopoiesis and platelet formation. Nature. 2011;480(7376):201-8.

39. Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub MA, Kasowski M, et al. Annotation of functional variation in personal genomes using RegulomeDB. Genome research. 2012;22(9):1790-7.

40. Zgheib NK, Akra-Ismail M, Aridi C, Mahfouz R, Abboud MR, Solh H, et al. Genetic polymorphisms in candidate genes predict increased toxicity with methotrexate therapy in Lebanese children with acute lymphoblastic leukemia. Pharmacogenetics and genomics. 2014;24(8):387-96.

41. Suthandiram S, Gan GG, Zain SM, Bee PC, Lian LH, Chang KM, et al. Effect of polymorphisms within methotrexate pathway genes on methotrexate toxicity and plasma levels in adults with hematological malignancies. Pharmacogenomics. 2014;15(11):1479-94.

42. Gregers J, Green H, Christensen IJ, Dalhoff K, Schroeder H, Carlsen N, et al. Polymorphisms in the ABCB1 gene and effect on outcome and toxicity in childhood acute lymphoblastic leukemia. The pharmacogenomics journal. 2015;15(4):372-9.

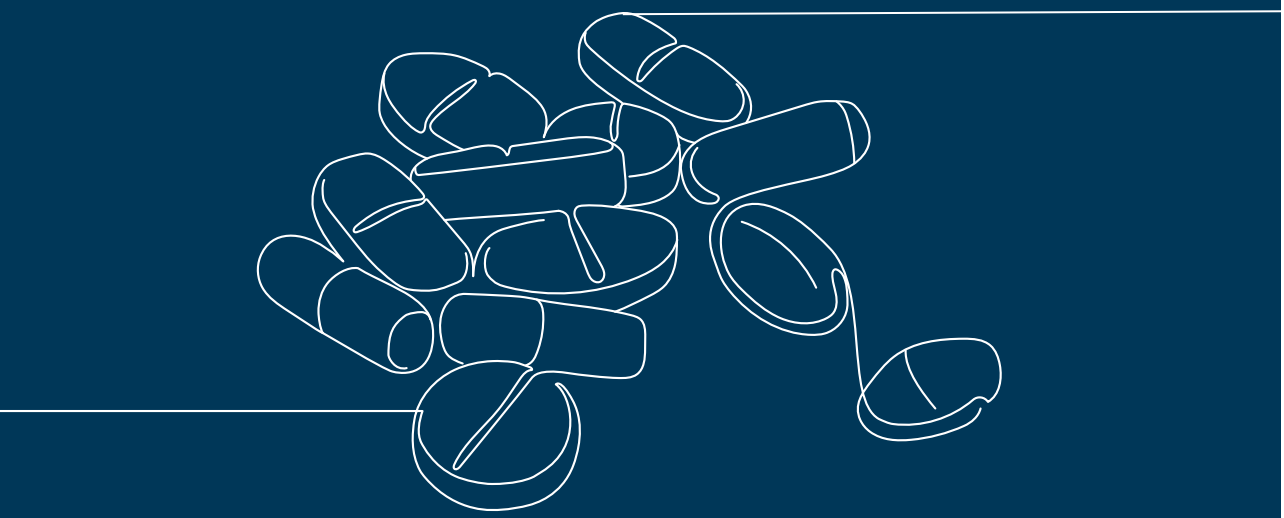
43. Tsujimoto S, Yanagimachi M, Tanoshima R, Urayama KY, Tanaka F, Aida N, et al. Influence of ADORA2A gene polymorphism on leukoencephalopathy risk in MTX-treated pediatric patients affected by hematological malignancies. Pediatric blood & cancer. 2016;63(11):1983-9.

44. Lui G, Treluyer JM, Fresneau B, Piperno-Neumann S, Gaspar N, Corradini N, et al. A Pharmacokinetic and Pharmacogenetic Analysis of Osteosarcoma Patients Treated With High-Dose Methotrexate: Data From the OS2006/Sarcoma-09 Trial. J Clin Pharmacol. 2018;58(12):1541-9.

45. Mikkelsen TS, Thorn CF, Yang JJ, Ulrich CM, French D, Zaza G, et al. PharmGKB summary: methotrexate pathway. Pharmacogenetics and genomics. 2011;21(10):679-86.

46. Tsurusawa M, Gosho M, Mori T, Mitsui T, Sunami S, Kobayashi R, et al. Statistical analysis of relation between plasma methotrexate concentration and toxicity in high-dose methotrexate therapy of childhood nonHodgkin lymphoma. Pediatric blood & cancer. 2015;62(2):279-84.

47. Holmboe L, Andersen AM, Morkrid L, Slordal L, Hall KS. High dose methotrexate chemotherapy: pharmacokinetics, folate and toxicity in osteosarcoma patients. British journal of clinical pharmacology. 2012;73(1):106-14.



CHAPTER 3

Supplementary materials

Analysis of drug metabolizing gene panel in osteosarcoma patients identifies association between variants in *SULT1E1*, *CYP2B6* and *CYP4F8* and methotrexate levels and toxicities

Table S1. 545 genetic variants in 176 genes included in genetic association analyses

Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP
ABCB1	rs10276036	*ADH7*	rs1573496	*CYP19A1*	rs700518	*EPHX1*	rs1051740	*PPARD*	rs1003973	*SLC7A7*	rs1805059
ABCB1	rs1045642	*ADH7*	rs971074	*CYP1A2*	rs2470890	*EPHX1*	rs1051741	*PPARD*	rs1883322	*SLC7A7*	rs1805061
ABCB1	rs1128503	*AHR*	rs10249788	*CYP1A2*	rs35694136	*EPHX1*	rs2292566	*PPARD*	rs2038067	*SLC7A7*	rs2281677
ABCB1	rs17064	*AHR*	rs2066853	*CYP1A2*	rs762551	*EPHX2*	rs41507953	*PPARD*	rs2076167	*SLC7A7*	rs8018462
ABCB1	rs2032588	*AKAP9*	rs2049900	*CYP1B1*	rs1056836	*FAAH*	rs324420	*PPARD*	rs2267665	*SLC7A8*	rs1884545
ABCB1	rs2214102	*AKAP9*	rs7785971	*CYP1B1*	rs1056837	*FMO1*	rs1126692	*PPARD*	rs2267667	*SLC7A8*	rs2236135
ABCB1	rs2235013	*ALB*	rs3756067	*CYP1B1*	rs1800440	*FMO1*	rs12954	*PPARD*	rs2267668	*SLC7A8*	rs2268873
ABCB1	rs2235033	*ALDH1A1*	rs13959	*CYP20A1*	rs1048013	*FMO1*	rs742350	*PPARD*	rs2267669	*SLC7A8*	rs2268877
ABCB1	rs2235040	*ALDH2*	rs886205	*CYP24A1*	rs2296241	*FMO1*	rs7877	*PPARD*	rs6457816	*SLC7A8*	rs7141505
ABCB1	rs3842	*ALDH3A1*	rs887241	*CYP24A1*	rs2762934	*FMO2*	rs2020860	*PPARD*	rs6901410	*SLC7A8*	rs8013529
ABCB1	rs9282564	*AOX1*	rs11678615	*CYP24A1*	rs6068816	*FMO2*	rs2020861	*PPARD*	rs6906237	*SLC7A8*	rs910795
ABCB11	rs2287622	*AOX1*	rs11684227	*CYP26A1*	rs10882140	*FMO2*	rs2020863	*PPARD*	rs6922548	*SLCO1A2*	rs10841795
ABCB11	rs3755163	*AOX1*	rs16834034	*CYP26A1*	rs4418728	*FMO2*	rs2020865	*PPARD*	rs7739752	*SLCO1A2*	rs4078
ABCB11	rs3770602	*AOX1*	rs3731722	*CYP26A1*	rs7905939	*FMO2*	rs2020868	*PPARD*	rs7751481	*SLCO1A2*	rs7957203
ABCB11	rs3770603	*AOX1*	rs6729738	*CYP2A13*	rs1709082	*FMO2*	rs2020869	*PPARG*	rs1152003	*SLCO1B1*	rs11045819
ABCB11	rs4148768	*AOX1*	rs7563682	*CYP2A6*	rs1137115	*FMO2*	rs2020870	*PPARG*	rs1801282	*SLCO1B1*	rs2291075
ABCB11	rs4148771	*APOA2*	rs5085	*CYP2A6*	rs28399433	*FMO2*	rs2307492	*PPARG*	rs3856806	*SLCO1B1*	rs2306283
ABCB11	rs4148771	*ARNT*	rs2228099	*CYP2A6*	rs4803381	*FMO2*	rs28369860	*PPARG*	rs9833097	*SLCO1B1*	rs4149056
ABCB11	rs4148776	*ARSA*	rs131713	*CYP2A6*	rs8192729	*FMO2*	rs7512785	*PTGIS*	rs5629	*SLCO1B1*	rs4149057
ABCB11	rs4668115	*ARSA*	rs743616	*CYP2A7*	rs3869579	*FMO2*	rs7515157	*QPRT*	rs13331798	*SLCO1B3*	rs2053098
ABCB11	rs473351	*ATP7B*	rs1051332	*CYP2B6*	rs2279343	*FMO3*	rs1736557	*QPRT*	rs3862476	*SLCO1B3*	rs3764006
ABCB11	rs495714	*ATP7B*	rs1061472	*CYP2B6*	rs3745274	*FMO3*	rs1800822	*RALBP1*	rs10898	*SLCO1B3*	rs4149117
ABCB11	rs496550	*ATP7B*	rs1801243	*CYP2B6*	rs4803418	*FMO3*	rs2066534	*RALBP1*	rs12680	*SLCO1B3*	rs7311358
ABCB11	rs497692	*ATP7B*	rs1801244	*CYP2B6*	rs4803419	*FMO3*	rs2266780	*RALBP1*	rs3322	*SLCO3A1*	rs1517618

Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP
ABCB11	rs7602171	*ATP7B*	rs1801249	*CYP2B6*	rs8192719	*FMO3*	rs2266782	*RPL13*	rs9930567	*SLCO3A1*	rs2190748
ABCB4	rs1202283	*ATP7B*	rs2277448	*CYP2C18*	rs2281891	*FMO3*	rs909530	*SLC10A1*	rs4646285	*SLCO3A1*	rs2283458
ABCB4	rs2097937	*ATP7B*	rs732774	*CYP2C18*	rs2860840	*FMO4*	rs2223477	*SLC10A2*	rs188096	*SLCO3A1*	rs3743369
ABCB4	rs2109505	*CA5P*	rs11150564	*CYP2C19*	rs12248560	*FMO5*	rs894469	*SLC10A2*	rs2301157	*SLCO3A1*	rs960440
ABCB4	rs2230028	*CA5P*	rs11859842	*CYP2C19*	rs4244285	*FMO6*	rs1736565	*SLC10A2*	rs2301159	*SLCO4A1*	rs2236553
ABCB4	rs2302387	*CA5P*	rs1364182	*CYP2C8*	rs10509681	*FMO6*	rs7886938	*SLC10A2*	rs279941	*SLCO4A1*	rs3787537
ABCB4	rs3747806	*CBR1*	rs1005695	*CYP2C8*	rs1058930	*FMO6*	rs7889839	*SLC10A2*	rs279942	*SLCO5A1*	rs1138541
ABCB4	rs4148805	*CBR1*	rs2835265	*CYP2C8*	rs11572080	*GSTA1*	rs4715332	*SLC10A2*	rs3803258	*SLCO5A1*	rs16936279
ABCB4	rs4148808	*CBR1*	rs3787728	*CYP2D6*	rs1058164	*GSTA2*	rs1803684	*SLC10A2*	rs7987433	*SLCO5A1*	rs3750266
ABCC1	rs212090	*CBR1*	rs998383	*CYP2D6*	rs1065852	*GSTA2*	rs2180314	*SLC13A1*	rs1880179	*SPG7*	rs12960
ABCC1	rs212091	*CBR3*	rs1056892	*CYP2D6*	rs1080983	*GSTA2*	rs6577	*SLC13A1*	rs2140516	*SPG7*	rs2292954
ABCC1	rs246221	*CBR3*	rs2835286	*CYP2D6*	rs1080985	*GSTA3*	rs512795	*SLC13A1*	rs2204295	*SPN*	rs4788172
ABCC1	rs4148380	*CBR3*	rs8133052	*CYP2D6*	rs1135840	*GSTA4*	rs13197674	*SLC13A1*	rs6962039	*SULT1B1*	rs11249460
ABCC1	rs8187858	*CCDC101*	rs11401	*CYP2D6*	rs16947	*GSTA4*	rs1802061	*SLC15A1*	rs1339067	*SULT1B1*	rs11731028
ABCC2	rs1137968	*CDA*	rs1048977	*CYP2D6*	rs28360521	*GSTA4*	rs367836	*SLC15A1*	rs2297322	*SULT1B1*	rs1604741
ABCC2	rs17222723	*CDA*	rs2072671	*CYP2D6*	rs3892097	*GSTA4*	rs405729	*SLC15A2*	rs1143670	*SULT1C4*	rs1402467
ABCC2	rs2273697	*CDA*	rs818202	*CYP2E1*	rs2070673	*GSTA4*	rs7496	*SLC15A2*	rs1143671	*SULT1E1*	rs1881668
ABCC2	rs3740066	*CES2*	rs11568311	*CYP2E1*	rs2515641	*GSTA5*	rs4715354	*SLC15A2*	rs1143672	*SULT1E1*	rs3736599
ABCC2	rs717620	*CES2*	rs11568314	*CYP2F1*	rs305968	*GSTA5*	rs7748890	*SLC15A2*	rs2257212	*SULT1E1*	rs3775770
ABCC2	rs8187707	*CES2*	rs4783745	*CYP2F1*	rs58285195	*GSTM2*	rs530021	*SLC15A2*	rs2293616	*SULT1E1*	rs3822172
ABCC3	rs1051640	*CHST1*	rs2028985	*CYP2J2*	rs890293	*GSTM2*	rs592792	*SLC16A1*	rs1049434	*SULT2B1*	rs1132054
ABCC3	rs11568591	*CHST1*	rs750398	*CYP2S1*	rs338599	*GSTM3*	rs1799735	*SLC16A1*	rs12727968	*SULT2B1*	rs2302948
ABCC3	rs2277624	*CHST1*	rs895729	*CYP39A1*	rs2277119	*GSTM3*	rs2234696	*SLC16A1*	rs7169	*SULT2B1*	rs2544794
ABCC3	rs4148416	*CHST1*	rs9787901	*CYP39A1*	rs7761731	*GSTM3*	rs7483	*SLC16A1*	rs9429505	*SULT4A1*	rs138056
ABCC4	rs1059751	*CHST10*	rs1530031	*CYP3A4*	rs2242480	*GSTM5*	rs11807	*SLC22A1*	rs1867351	*SULT4A1*	rs138057

Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP
ABCC4	rs1189466	*CHST10*	rs3748930	*CYP3A43*	rs17342647	*GSTM5*	rs1296954	*SLC22A1*	rs628031	*TPSG1*	rs909921
ABCC4	rs1678339	*CHST10*	rs3828193	*CYP3A43*	rs533486	*GSTO1*	rs4925	*SLC22A1*	rs683369	*UGT1A1*	rs1042640
ABCC4	rs2274405	*CHST11*	rs2463018	*CYP3A43*	rs800667	*GSTP1*	rs1138272	*SLC22A1*	rs72552763	*UGT1A1*	rs10929302
ABCC4	rs2274406	*CHST11*	rs2463437	*CYP3A5*	rs776746	*GSTP1*	rs1695	*SLC22A11*	rs1783811	*UGT1A1*	rs10929303
ABCC4	rs2274407	*CHST11*	rs2468110	*CYP3A7*	rs2257401	*GSTZ1*	rs1046428	*SLC22A11*	rs2078267	*UGT1A1*	rs111741722
ABCC4	rs3742106	*CHST11*	rs7847	*CYP4A11*	rs11211402	*GSTZ1*	rs7972	*SLC22A12*	rs11231825	*UGT1A1*	rs3755319
ABCC4	rs4148551	*CHST11*	rs903247	*CYP4B1*	rs2297809	*GSTZ1*	rs7975	*SLC22A13*	rs4679028	*UGT1A1*	rs4124874
ABCC4	rs4148553	*CHST13*	rs1056522	*CYP4B1*	rs2297810	*HMGCR*	rs5909	*SLC22A13*	rs9842091	*UGT1A1*	rs8330
ABCC5	rs3749442	*CHST13*	rs1873397	*CYP4B1*	rs3215983	*HNMT*	rs1050891	*SLC22A14*	rs149738	*UGT1A1*	rs887829
ABCC5	rs3805114	*CHST13*	rs3856650	*CYP4B1*	rs4646487	*HNMT*	rs4245861	*SLC22A14*	rs171248	*UGT1A3*	rs6706232
ABCC5	rs562	*CHST13*	rs6783962	*CYP4B1*	rs4646491	*HNMT*	rs4646333	*SLC22A14*	rs183574	*UGT1A4*	rs6755571
ABCC5	rs7636910	*CHST2*	rs3755739	*CYP4F11*	rs1060463	*MAT1A*	rs17102596	*SLC22A2*	rs316003	*UGT1A6*	rs1105880
ABCC5	rs939336	*CHST2*	rs4683739	*CYP4F11*	rs2305801	*MAT1A*	rs4934027	*SLC22A2*	rs316019	*UGT1A7*	rs7586110
ABCC6	rs2238472	*CHST2*	rs6664	*CYP4F11*	rs3765070	*MAT1A*	rs7087728	*SLC22A2*	rs624249	*UGT1A9*	rs3832043
ABCC6	rs8058694	*CHST3*	rs1871450	*CYP4F11*	rs8104361	*MAT1A*	rs9285726	*SLC22A3*	rs2292334	*UGT2A1*	rs11249454
ABCC6	rs8058696	*CHST3*	rs4148943	*CYP4F12*	rs593421	*NAT1*	rs4986993	*SLC22A3*	rs668871	*UGT2A1*	rs2288741
ABCC8	rs757110	*CHST3*	rs4148944	*CYP4F12*	rs609290	*NAT2*	rs1041983	*SLC22A4*	rs1050152	*UGT2A1*	rs4148301
ABCG1	rs1044317	*CHST3*	rs4148945	*CYP4F12*	rs609636	*NAT2*	rs1208	*SLC22A4*	rs272893	*UGT2A1*	rs4148304
ABCG1	rs1541290	*CHST3*	rs4148946	*CYP4F12*	rs688755	*NAT2*	rs1799929	*SLC22A5*	rs1045020	*UGT2B15*	rs1902023
ABCG1	rs3788007	*CHST3*	rs4148949	*CYP4F2*	rs2074900	*NAT2*	rs1799930	*SLC22A5*	rs274548	*UGT2B15*	rs3100
ABCG1	rs3788010	*CHST3*	rs4148950	*CYP4F2*	rs2108622	*NAT2*	rs1799931	*SLC22A5*	rs274558	*UGT2B15*	rs4148269
ABCG1	rs425215	*CHST3*	rs730720	*CYP4F2*	rs3093106	*NAT2*	rs1801280	*SLC22A7*	rs2242416	*UGT2B17*	rs28374627
ABCG1	rs492338	*CHST3*	rs731027	*CYP4F2*	rs3093153	*NNMT*	rs2852425	*SLC22A7*	rs2270860	*UGT2B4*	rs1131878
ABCG1	rs914189	*CHST5*	rs2641806	*CYP4F8*	rs2056822	*NQO1*	rs10517	*SLC22A8*	rs2276299	*UGT2B4*	rs13119049
ABCG2	rs2231142	*CHST5*	rs2738792	*CYP4F8*	rs4239614	*NQO1*	rs1800566	*SLC25A27*	rs9381468	*UGT2B4*	rs13142440

Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP	Gene	SNP
ABP1	rs1049793	*COMT*	rs4633	*CYP4F8*	rs4646523	*NQO1*	rs689453	*SLC25A27*	rs953062	*UGT2B4*	rs1966151
ABP1	rs10893	*COMT*	rs4680	*CYP4F8*	rs4808326	*NR1I2*	rs2276707	*SLC28A1*	rs2242046	*UGT2B7*	rs28365062
ABP1	rs12179	*CROT*	rs31651	*CYP4Z1*	rs7512729	*NR1I2*	rs3814055	*SLC28A1*	rs2242048	*UGT2B7*	rs7438284
ABP1	rs12539	*CROT*	rs31652	*CYP51A1*	rs7793861	*NR1I2*	rs6785049	*SLC28A1*	rs2290272	*UGT2B7*	rs7439366
ADH1A	rs12512110	*CROT*	rs7785206	*CYP51A1*	rs7797834	*NR1I3*	rs11584174	*SLC28A1*	rs2305367	*UGT2B7*	rs7662029
ADH1A	rs1826909	*CYP11B1*	rs1134095	*CYP7A1*	rs12542233	*NR1I3*	rs2501870	*SLC28A1*	rs8025045	*UGT2B7*	rs7668258
ADH1A	rs6811453	*CYP11B1*	rs4736312	*CYP7A1*	rs13251066	*NR1I3*	rs55802895	*SLC28A1*	rs8187758	*VKORC1*	rs17708472
ADH1C	rs1789915	*CYP11B1*	rs5297	*CYP7A1*	rs3808607	*NR3C1*	rs6196	*SLC28A2*	rs1060896	*VKORC1*	rs2359612
ADH4	rs1126670	*CYP11B1*	rs5303	*CYP7A1*	rs8192879	*PGAP3*	rs2952151	*SLC28A3*	rs10868138	*VKORC1*	rs2884737
ADH4	rs1126671	*CYP11B1*	rs7003319	*CYP7B1*	rs6980478	*PON1*	rs854560	*SLC28A3*	rs7853758	*VKORC1*	rs7294
ADH4	rs1126672	*CYP11B2*	rs4543	*CYP7B1*	rs6987861	*PON3*	rs11764079	*SLC28A3*	rs7867504	*VKORC1*	rs8050894
ADH4	rs3762894	*CYP17A1*	rs6162	*CYP8B1*	rs6771233	*PON3*	rs11770903	*SLC5A6*	rs1395	*VKORC1*	rs9923231
ADH5	rs1154400	*CYP17A1*	rs6163	*CYP8B1*	rs6774801	*PON3*	rs13226149	*SLC5A6*	rs7081	*VKORC1*	rs9934438
ADH5	rs2602836	*CYP17A1*	rs743572	*CYP8B1*	rs735320	*PON3*	rs17882539	*SLC6A6*	rs2341970	*XDH*	rs1884725
ADH6	rs10002894	*CYP19A1*	rs10046	*DPYD*	rs17376848	*PON3*	rs2072200	*SLC6A6*	rs9036	*XDH*	rs207440
ADH6	rs10008281	*CYP19A1*	rs1062033	*DPYD*	rs1801265	*POR*	rs17685	*SLC7A5*	rs1060253	*XDH*	rs2295475
ADH6	rs6830685	*CYP19A1*	rs4646	*DPYD*	rs2297595	*POR*	rs6965343	*SLC7A7*	rs1061040		

SNP, single nucleotide polymorphism

Table S2. Genetic variants associated with MTX plasma levels, 48 hours after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Coefficient	95% CI		P
SULT1E1	**rs3736599**	**4**	**70,725,821**	**A**	**0.08**	**-0.31**	**-0.46**	**-0.17**	**0.00003**
SLC6A6	rs2341970	3	14,474,225	T	0.25	-0.21	-0.33	-0.10	0.0004
CYP1B1	rs1056837	2	38,298,150	T	0.42	-0.17	-0.27	-0.07	0.0007
CYP1B1	rs1056836	2	38,298,203	G	0.42	-0.16	-0.26	-0.07	0.0008
GSTM2	rs530021	1	110,210,780	C	0.06	-0.27	-0.43	-0.10	0.002
SLC22A2	rs316003	6	160,645,832	G	0.19	-0.18	-0.30	-0.06	0.003
ABCB11	rs4148776	2	169,870,882	C	0.08	-0.21	-0.35	-0.06	0.006
CHST1	rs2028985	11	45,695,362	A	0.07	-0.25	-0.43	-0.07	0.007
CHST10	rs1530031	2	101,009,326	A	0.47	-0.16	-0.28	-0.04	0.007
CHST10	rs3828193	2	101,031,561	C	0.48	-0.13	-0.22	-0.04	0.007
CHST10	rs3748930	2	101,010,082	C	0.47	-0.16	-0.27	-0.04	0.008
PPARD	rs6901410	6	35,330,030	C	0.06	-0.24	-0.43	-0.05	0.013
GSTP1	rs1138272	11	67,353,579	T	0.11	-0.18	-0.33	-0.04	0.013
ABCB11	rs3755163	2	169,875,539	A	0.05	-0.21	-0.37	-0.04	0.017
ABCB11	rs4148771	2	169,875,534	delCT	0.05	-0.20	-0.37	-0.03	0.020
PPARD	rs6922548	6	35,353,523	G	0.06	-0.22	-0.40	-0.03	0.022
PPARD	rs7739752	6	35,339,035	T	0.06	-0.22	-0.40	-0.03	0.022
NR1I2	rs2276707	3	119,534,153	T	0.18	-0.14	-0.26	-0.02	0.022
CYP2C8	rs1058930	10	96,818,119	G	0.06	0.45	0.05	0.85	0.026
CYP2S1	rs338599	19	41,700,493	G	0.05	-0.22	-0.41	-0.02	0.027
SLC22A14	rs149738	3	38,367,984	G	0.46	0.11	0.01	0.22	0.031
SLC22A14	rs183574	3	38,363,837	C	0.46	0.11	0.01	0.22	0.031
SLC22A14	rs171248	3	38,363,393	C	0.46	0.11	0.01	0.22	0.031
GSTM3	rs1799735	1	110,280,254	delAGG	0.12	-0.17	-0.32	-0.01	0.035
POR	rs17685	7	75,616,105	A	0.29	-0.12	-0.23	0.00	0.041
SLC22A2	rs624249	6	160,679,400	T	0.42	0.14	0.01	0.28	0.042
ABCB4	rs2097937	7	87,030,903	C	0.24	-0.15	-0.30	-0.01	0.042
ABCB11	rs4148768	2	169,887,154	T	0.09	0.23	0.01	0.44	0.044
ABCC6	rs8058696	16	16,278,869	C	0.42	-0.10	-0.20	0.00	0.044
CYP2D6	rs1065852	22	42,526,694	T	0.23	-0.13	-0.25	0.00	0.046
CYP2A13	rs1709082	19	41,601,609	G	0.07	0.30	0.01	0.60	0.046
CYP2D6	rs28360521	22	42,528,976	A	0.24	-0.12	-0.23	0.00	0.048

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value; del, deletion.
Covariate included in statistical model is creatinine plasma levels at week 1.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower MTX plasma levels compared to carriers of the major allele.
The variant depicted in bold surpasses the Bonferroni corrected p-value threshold of 9.2×10^{-5}.
The following variants are in linkage disequilibrium (r^2>0.80): rs3755163 and rs4148771 (*ABCB11*), all depicted variants within *CHST10*, *CYP1B1*, *SLC22A14*, *PPARD* and *CYP2D6*.

Table S3. Genetic variants associated with creatinine plasma levels, 48 hours after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Coefficient	95% CI		P
ABCC1	rs246221	16	16,138,322	C	0.25	-4.86	-7.82	-1.89	0.001
VKORC1	rs7294	16	31,102,321	A	0.33	-4.51	-7.30	-1.73	0.001
GSTA1	rs4715332	6	52,669,185	G	0.42	5.68	2.13	9.24	0.002
ABCB4	rs2230028	7	87,056,176	G	0.11	-5.40	-9.16	-1.65	0.005
CYP3A43	rs17342647	7	99,459,256	T	0.08	7.62	2.32	12.92	0.005
CROT	rs7785206	7	87,021,023	C	0.10	-5.89	-10.01	-1.78	0.005
SLCO3A1	rs3743369	15	92,707,569	G	0.42	-4.51	-7.69	-1.32	0.006
SLC22A7	rs2242416	6	43,273,604	A	0.43	4.90	1.37	8.44	0.007
GSTA5	rs4715354	6	52,708,797	T	0.47	-3.87	-6.70	-1.04	0.007
CDA	rs1048977	1	20,945,055	T	0.34	3.62	0.90	6.34	0.009
ADH1A	rs12512110	4	100,195,815	A	0.08	6.22	1.53	10.90	0.009
VKORC1	rs9923231	16	31,107,689	A	0.47	4.27	1.05	7.50	0.009
VKORC1	rs8050894	16	31,104,509	C	0.48	4.12	1.01	7.23	0.009
VKORC1	rs9934438	16	31,104,878	T	0.47	4.18	1.02	7.35	0.010
ABCB11	rs2287622	2	169,830,328	T	0.41	4.90	1.05	8.76	0.013
SLC15A1	rs2297322	13	99,376,181	A	0.15	5.71	1.18	10.25	0.014
POR	rs17685*	7	75,616,105	A	0.29	-4.23	-7.59	-0.87	0.014
ABCC5	rs3749442	3	183,660,585	T	0.18	-5.12	-9.29	-0.94	0.016
ABCB11	rs4148768*	2	169,887,154	T	0.09	8.39	1.50	15.28	0.017
SLC16A1	rs9429505	1	113,454,571	C	0.24	4.72	0.83	8.61	0.017
ABCB1	rs9282564	7	87,229,440	G	0.10	-6.21	-11.33	-1.08	0.018
VKORC1	rs2884737	16	31,105,554	G	0.30	3.85	0.61	7.10	0.020
MAT1A	rs9285726	10	82,035,150	T	0.35	3.81	0.56	7.05	0.021
GSTP1	rs1138272*	11	67,353,579	T	0.11	6.77	1.00	12.54	0.021
NQO1	rs10517	16	69,743,760	T	0.13	-4.91	-9.12	-0.70	0.022
ABCB11	rs3770603	2	169,883,218	A	0.09	8.09	1.14	15.03	0.023
XDH	rs2295475	2	31,589,847	T	0.32	4.60	0.55	8.65	0.026
SLC6A6	rs9036	3	14,530,721	G	0.26	3.71	0.44	6.99	0.026
ADH7	rs1573496	4	100,349,669	C	0.11	-4.57	-8.73	-0.41	0.031
CYP4F8	rs4646523	19	15,726,487	G	0.38	3.10	0.27	5.93	0.032
SLC22A12	rs11231825	11	64,360,274	T	0.34	-4.12	-7.91	-0.33	0.033
FMO3	rs2066534	1	171,077,372	G	0.16	-4.05	-7.84	-0.26	0.036
ADH7	rs971074	4	100,341,861	A	0.12	-4.28	-8.30	-0.26	0.037
CYP1B1	rs1800440	2	38,298,139	G	0.14	-5.25	-10.29	-0.20	0.042
FMO2	rs2307492	1	171,168,545	C	0.07	-3.63	-7.16	-0.09	0.044
SPN	rs4788172	16	29,668,253	A	0.06	-5.08	-10.07	-0.09	0.046
POR	rs6965343	7	75,592,925	T	0.38	-3.21	-6.36	-0.05	0.046

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value.
Covariate included in statistical model is age.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower creatinine plasma levels compared to carriers of the major allele.
The following variants are in linkage disequilibrium (r^2>0.80): rs2230028 (*ABCB4*) and rs7785206 (*CROT*), rs4715332 (*GSTA1*) and rs4715354 (*GSTA5*), all depicted variants within *ABCB11*, *ADH7* and *VKORC1* (except rs7294 and rs2884737).
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, Table S2).

Table S4. Genetic variants associated with creatinine plasma levels, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Coefficient	95% CI		P
CYP1B1	rs1800440	2	38,298,139	G	0.14	-8.66	-13.07	-4.26	0.0001
CYP3A43	rs17342647	7	99,459,256	T	0.08	8.06	3.52	12.59	0.0005
SLC22A3	rs668871	6	160,769,811	T	0.47	-5.03	-8.56	-1.51	0.005
SLCO3A1	rs3743369	15	92,707,569	G	0.42	-4.40	-7.57	-1.23	0.006
SLC13A1	rs1880179	7	122,838,633	A	0.41	-3.44	-6.03	-0.86	0.009
SLC22A2	rs624249*	6	160,679,400	T	0.42	-4.24	-7.48	-0.99	0.011
SLCO5A1	rs16936279	8	70,584,809	C	0.12	-5.38	-9.61	-1.14	0.013
SLCO3A1	rs1517618	15	92,647,645	G	0.17	-5.12	-9.17	-1.06	0.013
CDA	rs2072671	1	20,915,701	C	0.33	-4.47	-8.03	-0.91	0.014
SLC13A1	rs2204295	7	122,838,090	G	0.40	-3.22	-5.81	-0.63	0.015
ATP7B	rs2277448	13	52,585,548	C	0.37	-4.30	-7.84	-0.77	0.017
SLC22A11	rs2078267	11	64,334,114	C	0.47	-4.30	-7.93	-0.68	0.020
CYP39A1	rs2277119	6	46,609,905	A	0.25	4.89	0.63	9.14	0.024
FMO2	rs2307492	1	171,168,545	C	0.07	-4.93	-9.24	-0.62	0.025
ATP7B	rs1051332	13	52,507,720	A	0.46	4.27	0.50	8.03	0.026
UGT2B7	rs7668258	4	69,962,078	C	0.42	3.52	0.39	6.65	0.028
UGT2B7	rs7662029	4	69,961,912	G	0.42	3.52	0.39	6.65	0.028
UGT2B7	rs7439366	4	69,964,338	C	0.42	3.48	0.35	6.61	0.029
UGT2B7	rs7438284	4	69,964,337	T	0.42	3.48	0.35	6.61	0.029
GSTP1	rs1138272*	11	67,353,579	T	0.11	6.15	0.58	11.71	0.030
VKORC1	rs7294	16	31,102,321	A	0.33	-3.34	-6.46	-0.22	0.036
CYP4A11	rs11211402	1	47,392,054	G	0.16	-4.20	-8.17	-0.23	0.038
VKORC1	rs2884737	16	31,105,554	G	0.30	3.57	0.19	6.95	0.039
SLC22A12	rs11231825	11	64,360,274	T	0.34	-3.77	-7.35	-0.19	0.039
VKORC1	rs8050894	16	31,104,509	C	0.48	3.40	0.17	6.62	0.039
CYP2A6	rs28399433	19	41,356,379	G	0.07	6.52	0.33	12.70	0.039
CROT	rs31652	7	87,029,687	G	0.15	-4.31	-8.49	-0.13	0.043
VKORC1	rs9934438	16	31,104,878	T	0.47	3.36	0.10	6.61	0.043
CYP4F12	rs609290	19	15,789,140	A	0.07	5.73	0.13	11.33	0.045
CYP4F12	rs609636	19	15,789,098	A	0.07	5.73	0.13	11.33	0.045
ABCC4	rs4148551	13	95,673,518	G	0.42	-3.42	-6.79	-0.05	0.047
ABCC4	rs3742106	13	95,673,791	G	0.42	-3.48	-6.93	-0.04	0.047
ABCC1	rs8187858	16	16,162,039	T	0.09	-4.68	-9.32	-0.03	0.048

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value.
Covariates included in statistical model are age and MTX cumulative dose.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower creatinine plasma levels compared to carriers of the major allele.
The following variants are in linkage disequilibrium (r^2>0.80): all depicted variants within *ABCC4, CYP4F12, SLC3A1, UGT2B7* and *VKORC1* (except rs7294 and rs2884737).
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, Table S2).

Table S5. Genetic variants associated with ALAT, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ALAT levels				Logistic multivariate GEE analysis of CTCAE v5 ALAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
CYP4F2	rs3093153	19	16,001,215	T	0.05	0.67	0.32	1.03	0.0002	2.32	0.91	3.73	0.001
PPARD	rs6906237	6	35,375,526	A	0.05	0.78	0.34	1.22	0.0005	1.24	0.12	2.37	0.030
CYP2E1	rs2070673	10	135,340,567	A	0.15	-0.44	-0.71	-0.18	0.001	-0.91	-1.67	-0.14	0.020
CYP2E1	rs2515641	10	135,351,362	T	0.09	-0.65	-1.04	-0.25	0.001	-1.43	-2.50	-0.35	0.009
PTGIS	rs5629	20	48,129,706	A	0.24	-0.38	-0.62	-0.15	0.002	-0.67	-1.19	-0.14	0.012
NAT1	rs4986993	8	18,080,747	T	0.27	-0.38	-0.61	-0.14	0.002	-0.66	-1.18	-0.13	0.015
CYP2A6	rs1137115	19	41,356,281	A	0.33	-0.30	-0.50	-0.10	0.004	-0.67	-1.17	-0.16	0.009
PPARD	rs6457816	6	35,362,848	C	0.07	0.64	0.19	1.08	0.005	1.00	-0.01	2.01	0.053
PPARD	rs6922548*	6	35,353,523	G	0.06	0.68	0.20	1.15	0.005	1.09	-0.02	2.20	0.055
PPARD	rs7739752*	6	35,339,035	T	0.06	0.68	0.20	1.15	0.005	1.09	-0.02	2.20	0.055
CYP1B1	rs1056836*	2	38,298,203	G	0.42	-0.29	-0.50	-0.08	0.006	-0.43	-0.95	0.08	0.101
SLCO1B1	rs11045819	12	21,329,813	A	0.25	0.34	0.09	0.59	0.007	0.58	-0.16	1.32	0.125
SLCO1B1	rs4149056	12	21,331,549	C	0.16	-0.43	-0.75	-0.12	0.007	-0.75	-1.56	0.05	0.067
CBR1	rs2835265	21	37,444,696	T	0.10	0.51	0.14	0.89	0.007	1.25	0.11	2.38	0.032
CYP1B1	rs1056837*	2	38,298,150	T	0.42	-0.28	-0.49	-0.07	0.009	-0.43	-0.94	0.09	0.106
CYP2D6	rs3892097	22	42,524,947	A	0.22	0.31	0.08	0.54	0.009	1.23	0.57	1.90	0.0003
SLC7A7	rs1805059	14	23,282,449	G	0.35	-0.29	-0.50	-0.07	0.010	-0.66	-1.09	-0.23	0.003
ABCC4	rs2274406	13	95,858,996	A	0.27	-0.37	-0.66	-0.09	0.010	-0.80	-1.46	-0.14	0.018
ABCC4	rs2274405	13	95,858,978	A	0.26	-0.37	-0.66	-0.09	0.010	-0.80	-1.46	-0.14	0.018
CYP2B6	rs2279343	19	41,515,263	G	0.30	-0.29	-0.51	-0.07	0.011	-0.30	-0.81	0.20	0.243
GSTP1	rs1138272*	11	67,353,579	T	0.11	0.42	0.09	0.75	0.014	0.82	-0.08	1.72	0.074
SLC28A1	rs2305367	15	85,476,441	A	0.41	0.34	0.06	0.62	0.017	0.66	0.13	1.19	0.014

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ALAT levels				Logistic multivariate GEE analysis of CTCAE v5 ALAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
ABCB4	rs1202283	7	87,082,292	T	0.50	0.27	0.04	0.49	0.019	0.41	-0.16	0.98	0.160
CYP2A6	rs8192729	19	41,350,996	A	0.08	0.43	0.07	0.79	0.019	1.75	0.66	2.84	0.002
PPARD	rs6901410*	6	35,330,030	C	0.06	0.62	0.10	1.15	0.020	1.02	-0.10	2.15	0.075
ALDH2	rs886205	12	112,204,427	G	0.21	-0.34	-0.63	-0.05	0.021	-0.45	-1.07	0.16	0.146
SULT1C4	rs1402467	2	108,994,808	G	0.24	-0.30	-0.56	-0.04	0.024	-0.45	-1.04	0.13	0.131
ABP1	rs10893	7	150,555,915	G	0.34	0.24	0.03	0.46	0.025	0.63	0.07	1.19	0.027
CYP7A1	rs8192879	8	59,403,576	A	0.42	-0.32	-0.61	-0.04	0.026	-0.47	-1.15	0.21	0.172
ABCC4	rs1059751	13	95,672,950	C	0.45	0.22	0.03	0.41	0.027	0.43	-0.08	0.94	0.100
ABCC4	rs4148553	13	95,673,135	A	0.45	0.22	0.02	0.41	0.029	0.42	-0.08	0.93	0.100
SLCO1B1	rs4149057	12	21,331,599	T	0.39	-0.26	-0.50	-0.02	0.034	-0.41	-0.96	0.15	0.152
SLC6A6	rs9036	3	14,530,721	G	0.26	0.22	0.01	0.42	0.035	0.62	0.06	1.18	0.029
SLCO5A1	rs1138541	8	70,584,628	A	0.26	0.27	0.02	0.52	0.036	0.34	-0.25	0.93	0.256
CHST2	rs3755739	3	142,837,272	A	0.24	-0.28	-0.54	-0.02	0.036	-0.77	-1.37	-0.17	0.012
CYP7A1	rs13251066	8	59,417,753	C	0.44	-0.30	-0.58	-0.01	0.039	-0.48	-1.14	0.17	0.147
NR1I2	rs3814055	3	119,500,035	T	0.35	-0.22	-0.43	-0.01	0.042	-0.26	-0.76	0.24	0.304
SLC22A4	rs1050152	5	131,676,320	T	0.42	0.23	0.01	0.45	0.042	0.66	0.19	1.14	0.006
ABP1	rs1049793	7	150,557,665	G	0.34	0.22	0.01	0.44	0.043	0.54	-0.01	1.09	0.052
ABP1	rs12179	7	150,557,622	A	0.34	0.22	0.01	0.44	0.043	0.54	-0.01	1.09	0.052
PON3	rs13226149	7	95,025,600	T	0.30	0.23	0.01	0.45	0.044	0.59	-0.04	1.21	0.066
ABCC4	rs4148551	13	95,673,518	G	0.42	-0.23	-0.45	0.00	0.046	-0.44	-0.98	0.10	0.111
SLC28A3	rs7853758	9	86,900,926	T	0.10	-0.49	-0.97	-0.01	0.046	-1.11	-1.97	-0.25	0.011
HMGCR	rs5909	5	74,656,175	A	0.08	0.46	0.01	0.92	0.047	0.67	-0.44	1.77	0.239
SLC7A7	rs1061040	14	23,242,828	A	0.10	-0.53	-1.05	-0.01	0.047	-0.91	-1.89	0.08	0.070

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ALAT levels				Logistic multivariate GEE analysis of CTCAE v5 ALAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
GSTM2	rs592792	1	110,211,956	T	0.19	0.35	0.00	0.69	0.047	0.76	-0.04	1.55	0.063
POR	rs17685*	7	75,616,105	A	0.29	-0.27	-0.54	0.00	0.049	-1.01	-1.71	-0.32	0.004
XDH	rs2295475	2	31,589,847	T	0.32	0.27	0.00	0.54	0.049	0.57	-0.11	1.25	0.101
ABCB4	rs2097937*	7	87,030,903	C	0.24	-0.28	-0.56	0.00	0.049	-0.44	-1.03	0.14	0.138

ALAT, alanine aminotransferase; MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value.

Covariates included in the linear and in the logistic GEE models are age and creatinine plasma levels at week 1.

Effect sizes and 95% CI are reported for the minor allele.

A coefficient below 0 indicates that carriers of minor allele have lower ALAT levels compared to carriers of the major allele.

The following variants are in linkage disequilibrium (r^2>0.80): rs2274406 and rs2274405 (*ABCC4*), rs1059751 and rs4148553 (*ABCC4*), all depicted variants within *ABP1*, *CYP1B1, CYP7A1, PPARD* (except rs6906237, which is linkage disequilibrium with rs7739752).

* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S6. Genetic variants associated with ASAT, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ASAT levels				Logistic multivariate GEE analysis of CTCAE v5 ASAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
ALDH2	rs886205	12	112,204,427	G	0.21	-0.43	-0.67	-0.20	0.0003	-0.53	-1.09	0.03	0.062
PTGIS	rs5629	20	48,129,706	A	0.24	-0.38	-0.60	-0.17	0.0004	-0.72	-1.20	-0.24	0.003
CYP4B1	rs2297810	1	47,280,859	A	0.11	-0.45	-0.70	-0.20	0.0005	-0.97	-1.68	-0.26	0.008
CYP2E1	rs2070673	10	135,340,567	A	0.15	-0.39	-0.63	-0.15	0.001	-1.01	-1.69	-0.33	0.004
CYP2A6	rs1137115	19	41,356,281	A	0.33	-0.26	-0.43	-0.09	0.002	-0.44	-0.88	0.00	0.050
CYP2B6	rs2279343	19	41,515,263	G	0.30	-0.30	-0.50	-0.10	0.003	-0.43	-0.87	0.02	0.059
GSTP1	rs1138272	11	67,353,579	T	0.11	0.44	0.14	0.75	0.004	0.81	0.12	1.49	0.021
FMO3	rs1800822	1	171,076,935	T	0.05	-0.54	-0.92	-0.15	0.006	-1.10	-2.02	-0.18	0.019
CYP1A2	rs35694136	15	75,039,613	delT	0.10	-0.32	-0.55	-0.09	0.006	-0.80	-1.41	-0.19	0.010
CYP4B1	rs4646491	1	47,280,884	T	0.10	-0.43	-0.75	-0.12	0.007	-0.93	-1.73	-0.14	0.021
CYP4B1	rs3215983	1	47,280,747	delAT	0.10	-0.43	-0.75	-0.12	0.007	-0.93	-1.73	-0.14	0.021
GSTM2	rs530021	1	110,210,780	C	0.06	-0.53	-0.92	-0.14	0.008	-1.33	-2.55	-0.11	0.033
CA5P	rs1364182	16	29,656,093	T	0.07	-0.49	-0.86	-0.12	0.009	-1.71	-3.09	-0.34	0.014
UGT2B15	rs3100	4	69,512,655	C	0.32	-0.22	-0.39	-0.06	0.009	-0.51	-0.87	-0.14	0.007
UGT1A7	rs7586110	2	234,590,527	G	0.35	0.25	0.06	0.45	0.010	0.65	0.27	1.04	0.001
NAT1	rs4986993	8	18,080,747	T	0.27	-0.30	-0.53	-0.07	0.011	-0.72	-1.21	-0.23	0.004
ABCC4	rs4148551	13	95,673,518	G	0.42	-0.21	-0.39	-0.04	0.014	-0.43	-0.81	-0.04	0.030
ABCB11	rs4148768	2	169,887,154	T	0.09	0.37	0.07	0.68	0.015	0.83	0.10	1.57	0.026
CYP4F11	rs2305801	19	16,045,141	A	0.20	0.24	0.04	0.44	0.017	0.31	-0.12	0.74	0.152
SLCO4A1	rs3787537	20	61,303,742	T	0.24	0.30	0.05	0.55	0.020	0.95	0.42	1.47	0.0004
CYP1B1	rs1056836	2	38,298,203	G	0.42	-0.23	-0.43	-0.04	0.021	-0.45	-0.86	-0.03	0.034
ABP1	rs10893	7	150,555,915	G	0.34	0.22	0.03	0.40	0.021	0.52	0.11	0.93	0.014

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ASAT levels				Logistic multivariate GEE analysis of CTCAE v5 ASAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
SLC22A4	rs1050152	5	131,676,320	T	0.42	0.22	0.03	0.41	0.021	0.58	0.17	0.99	0.005
RALBP1	rs10898	18	9,536,249	C	0.31	-0.26	-0.48	-0.04	0.022	-0.49	-0.98	-0.01	0.045
CYP2A6	rs4803381	19	41,357,344	A	0.40	-0.20	-0.37	-0.03	0.023	-0.34	-0.75	0.07	0.107
UGT2B7	rs7439366	4	69,964,338	C	0.42	-0.24	-0.45	-0.03	0.023	-0.49	-0.97	0.00	0.048
UGT2B7	rs7438284	4	69,964,337	T	0.42	-0.24	-0.45	-0.03	0.023	-0.49	-0.97	0.00	0.048
UGT2B7	rs7668258	4	69,962,078	C	0.42	-0.24	-0.45	-0.03	0.023	-0.49	-0.97	0.00	0.048
UGT2B7	rs7662029	4	69,961,912	G	0.42	-0.24	-0.45	-0.03	0.023	-0.49	-0.97	0.00	0.048
ABCC4	rs3742106	13	95,673,791	G	0.42	-0.20	-0.37	-0.03	0.025	-0.42	-0.80	-0.03	0.033
ABCB4	rs2230028	7	87,056,176	G	0.11	-0.42	-0.78	-0.05	0.025	-0.33	-1.08	0.41	0.383
SLC16A1	rs9429505	1	113,454,571	C	0.24	0.27	0.03	0.51	0.027	0.50	0.04	0.97	0.034
CYP1B1	rs1056837	2	38,298,150	T	0.42	-0.22	-0.42	-0.02	0.028	-0.44	-0.85	-0.02	0.040
SLC22A3	rs2292334	6	160,858,188	A	0.39	-0.24	-0.46	-0.03	0.028	-0.52	-1.02	-0.03	0.039
SLC10A1	rs4646285	14	70,263,648	A	0.09	-0.26	-0.50	-0.03	0.029	-0.43	-0.95	0.09	0.107
CYP2E1	rs2515641	10	135,351,362	T	0.09	-0.42	-0.81	-0.03	0.033	-0.92	-1.89	0.05	0.064
CROT	rs7785206	7	87,021,023	C	0.10	-0.43	-0.83	-0.03	0.033	-0.39	-1.21	0.42	0.341
CYP2B6	rs3745274	19	41,512,841	T	0.26	-0.24	-0.46	-0.02	0.033	-0.29	-0.75	0.17	0.215
SLCO1B1	rs11045819	12	21,329,813	A	0.25	0.24	0.02	0.45	0.035	0.29	-0.21	0.79	0.252
ABP1	rs1049793	7	150,557,665	G	0.34	0.20	0.01	0.38	0.037	0.47	0.06	0.88	0.023
ABP1	rs12179	7	150,557,622	A	0.34	0.20	0.01	0.38	0.037	0.47	0.06	0.88	0.023
CYP24A1	rs2762934	20	52,771,261	T	0.15	-0.21	-0.41	-0.01	0.039	-0.47	-0.95	0.00	0.052
CYP4F11	rs1060463	19	16,025,176	G	0.42	0.22	0.01	0.43	0.039	0.29	-0.13	0.71	0.174
PON3	rs13226149	7	95,025,600	T	0.30	0.22	0.01	0.43	0.039	0.55	0.10	1.00	0.018
GSTM2	rs592792	1	110,211,956	T	0.19	0.29	0.01	0.58	0.040	0.87	0.26	1.48	0.005

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with ASAT levels				Logistic multivariate GEE analysis of CTCAE v5 ASAT increase, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
UGT2B15	rs4148269	4	69,512,847	A	0.33	-0.18	-0.36	-0.01	0.040	-0.33	-0.69	0.02	0.068
ABCC4	rs1059751	13	95,672,950	C	0.45	0.18	0.00	0.35	0.044	0.45	0.07	0.83	0.021
ABCC4	rs4148553	13	95,673,135	A	0.45	0.17	0.00	0.34	0.047	0.44	0.06	0.82	0.023
ABCB4	rs2097937	7	87,030,903	C	0.24	-0.25	-0.49	0.00	0.048	-0.02	-0.49	0.44	0.922
UGT1A1	rs3755319	2	234,667,582	C	0.40	0.20	0.00	0.41	0.050	0.56	0.14	0.97	0.008

ASAT, aspartate aminotransferase; MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value; del, deletion.

Covariates included in the linear and in the logistic GEE models are age and creatinine plasma levels at week 1.

Effect sizes and 95% CI are reported for the minor allele.

A coefficient below 0 indicates that carriers of minor allele have lower ASAT levels compared to carriers of the major allele.

The following variants are in linkage disequilibrium (r^2>0.80): rs2230028 (*ABCB4*) and rs7785206 (*CROT*), all depicted variants within *CYP4B1*, *CYP1B1*, *UGT2B15*, *UGT2B7* and *ABP1*.

* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S7. Genetic variants associated with hemoglobin, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with hemoglobin levels				Logistic multivariate GEE analysis of CTCAE v5 anemia grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
CYP2C8	rs1058930	10	96,818,119	G	0.06	-0.37	-0.57	-0.18	0.0002	0.77	0.36	1.19	0.0003
SLC22A1	rs683369	6	160,551,204	G	0.23	-0.20	-0.31	-0.08	0.0008	0.38	0.13	0.63	0.003
CYP2C19	rs4244285	10	96,541,616	A	0.15	-0.25	-0.40	-0.10	0.0008	0.54	0.22	0.86	0.001
CYP2C18	rs2281891	10	96,493,058	T	0.15	-0.25	-0.39	-0.10	0.001	0.54	0.22	0.86	0.001
ABCB11	rs4148768	2	169,887,154	T	0.09	-0.28	-0.46	-0.10	0.003	0.61	0.24	0.98	0.001
SLC6A6	rs2341970	3	14,474,225	T	0.25	0.20	0.05	0.35	0.008	-0.33	-0.68	0.03	0.076
CHST3	rs4148944	10	73,769,543	A	0.11	-0.22	-0.38	-0.05	0.010	0.52	0.16	0.88	0.005
SLC22A1	rs628031	6	160,560,845	A	0.45	-0.13	-0.23	-0.03	0.010	0.24	-0.04	0.52	0.090
CYP1A2	rs762551	15	75,041,917	C	0.23	0.16	0.04	0.28	0.012	-0.27	-0.60	0.06	0.106
QPRT	rs3862476	16	29,687,360	C	0.07	0.28	0.06	0.50	0.012	-0.83	-1.41	-0.26	0.004
ABCB11	rs3770603	2	169,883,218	A	0.09	-0.24	-0.43	-0.04	0.017	0.47	0.09	0.86	0.015
FMO2	rs2307492	1	171,168,545	C	0.07	0.22	0.04	0.40	0.017	-0.38	-0.98	0.23	0.221
CYP3A43	rs17342647	7	99,459,256	T	0.08	-0.17	-0.32	-0.03	0.017	0.48	0.07	0.89	0.021
SLC10A2	rs279941	13	103,698,168	T	0.13	-0.20	-0.36	-0.03	0.017	0.38	-0.05	0.80	0.084
AOX1	rs16834034	2	201,542,014	G	0.09	-0.16	-0.29	-0.03	0.018	0.28	-0.01	0.57	0.057
CYP4B1	rs4646491	1	47,280,884	T	0.10	0.23	0.04	0.42	0.018	-0.67	-1.24	-0.09	0.023
CYP4B1	rs3215983	1	47,280,747	delAT	0.10	0.23	0.04	0.42	0.018	-0.67	-1.24	-0.09	0.023
CYP11B1	rs7003319	8	143,954,747	A	0.42	0.12	0.02	0.23	0.019	-0.24	-0.52	0.04	0.089
CYP11B1	rs1134095	8	143,954,290	C	0.42	0.12	0.02	0.23	0.019	-0.24	-0.52	0.04	0.089
CYP11B1	rs4736312	8	143,953,937	T	0.42	0.12	0.02	0.23	0.019	-0.24	-0.52	0.04	0.089
ADH4	rs1126672	4	100,047,812	T	0.26	0.17	0.03	0.32	0.019	-0.33	-0.68	0.03	0.069
CYP1B1	rs1056837	2	38,298,150	T	0.42	0.14	0.02	0.25	0.021	-0.25	-0.56	0.05	0.104

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with hemoglobin levels				Logistic multivariate GEE analysis of CTCAE v5 anemia grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
QPRT	rs13331798	16	29,747,291	G	0.21	0.16	0.02	0.29	0.022	-0.45	-0.80	-0.10	0.012
CYP11B1	rs5303	8	143,955,095	T	0.42	0.12	0.02	0.23	0.023	-0.23	-0.52	0.05	0.102
SLC10A2	rs188096	13	103,705,044	T	0.13	-0.19	-0.36	-0.03	0.024	0.38	-0.05	0.81	0.084
CHST3	rs4148945	10	73,769,590	T	0.49	0.13	0.01	0.25	0.028	-0.32	-0.62	-0.03	0.032
SLC15A1	rs1339067	13	99,356,612	T	0.39	-0.12	-0.22	-0.01	0.029	0.41	0.13	0.68	0.004
CYP2A13	rs1709082	19	41,601,609	G	0.07	-0.19	-0.37	-0.02	0.030	0.45	0.02	0.88	0.040
VKORC1	rs2884737	16	31,105,554	G	0.30	0.15	0.01	0.29	0.030	-0.22	-0.55	0.10	0.181
SLC7A7	rs2281677	14	23,284,572	T	0.39	-0.12	-0.24	-0.01	0.030	0.27	-0.01	0.55	0.057
CHST3	rs1871450	10	73,772,014	A	0.49	0.13	0.01	0.25	0.035	-0.32	-0.62	-0.02	0.037
CHST3	rs4148950	10	73,771,706	A	0.49	0.13	0.01	0.25	0.035	-0.32	-0.62	-0.02	0.037
CYP1B1	rs1056836	2	38,298,203	G	0.42	0.12	0.01	0.24	0.037	-0.23	-0.54	0.07	0.134
SLCO1B1	rs11045819	12	21,329,813	A	0.25	0.14	0.01	0.27	0.038	-0.39	-0.72	-0.05	0.024
SLC7A7	rs1805059	14	23,282,449	G	0.35	-0.13	-0.25	-0.01	0.038	0.31	0.02	0.60	0.038
ADH4	rs1126670	4	100,052,733	G	0.28	0.15	0.01	0.29	0.039	-0.28	-0.61	0.05	0.101
ADH4	rs1126671	4	100,048,414	A	0.28	0.15	0.01	0.29	0.039	-0.28	-0.61	0.05	0.101
SLC7A7	rs8018462	14	23,282,110	T	0.44	-0.12	-0.23	-0.01	0.040	0.26	-0.02	0.53	0.068
GSTO1	rs4925	10	106,022,789	A	0.33	-0.12	-0.24	-0.01	0.040	0.33	0.04	0.62	0.024
CYP26A1	rs10882140	10	94,842,213	G	0.45	0.11	0.01	0.22	0.040	-0.20	-0.49	0.09	0.181
SULT1E1	rs3736599	4	70,725,821	A	0.08	0.17	0.00	0.33	0.043	-0.53	-0.95	-0.11	0.014
GSTM2	rs530021	1	110,210,780	C	0.06	0.23	0.01	0.46	0.043	-0.84	-1.62	-0.06	0.034
CHST3	rs730720	10	73,772,762	T	0.50	0.12	0.00	0.24	0.046	-0.29	-0.58	0.00	0.053
CHST3	rs731027	10	73,772,336	C	0.50	0.12	0.00	0.24	0.046	-0.29	-0.58	0.00	0.053
CHST3	rs4148943	10	73,769,507	T	0.50	0.12	0.00	0.24	0.046	-0.29	-0.58	0.00	0.053

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with hemoglobin levels				Logistic multivariate GEE analysis of CTCAE v5 anemia grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
ABCB1	rs2214102	7	87,229,501	A	0.11	-0.16	-0.31	0.00	0.047	0.17	-0.26	0.60	0.433
GSTP1	rs1695	11	67,352,689	G	0.36	0.12	0.00	0.23	0.049	-0.17	-0.49	0.14	0.274

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value; del, deletion.
Covariates included in the linear GEE model are sex, MTX cumulative and creatinine plasma levels at week 1. Covariates included in the logistic GEE model are sex, age and creatinine plasma levels at week 1.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower hemoglobin levels compared to carriers of the major allele.
The following variants are in linkage disequilibrium (r^2>0.80): all depicted variants within *CYP4B1*, *ABCB11*, *CYP1B1*, *ADH4*, *CYP11B1*, *CHST3* (except rs4148944), *SLC10A2* and *SLC7A7* (except rs8018462).
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S8. Genetic variants associated with thrombocyte counts, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with thrombocyte counts				Logistic multivariate GEE analysis of CTCAE v5 platelet count decrease, grade 0 compared to grade 1-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
CYP2B6	**rs4803418**	**19**	**41,511,803**	**G**	**0.31**	**-0.19**	**-0.27**	**-0.10**	**0.00003**	**0.32**	**0.01**	**0.63**	**0.046**
CYP4F8	**rs4808326**	**19**	**15,726,147**	**A**	**0.10**	**0.19**	**0.10**	**0.29**	**0.00006**	**-0.97**	**-1.55**	**-0.39**	**0.001**
CYP2B6	**rs4803419**	**19**	**41,512,792**	**T**	**0.32**	**-0.19**	**-0.28**	**-0.09**	**0.00009**	**0.32**	**0.00**	**0.65**	**0.051**
SLC13A1	rs6962039	7	122,754,358	T	0.24	-0.18	-0.29	-0.07	0.001	0.59	0.22	0.95	0.002
FMO2	rs2020870	1	171,154,959	G	0.08	0.23	0.09	0.37	0.001	-0.74	-1.31	-0.17	0.011
CYP2B6	rs2279343	19	41,515,263	G	0.30	0.16	0.05	0.27	0.003	-0.28	-0.67	0.11	0.165
SLC7A8	rs2268877	14	23,636,757	C	0.22	-0.17	-0.29	-0.06	0.004	0.49	0.08	0.89	0.018
NR1I2	rs2276707	3	119,534,153	T	0.18	0.15	0.05	0.26	0.004	-0.64	-1.10	-0.17	0.007
SLC16A1	rs9429505	1	113,454,571	C	0.24	-0.17	-0.29	-0.05	0.005	0.44	0.06	0.81	0.023
CYP4F11	rs1060463	19	16,025,176	G	0.42	-0.13	-0.22	-0.04	0.006	0.46	0.12	0.81	0.008
CYP4F11	rs3765070	19	16,040,292	T	0.41	-0.13	-0.22	-0.04	0.007	0.45	0.11	0.79	0.010
ADH4	rs1126672	4	100,047,812	T	0.26	0.16	0.04	0.27	0.007	-0.47	-0.86	-0.07	0.020
FMO3	rs1736557	1	171,080,080	A	0.10	0.16	0.04	0.28	0.008	-0.46	-0.98	0.05	0.078
SULT2B1	rs2302948	19	49,096,065	T	0.23	-0.19	-0.33	-0.05	0.008	0.69	0.23	1.15	0.003
UGT1A9	rs3832043	2	234,580,463	T	0.38	0.16	0.04	0.29	0.009	-0.44	-0.81	-0.06	0.022
SLC28A1	rs2305367	15	85,476,441	A	0.41	-0.14	-0.24	-0.03	0.009	0.42	0.08	0.77	0.016
ABCC5	rs939336	3	183,685,534	T	0.39	-0.11	-0.19	-0.02	0.012	0.24	-0.07	0.55	0.130
CYP2B6	rs8192719	19	41,518,773	T	0.25	0.14	0.03	0.25	0.015	-0.20	-0.59	0.19	0.317
CYP2B6	rs3745274	19	41,512,841	T	0.26	0.14	0.03	0.25	0.015	-0.23	-0.62	0.17	0.267
ADH1A	rs6811453	4	100,194,977	T	0.38	0.13	0.02	0.23	0.015	-0.31	-0.67	0.05	0.087
ADH1A	rs1826909	4	100,217,743	A	0.38	0.13	0.02	0.23	0.017	-0.31	-0.66	0.05	0.091

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with thrombocyte counts				Logistic multivariate GEE analysis of CTCAE v5 platelet count decrease, grade 0 compared to grade 1-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
ABP1	rs12539	7	150,558,366	T	0.21	0.14	0.02	0.26	0.018	-0.38	-0.80	0.05	0.083
ADH4	rs1126670	4	100,052,733	G	0.28	0.14	0.02	0.25	0.019	-0.37	-0.77	0.03	0.070
ADH4	rs1126671	4	100,048,414	A	0.28	0.14	0.02	0.25	0.019	-0.37	-0.77	0.03	0.070
CYP4F11	rs2305801	19	16,045,141	A	0.20	-0.13	-0.23	-0.02	0.020	0.42	0.04	0.80	0.029
CYP4F11	rs8104361	19	16,034,714	T	0.20	-0.13	-0.25	-0.02	0.022	0.37	-0.03	0.78	0.068
CYP2C19	rs12248560	10	96,521,657	T	0.27	0.12	0.02	0.21	0.022	-0.30	-0.67	0.08	0.119
ALB	rs3756067	4	74,269,645	A	0.39	-0.12	-0.22	-0.02	0.024	0.58	0.23	0.93	0.001
ABCC5	rs7636910	3	183,699,516	G	0.42	0.11	0.01	0.20	0.025	-0.18	-0.50	0.14	0.274
GSTM2	rs530021	1	110,210,780	C	0.06	0.18	0.02	0.34	0.025	-0.98	-1.75	-0.21	0.013
SLC5A6	rs1395	2	27,424,636	C	0.32	0.11	0.01	0.20	0.027	-0.22	-0.55	0.11	0.192
FMO2	rs2020863	1	171,174,531	G	0.07	-0.22	-0.43	-0.02	0.031	0.48	-0.26	1.22	0.203
ADH5	rs1154400	4	100,010,010	G	0.29	0.12	0.01	0.23	0.034	-0.38	-0.74	-0.02	0.036
CYP2F1	rs58285195	19	41,622,205	C	0.05	-0.27	-0.53	-0.02	0.035	0.91	0.11	1.72	0.025
SLC7A8	rs1884545	14	23,652,004	T	0.10	-0.16	-0.32	-0.01	0.036	0.32	-0.26	0.90	0.276
CYP1B1	rs1056837	2	38,298,150	T	0.42	0.11	0.01	0.22	0.038	-0.16	-0.51	0.19	0.378
FMO2	rs2020869	1	171,178,152	G	0.12	0.15	0.01	0.30	0.040	-0.48	-1.03	0.06	0.084
HNMT	rs4646333	2	138,773,229	A	0.22	-0.13	-0.25	-0.01	0.040	0.32	-0.08	0.73	0.120
HNMT	rs1050891	2	138,771,760	G	0.22	-0.13	-0.25	-0.01	0.040	0.32	-0.08	0.73	0.120
CYP19A1	rs10046	15	51,502,986	C	0.49	-0.12	-0.24	-0.01	0.041	0.64	0.23	1.05	0.002
SLC22A2	rs624249	6	160,679,400	T	0.42	-0.11	-0.22	0.00	0.042	0.47	0.09	0.86	0.016
ADH1A	rs12512110	4	100,195,815	A	0.08	-0.17	-0.34	-0.01	0.043	0.37	-0.26	1.00	0.250
CYP4F8	rs2056822	19	15,739,597	A	0.29	0.11	0.00	0.21	0.044	-0.34	-0.70	0.01	0.060
CYP4F8	rs4239614	19	15,740,220	C	0.29	0.11	0.00	0.21	0.044	-0.35	-0.71	0.01	0.055

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with thrombocyte counts				Logistic multivariate GEE analysis of CTCAE v5 platelet count decrease, grade 0 compared to grade 1-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
HNMT	rs4245861	2	138,772,694	T	0.23	-0.13	-0.25	0.00	0.047	0.33	-0.08	0.74	0.119
UGT2B7	rs28365062	4	69,964,271	G	0.10	0.14	0.00	0.29	0.047	-0.59	-1.17	-0.01	0.045

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value.
Covariates included in linear GEE model are sex and creatinine plasma levels at week 1. Covariate included in the logistic GEE model is creatinine plasma levels at week 1.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower thrombocyte counts compared to carriers of the major allele.
Variants depicted in bold surpass the Bonferroni corrected p-value threshold of 9.2×10^{-5}.
The following variants are in linkage disequilibrium ($r^2>0.80$): rs1126672 (*ADH4*) and rs115440 (*ADH5*), rs4803418 and rs4803419 (*CYP2B6*), rs1060463 and rs3765070 (*CYP4F11*), rs2305801 and rs8104361 (*CYP4F11*), all depicted variants within *HNMT*, *ADH1A*, *ADH4* and *CYP4F8* (except rs4808326).
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S9. Genetic variants associated with leucocyte counts, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with leukocyte counts				Logistic multivariate GEE analysis of CTCAE v5 white blood cell decrease, grade 0-1 compared to grade 2-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
CYP2B6	rs4803418	19	41,511,803	G	0.31	-0.12	-0.19	-0.06	0.0003	0.36	0.06	0.66	0.019
ALB	rs3756067	4	74,269,645	A	0.39	-0.13	-0.21	-0.06	0.0007	0.62	0.23	1.00	0.002
CYP2B6	rs4803419	19	41,512,792	T	0.32	-0.11	-0.18	-0.04	0.003	0.24	-0.07	0.55	0.133
GSTZ1	rs7972	14	77,793,237	A	0.08	0.18	0.06	0.30	0.004	-0.70	-1.30	-0.11	0.020
ABCC6	rs8058696	16	16,278,869	C	0.42	0.10	0.03	0.17	0.008	-0.38	-0.76	-0.01	0.044
CYP2F1	rs58285195	19	41,622,205	C	0.05	-0.24	-0.42	-0.06	0.009	0.97	0.39	1.56	0.001
UGT2B15	rs1902023	4	69,536,084	T	0.50	0.10	0.03	0.18	0.009	-0.38	-0.73	-0.03	0.035
NR1I2	rs3814055	3	119,500,035	T	0.35	0.09	0.02	0.16	0.009	-0.55	-0.87	-0.23	0.001
UGT2B4	rs1131878	4	70,345,904	G	0.38	-0.10	-0.17	-0.02	0.013	0.35	0.03	0.68	0.035
SLCO5A1	rs1138541	8	70,584,628	A	0.26	-0.11	-0.19	-0.02	0.014	0.46	0.11	0.81	0.009
ABCC6	rs8058694	16	16,278,863	C	0.42	0.09	0.02	0.17	0.017	-0.37	-0.74	0.00	0.052
PTGIS	rs5629	20	48,129,706	A	0.24	0.10	0.02	0.19	0.017	-0.51	-0.92	-0.11	0.013
NAT2	rs1041983	8	18,257,795	T	0.36	-0.09	-0.17	-0.01	0.031	0.44	0.08	0.80	0.015
VKORC1	rs2884737	16	31,105,554	G	0.30	0.09	0.01	0.18	0.037	-0.32	-0.67	0.02	0.069
SLC13A1	rs6962039	7	122,754,358	T	0.24	-0.10	-0.20	-0.01	0.037	0.38	0.00	0.76	0.050
ADH6	rs6830685	4	100,143,184	C	0.21	-0.10	-0.19	0.00	0.039	0.17	-0.22	0.56	0.404
GSTM3	rs1799735	1	110,280,254	delAGG	0.12	0.13	0.01	0.25	0.041	-0.57	-1.36	0.22	0.159
UGT2B4	rs13119049	4	70,346,565	A	0.31	-0.09	-0.18	0.00	0.046	0.37	0.00	0.73	0.050
SPG7	rs2292954	16	89,613,123	G	0.22	-0.08	-0.16	0.00	0.049	0.24	-0.11	0.58	0.174
HNMT	rs4245861	2	138,772,694	T	0.23	-0.09	-0.19	0.00	0.049	0.18	-0.21	0.58	0.365
UGT2B4	rs13142440	4	70,346,564	A	0.31	-0.09	-0.18	0.00	0.049	0.37	0.00	0.74	0.049

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value; del, deletion.
Covariates included in the linear and the logistic GEE model are age and creatinine plasma levels at week 1.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower leukocyte counts compared to carriers of the major allele.
The following variants are in linkage disequilibrium (r^2>0.80): all depicted variants within *ABCC6, CYP2B6* and *UGT2B4* (except rs1131878).
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S10. Genetic variants associated with neutrophil counts, 1 week after MTX infusion (P<0.05)

Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with neutrophil counts				Logistic multivariate GEE analysis of CTCAE v5 neutrophil count decrease, grade 0-2 compared to grade 3-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
PTGIS	rs5629	20	48,129,706	A	0.24	0.19	0.08	0.29	0.0008	-0.63	-1.09	-0.17	0.007
UGT2B4	rs1131878	4	70,345,904	G	0.38	-0.16	-0.25	-0.07	0.0008	0.50	0.12	0.88	0.010
UGT2B4	rs13119049	4	70,346,565	A	0.31	-0.17	-0.28	-0.06	0.002	0.54	0.13	0.96	0.011
ALB	rs3756067	4	74,269,645	A	0.39	-0.17	-0.28	-0.06	0.003	0.53	0.14	0.93	0.008
UGT2B4	rs13142440	4	70,346,564	A	0.31	-0.17	-0.28	-0.06	0.003	0.52	0.10	0.94	0.015
UGT2B4	rs1966151	4	70,346,127	C	0.25	0.15	0.05	0.26	0.005	-0.41	-0.86	0.05	0.080
ADH6	rs6830685	4	100,143,184	C	0.21	-0.18	-0.31	-0.05	0.005	0.74	0.35	1.12	0.000
CYP2B6	rs4803418	19	41,511,803	G	0.31	-0.14	-0.24	-0.04	0.007	0.22	-0.14	0.57	0.227
CA5P	rs1364182	16	29,656,093	T	0.07	0.25	0.07	0.43	0.007	-0.37	-1.06	0.32	0.296
SLCO5A1	rs1138541	8	70,584,628	A	0.26	-0.17	-0.29	-0.05	0.007	0.25	-0.17	0.68	0.239
CYP2A13	rs1709082	19	41,601,609	G	0.07	-0.20	-0.35	-0.05	0.008	0.59	0.03	1.15	0.040
CYP4Z1	rs7512729	1	47,578,471	C	0.41	0.14	0.04	0.25	0.009	-0.32	-0.68	0.04	0.080
NR1I2	rs3814055	3	119,500,035	T	0.35	0.14	0.04	0.25	0.009	-0.55	-1.00	-0.09	0.019
CDA	rs2072671	1	20,915,701	C	0.33	-0.15	-0.26	-0.04	0.010	0.20	-0.17	0.57	0.288
CDA	rs2072671	1	20,915,701	C	0.33	-0.15	-0.26	-0.04	0.010	0.20	-0.17	0.57	0.288
CA5P	rs11150564	16	29,666,736	T	0.44	-0.14	-0.24	-0.03	0.012	0.56	0.17	0.96	0.005
ADH6	rs10002894	4	100,141,240	C	0.22	-0.16	-0.29	-0.03	0.015	0.74	0.35	1.13	0.0002
CYP2B6	rs4803419	19	41,512,792	T	0.32	-0.12	-0.22	-0.01	0.026	0.18	-0.18	0.53	0.327
CHST1	rs9787901	11	45,696,153	T	0.08	0.21	0.02	0.40	0.028	-0.97	-1.84	-0.10	0.029
CYP2F1	rs58285195	19	41,622,205	C	0.05	-0.32	-0.60	-0.03	0.030	0.92	0.20	1.64	0.013
UGT2B15	rs1902023	4	69,536,084	T	0.50	0.14	0.01	0.26	0.030	-0.36	-0.79	0.07	0.099
CYP2S1	rs338599	19	41,700,493	G	0.05	0.30	0.02	0.58	0.034	-0.48	-1.44	0.48	0.325

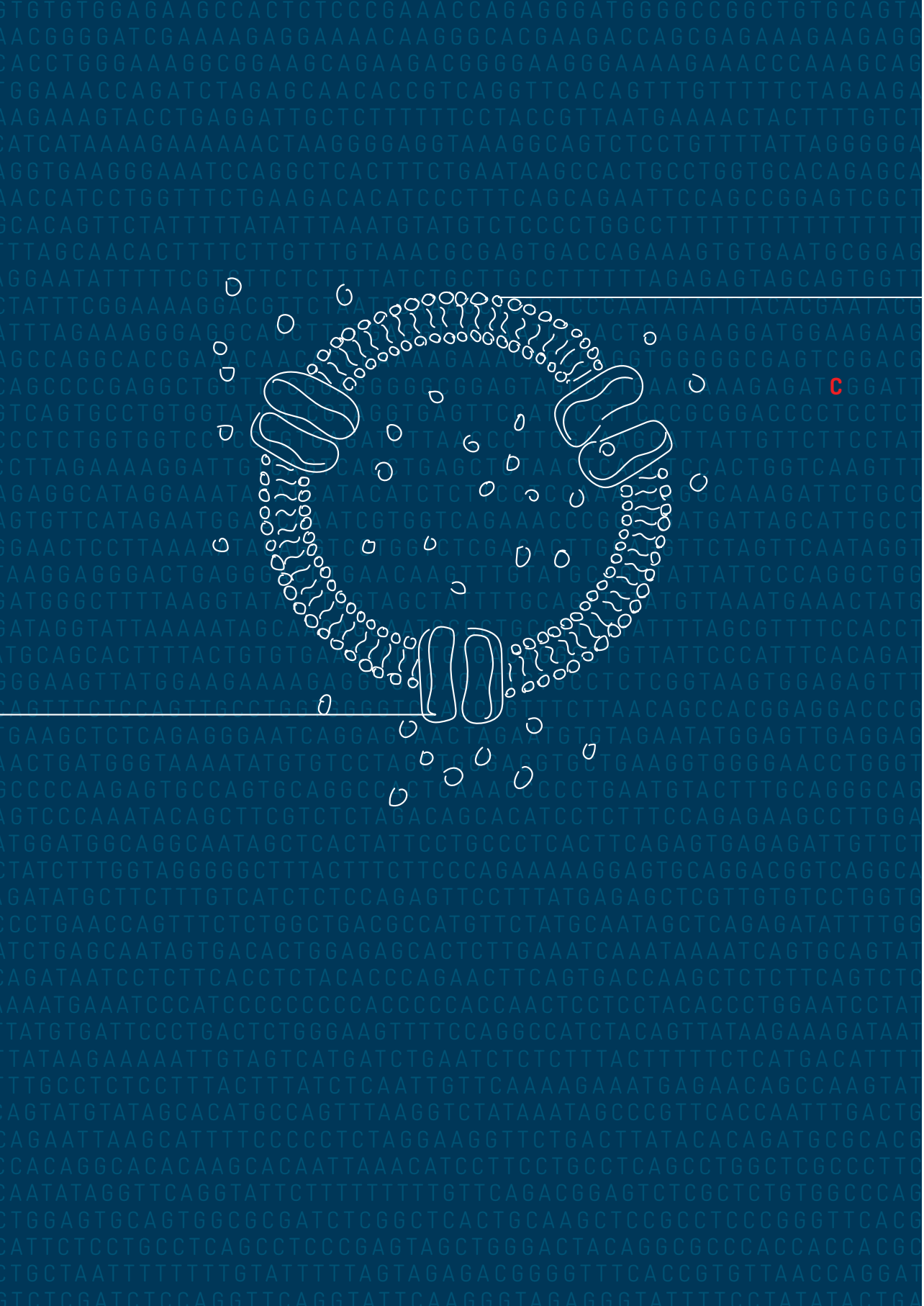
Gene	SNP	Chr	Position	Minor allele	MAF	Linear multivariate GEE analysis with neutrophil counts				Logistic multivariate GEE analysis of CTCAE v5 neutrophil count decrease, grade 0-2 compared to grade 3-4			
						Coefficient	95% CI		P	Coefficient	95% CI		P
SLC22A1	rs628031	6	160,560,845	A	0.45	-0.12	-0.24	-0.01	0.038	0.22	-0.20	0.65	0.304
QPRT	rs13331798	16	29,747,291	G	0.21	0.14	0.01	0.27	0.039	-0.63	-1.12	-0.14	0.011
SLC10A1	rs4646285	14	70,263,648	A	0.09	0.22	0.01	0.42	0.042	-0.37	-1.20	0.46	0.378
CYP4F11	rs2305801	19	16,045,141	A	0.20	-0.14	-0.27	0.00	0.044	0.52	0.07	0.97	0.023
ABCC6	rs8058696	16	16,278,869	C	0.42	0.12	0.00	0.23	0.049	-0.35	-0.80	0.09	0.122
SLC28A1	rs2290272	15	85,447,431	A	0.32	-0.11	-0.23	0.00	0.049	0.35	-0.04	0.74	0.080

MTX, methotrexate; SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; 95% CI, 95% confidence interval; P, p-value.
Covariates included in linear GEE model are MTX cumulative dose and creatinine plasma levels at week 1. Covariate included in the logistic GEE model is creatinine plasma levels at week 1.
Effect sizes and 95% CI are reported for the minor allele.
A coefficient below 0 indicates that carriers of minor allele have lower neutrophil counts compared to carriers of the major allele.
The following variants are in linkage disequilibrium (r^2>0.80): rs13119094 and rs13142440 (*UGT2B4*), all depicted variants within *ADH6* and *CYP2B6*.
* variant is also associated with MTX plasma levels at 48 hours (P<0.05, *Table S2*).

Table S11. National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, used to grade laboratory toxicity markers

CTCAE Term	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Alanine aminotransferase increased	<ULN if baseline was normal; <1.5 x baseline if baseline was abnormal	>ULN - 3.0 x ULN if baseline was normal; 1.5 - 3.0 x baseline if baseline was abnormal	>3.0 - 5.0 x ULN if baseline was normal; >3.0 - 5.0 x baseline if baseline was abnormal	>5.0 - 20.0 x ULN if baseline was normal; >5.0 - 20.0 x baseline if baseline was abnormal	>20.0 x ULN if baseline was normal; >20.0 x baseline if baseline was abnormal	-
Aspartate aminotransferase increased	<ULN if baseline was normal; <1.5 x baseline if baseline was abnormal	>ULN - 3.0 x ULN if baseline was normal; 1.5 - 3.0 x baseline if baseline was abnormal	>3.0 - 5.0 x ULN if baseline was normal; >3.0 - 5.0 x baseline if baseline was abnormal	>5.0 - 20.0 x ULN if baseline was normal; >5.0 - 20.0 x baseline if baseline was abnormal	>20.0 x ULN if baseline was normal; >20.0 x baseline if baseline was abnormal	-
Anemia	Hgb >LLN	Hgb <LLN - 10.0 g/dL; <LLN - 6.2 mmol/L; <LLN - 100 g/L	Hgb <10.0 - 8.0 g/dL; <6.2 - 4.9 mmol/L; <100 - 80 g/L	Hgb <8.0 g/dL; <4.9 mmol/L; <80 g/L; transfusion indicated	Life-threatening consequences; urgent intervention indicated	Death
Platelet count decreased	>LLN	<LLN - 75,000/mm3; <LLN - 75.0 x 10e9 / L	<75,000 - 50,000/mm3; <75.0 - 50.0 x 10e9 /L	<50,000 - 25,000/mm3; <50.0 - 25.0 x 10e9 /L	<25,000/mm3; <25.0 x 10e9 /L	-
White blood cell decreased	>LLN	<LLN - 3000/mm3; <LLN - 3.0 x 10e9 /L	<3000 - 2000/mm3; <3.0 - 2.0 x 10e9 /L	<2000 - 1000/mm3; <2.0 - 1.0 x 10e9 /L	<1000/mm3; <1.0 - 10e9 /L	-
Neutrophil count decreased	>LLN	<LLN - 1500/mm3; <LLN - 1.5 x 10e9 /L	<1500-1000/mm3; <1.5 - 1.0 x 10e9 /L	<1000 - 500/mm3; <1.0 - 0.5 x 10e9 /L	<500/mm3; <0.5 - 10e9 /L	-
Creatinine increased	<ULN	>ULN - 1.5 x ULN	>1.5 - 3.0 x baseline; >1.5 - 3.0 x ULN	>3.0 x baseline; >3.0 - 6.0 x ULN	>6.0 x ULN	-

ULN, upper limit of normal; LLN, lower limit of normal; Hgb, hemoglobin.
Grades depicted by a green cell are considered 'controls' in binary genetic association analyses. Grades depicted by a red cell are considered 'cases'.


C

CHAPTER 4

Novel pharmacogenetic association of *SLC7A8* locus with early disease progression in osteosarcoma leads to discovery of doxorubicin as a LAT2 substrate.

Evelien G. E. Hurkmans[1], Jan B. Koenderink[2], Jeroen J. M. W. van den Heuvel[2], Yvonne M. H. Versleijen-Jonkers[3], Melissa H. S. Hillebrandt-Roeffen[3], Johanne M. Groothuismink[1], Hanneke I. Vos[1], Winette T. A. van der Graaf[3,4], Uta Flucke[5], Grigor Muradjan[2], Hendrik W. B. Schreuder[6], Melanie M. Hagleitner[7], Han G. Brunner[1], Hans Gelderblom[8], Anne-Marie Cleton-Jansen[9], Henk-Jan Guchelaar[10], Eveline S. J. M. de Bont[11], Daan J. Touw[12], G. Jan Nijhoff[12], Leontien C. M. Kremer[13], Huib Caron [13], Rachael Windsor[14], Ana Patiño-García[15], Anna González-Neira[16], Federica Saletta[17], Geoff McCowage[18], Sumanth Nagabushan[18,19], Daniel Catchpoole[17], D. Maroeska W. M. te Loo[20]*, Marieke J. H. Coenen[1]*

[1] Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands
[2] Department of Pharmacology and Toxicology, Radboud university medical center, Nijmegen, The Netherlands
[3] Department of Medical Oncology, Radboud university medical center, Nijmegen, The Netherlands
[4] Department of Medical Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
[5] Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands
[6] Department of Orthopedics, Radboud university medical center, Nijmegen, The Netherlands
[7] Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands
[8] Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands
[9] Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[10] Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, The Netherlands
[11] Department of Pediatrics, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, The Netherlands
[12] Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, Groningen, The Netherlands.
[13] Department of Pediatrics, Amsterdam University Medical Centers, Emma Children's Hospital, Amsterdam, Netherlands
[14] Pediatric & Adolescent Division, University College London Hospitals NHS Foundation Trust, London, United Kingdom
[15] Department of Pediatrics, Clínica Universidad de Navarra, Solid Tumor Program, CIMA, Pamplona, Spain.
[16] Human Genotyping Unit-CeGen, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.
[17] Children's Cancer Research Unit, The Children's Hospital at Westmead, Sydney NSW, Australia
[18] Cancer Centre for Children, The Children's Hospital at Westmead, Sydney NSW, Australia.
[19] Discipline of Child and adolescent health, University of Sydney, Sydney NSW, Australia
[20] Department of Pediatrics, Amalia Children's Hospital, Radboud university medical center, Nijmegen, The Netherlands
* These authors contributed equally

Submitted in adapted version

Abstract

Despite (neo)adjuvant chemotherapy with cisplatin, doxorubicin and methotrexate, some patients with primary osteosarcoma progress during first-line systemic treatment and have a poor prognosis. In this study, we investigated whether patients with early disease progression (EDP), are characterized by a distinctive pharmacogenetic profile.

Germline DNA from 287 Dutch high-grade osteosarcoma patients was genotyped using the DMET Plus array (containing 1,936 genetic markers in 231 drug metabolism and transporter genes). Associations between genetic variants and EPD were assessed using logistic regression models and associated variants ($P<0.05$) were validated in independent cohorts of 146 (Spain and UK) and 28 patients (Australia). The functional relevance of the top hits was explored by immunohistochemistry staining and an *in vitro* transport models.

In the association analyses, EPD was significantly associated with an *SLC7A8* locus and was independently validated (meta-analysis validation cohorts: OR 0.19 [0.06-0.55], P=0.002). *SLC7A8* encodes for the L-type amino acid transporter 2 (LAT2). Transport assays in HEK293 cells overexpressing LAT2 showed that doxorubicin, but not cisplatin and methotrexate, is a substrate for LAT2 ($p < 0.0001$). Finally, *SLC7A8* mRNA expression analysis and LAT2 immunohistochemistry of osteosarcoma tissue showed that the lack of LAT2 expression is a prognostic factor of poor prognosis and reduced overall survival in patients without metastases ($p = 0.0099$ and $p = 0.14$, resp.).

This study identified a novel locus in *SLC7A8* to be associated with EPD in osteosarcoma. Functional studies indicate LAT2-mediates uptake of doxorubicin, which could give new opportunities to personalize treatment of osteosarcoma patients.

Introduction

Osteosarcoma is a malignant bone tumor that mainly affects children and adolescents. Although the number of people affected by osteosarcoma is low (worldwide 3-4 patients per million), the disease is ranked as one of the most frequent causes of cancer-related death in young patients (1). The disease has a great impact on the patient's life, as treatment requires an intensive combination of chemotherapy, often disabling surgery, and prolonged periods of rehabilitation. Despite this harsh treatment regimen, some patients fail to respond, showing no response or even tumor growth during primary treatment (early disease progression – EPD). Recognition of patients that do not benefit from current chemotherapy schedules at an early phase in treatment is therefore important.

Although the genetics and biology of the tumor are likely to contribute to the heterogeneous response to treatment, we postulate that germline variants in drug metabolizing enzymes or transporters might also contribute to this observed heterogeneity. Pharmacogenetics holds the promise to identify germline genetic variants predictive of drug response in individual patients. Most studies aimed at identifying germline genetic variants predictive of treatment outcome in osteosarcoma have considered survival as the main clinical endpoint. However, patients with EPD, who may have a distinctive pharmacogenetic profile, have not been widely studied as a subgroup. The few studies comparing complete or partial responders investigated only a few candidate genes involved in DNA repair (*CCNH, ERCC1/2/5/6*, *MMS19L* and *XPC*) and *GSTP1,* a gene involved in detoxification of exogenous and endogenous compounds (2-5). Significant associations of genetic variants in *ERCC2/5*, *MMS19L*, *XPG* and *GSTP1* with clinical response have been identified. However, validation in additional samples is necessary to confirm these associations as the results are conflicting (4-6).

We performed a screening of 1,936 genetic variants in 231 drug metabolism and transporter genes in a group of osteosarcoma patients with the objective of discovering a relationship with suboptimal drug response during the first-line treatment, followed by validation in independent cohorts of patients. Finally, we studied the top hit gene by performing functional assays to elucidate the biological mechanism behind the association.

Materials and methods

Patient cohorts

A discovery cohort of 287 osteosarcoma patients was retrospectively collected at the Radboud university medical center (Nijmegen), the University Medical Center of Groningen (Groningen), Leiden University Medical Center (Leiden) and the Emma Children's Hospital/Academic Medical Center (Amsterdam), The Netherlands. All patients were treated between 1978 and 2013 and the clinical data were retrospectively collected from medical records. Eligibility criteria were: histological diagnosis of primary high-grade osteosarcoma with or without metastatic disease, age ≤45 years, treatment with cisplatin and doxorubicin-based chemotherapy (also neoadjuvant), and self-reported Caucasian ethnicity. Patients were treated either according to institutional standard therapy consisting of cisplatin (maximum cumulative dose 600 mg/m^2) and doxorubicin (maximum cumulative dose 450 mg/m^2), or according to the standard schedule as given in the EURAMOS-1 trial, which consisted of cisplatin (480 mg/m^2)/ doxorubicin (450 mg/m^2) and additionally high-dose methotrexate (MTX; 144 g/m^2), with or without additional ifosfamide/etoposide or interferon-α (7).

A combined cohort of 146 high-grade osteosarcoma patients treated with cisplatin and doxorubicin-based chemotherapy from Spain (*N*=95) and England (*N*=51) was used for independent validation of the positive findings in the discovery cohort (8, 9). Information on treatment has been reported previously (8, 9). In a second validation phase, a cohort of 28 high-grade osteosarcoma patients, treated with cisplatin and doxorubicin-based chemotherapy from Australia (Sydney Children's Tumour Bank Network), was included. Patients were treated with a cisplatin cumulative dose of 480 mg/m^2 or 600 mg/m^2 and doxorubicin cumulative dose of 450 mg/m^2. The same inclusion criteria as used in the discovery cohort were applied, with the exception of ethnicity.

The study was approved by the Institutional review board of the Radboud university medical center, and approval for inclusion of patients in other institutions was obtained from the corresponding institutional ethics committees. All patients and/or parents provided written informed consent.

Response definition

The clinical (radiological) response to treatment was based on imaging results (CT/MRI/X-ray) reviewed by local expert radiologists. EPD was defined as: (1) growth of the primary tumor (>20%) and/or metastases (>20%), or development of new lesions, in the time from start of primary treatment until 3 months after end of adjuvant chemotherapy or end of first-line treatment in case of primary metastatic disease, and/or (2) inadequacy to reach complete remission at the end of (surgical and chemotherapeutic) therapy for primary localized or primary metastatic osteosarcoma. The opposite extremes, patients showing an adequate drug response with no signs of relapse were considered controls. Thus, patients with recurrent disease, defined as local or distant relapse later than 3 months after end of primary treatment to end of follow-up, were excluded from the analysis.

Genotyping methods

For the discovery cohort, germline DNA was isolated from blood using the QIAamp DNA Blood Midi kit (Qiagen, Venlo, The Netherlands), or from saliva using the Oragene saliva collection kit (DNA Genotek, Kanata, Ontario, Canada) according to the manufacturer's protocols. From patients who had died before inclusion in this pharmacogenetic study, DNA was isolated from normal formalin-fixed, paraffin-embedded (FFPE) tissue as described previously (10, 11). However, for 16 patients of the initial Dutch patient cohort, DNA yield isolated from FFPE tissue was too low for array genotyping. These patients were deceased with often progressive disease. Therefore, the exclusion of these patients was non-random, possibly inducing bias. The limited available DNA was used to manually genotype the top SNPs from the study to assess the effect of exclusion on the main findings. The DNA samples from all other patients were genotyped for 1,936 genetic variants using the Affymetrix DMET Plus array according to the manufacturer's instructions (Affymetrix UK Ltd, High Wycombe, UK). Genotypes were calculated with DMET console software 1.3 using the Dynamic Genotype Boundaries version 2 algorithm. Variants were excluded from analysis if the genotype cluster plots were considered unreliable, being plots with genotype calling showing merged clusters without distinct cluster boundaries. Additional stringent evaluation of the genotype clustering in combination with expected genotype frequencies was carried out for variants significant in association analysis. Quality control was carried out on the total cohort of 316 genotyped patients. Samples and variants were excluded if call rates <0.9, minor allele frequency (MAF) <0.01 and/or deviating from Hardy-Weinberg equilibrium (HWE) (*P*-value <0.0001). The five copy number variants, 46 X-chromosomal variants and one tri-allelic variant present on the array were not included in the analyses.

Isolation of germline DNA in the validation cohort has been previously reported (8, 9); from the Australian validation cohort germline DNA was isolated from blood using the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions. Genotyping of the validation cohort was performed for seven of the ten significant variants in the discovery cohort, excluding three variants that were in linkage disequilibrium with any of the seven variants (based on $r^2 \geq 0.80$) and that had higher *P*-values than their linked variants. Genotyping of the second validation cohort was subsequently performed for the five variants that were significant in the first validation stage and that showed the same direction of effect in the discovery and validation cohorts. KASP-On-Demand (KOD) assays were used for *CYP4F12* rs688755, *SLC22A5* rs274548, *FMO6* rs7886938 and *SLC7A8* rs8013529; KASP-By-Design (KBD) assays were used for *CYP8B1* rs6771233, *SLC22A2* rs316003, and *SLC7A8* rs1884545, all according to the manufacturer's protocol (LGC Genomics, Hoddesdon, UK). Fluorescence was measured with a 7500FAST Real-Time PCR System (ThermoFisher, Nieuwegein, The Netherlands). Genotypes were scored using the algorithm and software (v2.0.6) supplied by ThermoFisher. Blanks (3%) as well as duplicates between plates were included as quality controls for genotyping.

Statistical analysis

Statistical differences in demographic data between patients with EPD and control patients were assessed by the Fisher exact, Pearson chi-square or Mann-Whitney U tests as appropriate using SPSS v22 (SPSS Inc., Chicago, Ill, USA). To assess the effect of a genetic variant on EPD, the data were dichotomized to EPD yes/no Associations between genetic variants and EPD were assessed by multivariable logistic regression analysis in PLINK using the command --logistic (additive model) (PLINK v1.07) (12). For genetic variants significantly associated with EPD in the discovery cohort, we assessed potential associations with two other clinical endpoints: recurrent disease (using PLINK), and 5-year disease free survival (DFS) (time interval from diagnosis to either progression or recurrence) using Cox proportional hazards models in SPSS. These variants were excluded from subsequent analysis, to filter out those variants that were not specific for the inadequate drug response observed in EPD patients. Reported *P*-values are two-sided and are considered statistically significant if <0.05 in the genetic analyses (<0.05 for selection of clinical covariables). No correction for multiple testing was performed because of the exploratory nature of the study. Meta-analysis of the association analysis results of the discovery and validation cohorts, and of all three cohorts including the second validation cohort, was performed using a fixed effects or random effects (in case of large heterogeneity: I^2>50) model in PLINK.

Data availability

Summary statistics of the discovery analysis will be available at RIS (Research Information Services), the current research information system of Radboud University.

Cell culture and transduction

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX with 10% (*v/v*) Fetal Bovine Serum (FBS) at 37°C, 5% CO_2 until 70-80% confluence. Cells were diluted 1:5 and ~300,000 cells per well were seeded into Poly-D-lysine coated 24-wells plates. Twenty-four hours after seeding, cells were transduced with 60-90µL of recombinant baculoviruses and sodium butyrate (3 mM) was added to a final volume of 600 µL. These baculoviruses were modified to induce protein overexpression in HEK293 cells as previously described (13). Briefly, cDNA of the control, LAT2 or 4F2 was cloned downstream of a CMV promotor in the baculoviruses using a Bac-to-Bac gateway system (Invitrogen, Breda, The Netherlands). The sequences of the cDNA are equal to the reference genome (GRCh37/hg19) without genetic variants. During transduction, the baculoviruses induced expression of EYFP (negative control), LAT2 or 4F2. For expressing both LAT2 and 4F2, cells were transduced with both baculoviruses. After transduction, cells were incubated for 48-72h at 37°C, 5% CO_2.

Transport assay

After HEK293 cell transduction, the transport assay was performed in a 24-wells plate. Culture medium was removed and cells were washed with Na^+-free buffer (37°C) containing 125 mM choline chloride, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO4, 1.3 mM $CaCl_2$, 5.6 mM glucose and 25 mM HEPES at pH 7.4, according to Khunweeraphong *et al*. (14). Thereafter, cells were exposed to a radiolabeled compound (^{3}H-methotrexate or ^{3}H-alanine) or an unlabeled compound (cisplatin or doxorubicin) diluted in Na^+-Free HBSS-HEPES buffer at 37°C. When studying inhibition, cells were exposed to a mixture of a substrate and an inhibitor. ^{3}H-L-alanine and BCH (2-Aminobicyclo(2.2.1)heptane-2-carboxylic acid) were used as known substrate and inhibitor, respectively, to validate the model. Exposure time and concentrations are specified per experiment in the results section. After incubation, cells were washed with ice cold Na^+-free buffer containing 0.5% BSA (*m/v*) (4°C) and with Na^+-free buffer only (4°C). Ultimately, cells were lysed in 1-0.1M NaOH or 0.1% Triton X-100.

To determine uptake of radiolabeled substrates, cell lysates were mixed with 4 mL OPTI-FLUOR (PerkinElmer, Waltham, MA, USA) and analyzed with the Hidex automatic TDCR liquid scintillation counter (Turku, Finland). Doxorubicin uptake was measured in lysed cells with 0.1% Triton X-100 by determining the fluorescence of doxorubicin using the Victor X3 multimode plate reader at an excitation and emission wavelength of 485 nm and 590 nm, respectively (PerkinElmer Nederland B.V., Groningen, The Netherlands). Cisplatin uptake concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS). For this, a Thermo Scientific (Waltham, Massachusetts, USA) iCAP TQ mass spectrometer was used (in SQ modus). The system operated under the standard conditions (specified in supplementary methods). Measurements were performed in a matrix of 0.1 M NaOH, which is consistent with the lysis buffer of cells in the transport experiment (Merck - Darmstadt, Germany).

The protein concentration was determined (Bio-Rad Laboratories) and measured using a Benchmark Plus plate reader (595 nm). The transport assays were corrected for protein content in the wells. The mean ± SD of three experiments with cells transduced by independently produced baculoviruses was plotted with GraphPad Prism version 8. Significant uptake was determined using a one-way ANOVA, comparing conditions to control, with Dunnett's post-test. To determine if inhibition was significant, relative substrate uptake in the presence of inhibitor was compared to a solvent only control using an independent t-test.

SDS-page and western blot

LAT2 protein expression was determined with SDS-PAGE and western blotting. HEK293 cells were transduced according to the same protocol as in preparation for the transport assays, but in a T75 flask. Subsequently, cells were lysed and the total membrane fraction (20000g) was run on a SDS-Page and transferred to a Nitrocellulose membrane. LAT2 was visualized with the Odyssey (LI-COR) and the SLC7A8 mouse monoclonal antibody, clone UMAB70 (OriGene, Analog No: UM570058)

Immunohistochemistry

Immunohistochemistry (IHC) of eight tissue micro arrays (TMAs) (one from the Australian cohort and seven from the Dutch cohort) was performed to assess LAT2 protein expression in tumor tissue of osteosarcoma patients (UMAB70 antibody, OriGene Technologies, Inc., Rockville, USA). In addition, IHC was performed for p-mTOR expression to assess possible LAT2 mediated mTOR phosphorylation (#2976, Cell Signaling Technology, Danvers, USA). More detailed methods on the TMAs and IHC staining are available in the supplementary methods section. Only tissue samples from diagnostic biopsies, that were chemotherapy naive, were analyzed. TMAs were scored by two independent observers (EH, YVJ, FS) on percentage of cells with expression (none, <10%, 10-50% or >50%) and intensity of expression (0, + or ++). Differences between observers were resolved by consensus. Eventually, expression data were dichotomized and tissues with expression ≥10% of cells were considered positive and tissues with expression in < 10% of cells were considered negative for LAT2 or p-mTOR expression.

SLC7A8 mRNA expression analysis

SLC7A8 mRNA expression data of osteosarcoma tissue of a previous study by Kuijjer *et al*. was accessed through the 'R2: Genomics Analysis and Visualization Platform' (15, 16). The R2 online interface was utilized for the visualization of the data in a Kaplan Meier curve, with subsequent Log-Rank test for significance.

Results

Patient population

The genetic study was carried out following a three-stage design, including a discovery cohort, and two independent validation cohorts. Of the 287 eligible patients in the discovery cohort, four patients were excluded based on a genotype call rate lower than 0.9, leaving 283 patients for analysis. From the first validation cohort, all 146 patients were successfully genotyped, while from the second, one of the 28 patients was excluded based on genotyping failure for all variants, leaving 27 patients for analysis. The patient characteristics of the three cohorts are provided in *Table 1*. EPD was observed in 13.8%, 12.3% and 18.5% of patients in the discovery, first and second validation cohorts, respectively.

In the discovery cohort, of all clinical variables included in *Table 1*, male gender (P=0.003), the presence of primary metastases (P<0.001), and poor histologic response (P<0.001) were significantly associated with the occurrence of EPD. Therefore, these were included as clinical covariates in the genetic analyses, with the exception of the histologic response. Histologic response and progression are both a reflection of the response to chemotherapy, inclusion of the histologic response as covariate would unintentionally remove variation between EPD and control patients and would therefore result in overcorrection. Despite that the association with increased age at diagnosis was not significant (P=0.058), age was included as a covariate since it was previously identified as a prognostic factor in osteosarcoma (17).

Table 1. Clinical characteristics of the osteosarcoma patients of the discovery and validation cohorts

	Discovery cohort		Validation cohort		Second validation cohort	
	Progression (*N*=39)	**Controls (*N*=168)**	**Progression (*N*=18)**	**Controls (*N*=93)**	**Progression (*N*=5)**	**Controls (*N*=19)**
Age at diagnosis, median (range)	17.2 (6.8-44.7)	15.2 (3.4-45.8)	15.4 (1.9-37.0)	15.0 (6.1-39.0)	15.3 (8.7-16.8)	14.2 (1.1-16.3)
Male sex, *n* (%)	29 (74.4%)	81 (48.2%)	11 (61.1%)	47 (50.5%)	4 (80.0%)	7 (36.8%)
Axial tumor, *n* (%)	4 (10.3%)	5 (2.98%)	1 (5.6%)	2 (2.2%)	1 (20.0%)	0 (0%)
Primary metastases, *n* (%)	16 (41.0%)	16 (9.52%)	9 (50.0%)	8 (8.6%)	3 (60.0%)	1 (5.3%)
Cumulative dose (mg/m^2) Cisplatin[a], median (range) Doxorubicin, median (range)	 400 (100-600) 450 (150-450)	 480 (200-720) 450 (150-455)	 354 (175-1,064) 242 (143-950)	 474 (0-1,038) 385 (90-623)	 480 (480-600) 450 (450-450)	 480 (480-600) 450 (450-450)
MTX treatment[b], *n* (%)	27 (69.2%)	90 (53.6%)	16 (100%)	86 (96.6%)	5 (100%)	15 (78.9%)
Poor histologic response[c], *n* (%)	28 (82.4%)	79 (49.4%)	10 (62.5%)	37 (41.6%)	4 (100%)	4 (44.4%)
5-year overall survival	26.8%	100%	33.3%	98.9%	30.0%	100%

MTX, methotrexate

Patients with recurrent disease are not included in the table: discovery cohort *N*=76, validation cohort *N*=35, second validation cohort *N*=3.

[a]Number of patients with cumulative dose of cisplatin data available; discovery cohort: progression *N*=39, controls *N*=165; additional cohort: all patients

[b]Number of patients with MTX treatment data available; discovery cohort: all patients; validation cohort: progression *N*=16, controls *N*= 89; second validation cohort: progression *N*=5, controls *N*=19.

[c]Number of patients with histologic response data available; discovery cohort: progression *N*=34, controls *N*=160; validation cohort: progression *N*=16, controls *N*=89; second validation cohort: progression *N*=4, recurrence *N*=2, controls *N*= 9.

Table S1 shows demographic data of the additional cohort of patients that was non-randomly excluded from the initial association analysis due to limited availability of DNA.

In the discovery cohort, 11 (28.2%) of 39 EPD patients; nine with follow-up of greater than 5 years. Seven patients were classified as EPD because of primary tumor progression, which was in all cases followed by interruption of chemotherapy and early surgery. The other four patients showed progression of distant metastases (N=3), or progression of both primary tumor and metastases (N=1); in three of these cases surgical removal of the metastatic lesions was performed. In the validation cohort, six (all with follow-up of >5 years) of 18 patients survived EPD of the primary tumor (N=1), metastases (N=3), local recurrence (N=1) or unknown (N=1). In the second validation cohort, two of five patients survived EPD either in the form of local recurrence (follow-up <1 year) or metastases.

Association analyses in the discovery cohort

Of the 1,884 variants included in the quality control, 90 variants were excluded based on unreliable cluster plots, 28 variants were excluded because of genotype call rates of <0.9 and 1,056 variants because of a MAF of <0.01. All remaining variants were in HWE. After quality control, 710 variants were included in the analysis of the discovery cohort. In multivariable logistic regression analysis, comparing patients with EPD to controls and including sex, age at diagnosis, and the presence of primary metastases as covariates, 11 genetic variants were significantly associated with EPD, after filtering of variants also associated with recurrent disease or 5-year DFS. The genotype cluster plot of one of these variants, *CYP2B6* rs2279341, was considered unreliable after additional stringent evaluation of the clustering combined with expected genotype frequencies, leading to 10 remaining variants in 6 genes (*Table 2, Table S2*).

Validation results

In the validation cohort, seven variants were investigated based on the results of the discovery cohort. All variants were in HWE (P>0.05) and showed an average call rate of 0.98. Upon multivariable logistic regression analysis of the validation cohort similar to the discovery cohort, two of the seven genetic variants showed a significant association with EPD (*Table 2*). For these variants, rs8013529 and rs1884545 located in the Solute Carrier Family 7 (Amino Acid Transporter Light Chain, L System) Member 8 *(SLC7A8)* gene, a protective effect was observed for patients carrying the T allele in case of rs1884545 (P=0.020) or the C allele in case of rs8013529. The effect remained significant with addition of inclusion site (Spain or England) as covariate (P=0.018).

Table 2. Results of the association analysis in the discovery and validation cohorts. In the discovery cohort, 710 genetic variants in drug metabolizing enzymes and transporters were assessed for their association to progressive disease in patients with osteosarcoma. Variants with a significant association were manually genotyped and analyzed in the validation cohorts. The results were combined in meta-analyses.

					Discovery			Validation						Second validation		
								Validation cohort			Meta-analysis			Meta-analysis overall		
SNP	**Gene**	**Chromo-some**	**MAF**	**Minor allele**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
rs8013529	*SLC7A8*	14	0.128	C	0.23	0.06-0.81	0.023	0.05	0.005-0.63	0.020	0.16	0.05-0.52	0.002	0.19	0.06-0.55	0.002
rs1884545	*SLC7A8*	14	0.127	T	0.22	0.06-0.78	0.019	0.05	0.005-0.63	0.020	0.16	0.05-0.50	0.002	0.22	0.07-0.63	0.005
rs6771233	*CYP8B1*	3	0.332	A	1.95	1.08-3.54	0.027	1.70	0.78-3.72	0.184	1.86	1.16-2.98	0.010	1.91	1.20-3.05	0.006
rs6774801	*CYP8B1*	3	0.330	A	1.95	1.08-3.54	0.027	NA	NA	NA	NA	NA	NA	NA	NA	NA
rs316003	*SLC22A2*	6	0.205	G	0.44	0.20-0.97	0.042	0.58	0.20-1.69	0.318	0.48	0.25-0.92	0.026	0.49	0.26-0.91	0.023
rs274548	*SLC22A5*	5	0.146	T	2.13	1.01-4.48	0.048	1.75	0.59-5.18	0.311	2.00	1.08-3.69	0.027	1.94	1.08-3.52	0.028
rs7886938	*FMO6*	1	0.170	A	2.14	1.06-4.32	0.033	0.83	0.22-3.17	0.788	1.75	0.94-3.25	0.078	NA	NA	NA
rs7889839	*FMO6*	1	0.170	G	2.14	1.06-4.32	0.033	NA	NA	NA	NA	NA	NA	NA	NA	NA
rs688755[a]	*CYP4F12*	19	0.228	C	2.06	1.07-3.98	0.031	0.73	0.29-1.83	0.499	1.29	0.47-3.57	0.623	NA	NA	NA
rs593421	*CYP4F12*	19	0.221	C	2.02	1.06-3.84	0.033	NA	NA	NA	NA	NA	NA	NA	NA	NA

OR, odds ratio; 95% CI, 95% confidence interval; NA, not analyzed.
OR and 95% CI are reported for the minor allele, i.e. an OR<1 indicates risk of progression for the major allele.
Variants within *CYP4F12*, *CYP8B1* and *FMO6* are in linkage disequilibrium (LD) with r^2>0.80. LD statistic in this cohort for variants in *SLC7A8* are r^2=0.78 and D'=0.89.

[a] I^2>50.

Meta-analysis

A meta-analysis of the association analysis results was performed of the seven variants genotyped in both the discovery and validation cohort. Two variants in the *SLC7A8* locus independently validated and the effect further reinforces when combined in a meta-analysis. The rs1884545 variant showed a significant protective effect in patients with EPD with an odds ratio (OR) of 0.16 (95% confidence interval 0.05-0.50) (*Table 2, Table S3*). In both the discovery and validation cohorts, none of the patients with EPD were homozygous for the T allele, whereas in the control groups of the discovery and validation cohort, 1.2% and 2.2% of patients were homozygous, respectively. In addition, a substantially lower percentage of patients were heterozygous in the EPD group (7.7% in discovery and 5.6% in validation) compared to controls (25.0% and 24.2%). As linkage disequilibrium between the two *SLC7A8* variants is high (R^2 = 0.78), these numbers were comparable for the C allele of rs8013529. In addition to the *SLC7A8* variants, three other variants (*CYP8B1* rs6771233, *SLC22A2* rs316003, *SLC22A5* rs274548) were significantly associated with EPD in the meta-analysis, showing a stronger association compared to the results of the discovery or validation cohorts alone.

Based on the meta-analysis results of the discovery and validation cohort, five variants were investigated in a second validation cohort, including 27 additional patients. In a meta-analysis including all samples investigated, a significant association of *SLC7A8* rs1884545 with an OR of 0.22 (0.07-0.63) and of *SLC7A8* rs8013529 with an OR of 0.19 (0.06 – 0.55) was observed, corresponding to an approximately four to five-fold protective effect. Despite that, the association did not become stronger compared to the meta-analysis without these additional 27 patients (*Table 2*). The other variants, *SLC22A5* rs274548, *CYP8B1* rs6771233, and *SLC22A2* rs316003, remained significantly associated with EPD, with the latter two showing a stronger association.

Validation of LAT2 overexpression model

SLC7A8 codes for the L-type amino acid transporter 2 (LAT2), which mainly transports neutral amino acids and is also involved in the transport of multiple medicines, e.g. gabapentin and L-DOPA. This transporter is most active when it is bound to its heterodimer 4F2. In order to study the role of the LAT2 transporter in chemotherapeutic treatment of osteosarcoma, an *in vitro* LAT2-4F2 overexpression cell model was developed in HEK293 cells. The Western blot with the anti-LAT2 antibody in *Figure 1A* only shows bands in the LAT2-HEK293 cells and the LAT2-4F2-HEK293 cells, indicating a LAT2 overexpression effect. Radiolabeled ^{3}H-L-Alanine was used to functionally test and characterize LAT2-mediated transport in this model. *Figure 1B* shows that overexpression of LAT2 alone causes significant (P=0.0001) uptake compared to control-transduced cells when cells are exposed to 0.017 µM ^{3}H-L-alanine for 2 minutes. It was confirmed that 4F2-HEK293 cells do not have any transport activity. Overexpression of both LAT2 and 4F2 further enhanced L-alanine uptake (P<0.0001).

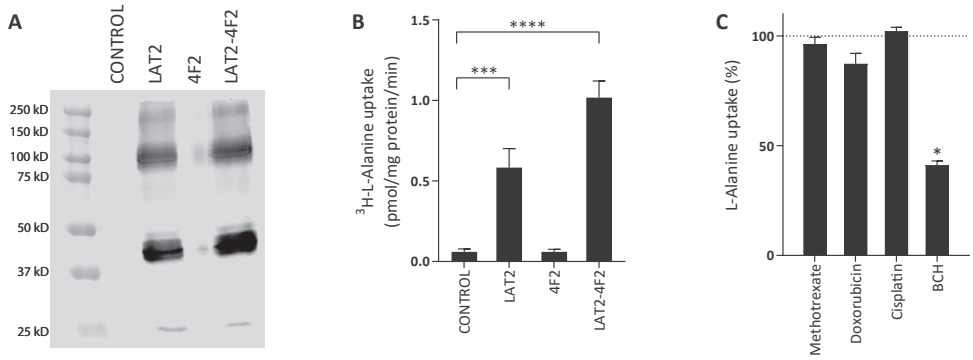


Figure 1. Western blot (A) and functional validation (B and C) of the transport assay in HEK293 cells overexpressing the L-type amino acid transporter 2 (LAT2). Figure B confirms ^{3}H-L-alanine as a substrate of LAT2 and LAT2-4F2, after exposure to 0.018 µM of ^{3}H-L-Alanine for 2 minutes at 37˚C. Data is expressed as mean ± SD (N=3). Figure C shows L-alanine (10 µM) uptake, in the presence of cisplatin (1 mM), doxorubicin (1 mM), methotrexate (1 mM) or BCH (1 mM), after 1 minute incubation at 37˚C. BCH is a known inhibitor of LAT2-4F2 and was therefore included as a positive control. Inhibition was expressed as a percentage ± SD of L-alanine uptake with solvent control only, which was fixed at 100% (N=3).

To further characterize transport, time and concentration dependent uptake was studied in a time curve and Michaelis-Menten curve (*Figure S1*). As shown in *Figure S1a*, the uptake rate is linear until two minutes, and therefore, the Michaelis-Menten curve was performed with an incubation time of one minute. The K_m for LAT2-4F2 mediated L-alanine uptake was estimated at 598 µM, with 95% confidence interval of 304 µM – 892 µM (*Figure S1b*).

Interaction of LAT2-4F2 with cisplatin, doxorubicin and methotrexate

The association of a locus in the *SLC7A8* gene to EPD may be caused by an interaction of LAT2 with chemotherapeutics that are used in the treatment of osteosarcoma. First, potential inhibition of LAT2-4F2-mediated L-alanine transport was measured for cisplatin, doxorubicin and methotrexate. BCH is a known amino-acid transport inhibitor and inhibitor of LAT2 and was used as a positive control. BCH (1 mM) significantly (p=0.01) inhibited L-alanine (10 µM) uptake transport after 1 minute exposure at 37˚C. Under the same conditions, cisplatin (1 mM), doxorubicin (1 mM) or methotrexate (1 mM) did not significantly inhibit L-Alanine (10 µM) transport in this cell model (*Figure 1C*).

Finally, transport assays were performed to assess if methotrexate, cisplatin or doxorubicin are substrates of LAT2-4F2 (*Figure 2*). No significantly increased uptake of ^{3}H-methotrexate by LAT2-4F2 (P=0.1) was found compared to the control. The accumulation of cisplatin was measured as platinum uptake by ICP-MS. As this technique was not previously used in this assay, a positive control transporter (OCT2) was also included. The uptake of cisplatin by OCT2 compared to control was significant ($p < 0.0001$,

Figure S2), but no uptake of cisplatin by LAT2-4F2 was measured. Finally, doxorubicin was identified as a substrate of LAT2-4F2 ($p < 0.0001$, *Figure 2B*) and transport of doxorubicin (10 μM) was inhibited to 81% (SD = 5.2%) by BCH (1 mM) under the same experimental conditions ($p = 0.0032$, *data not shown*). Altogether, doxorubicin, and not methotrexate or cisplatin, was shown to be a substrate for LAT2 which gives novel implications for the role of LAT2 in osteosarcoma therapy response to doxorubicin.

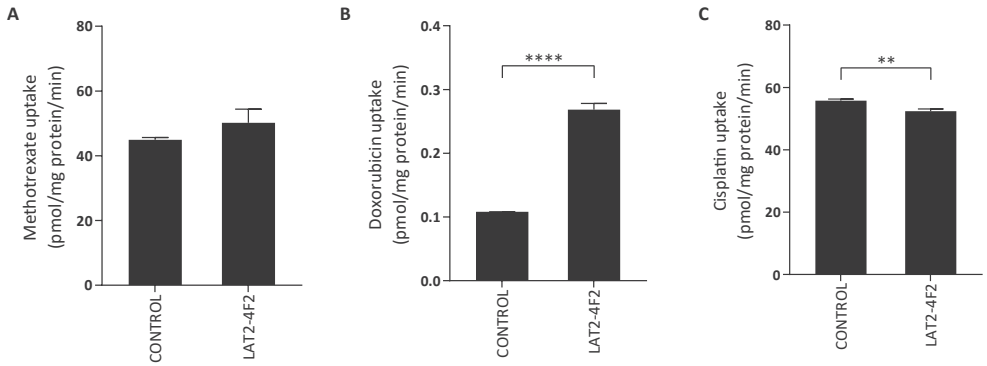


Figure 2. Transport assay to assess if methotrexate (A), doxorubicin (B) or cisplatin (C) are substrates for LAT2-4F2. HEK293 cells were incubated with methotrexate (500 μM), doxorubicin (500 μM) and cisplatin (330 μM) for 30 minutes at 37˚C. No significant difference in uptake of methotrexate or cisplatin was found in LAT2-4F2-HEK293 cells ($p = 0.1$, $p=0.0028$, resp.) compared control cells. Doxorubicin uptake was significant in LAT2-2F4-HEK293 cells ($p < 0.0001$). All data is expressed as mean ± SD (N=3).

Immunohistochemistry and mRNA expression analysis

Protein expression analysis through IHC and mRNA expression analysis of osteosarcoma tissue at diagnosis was performed to assess if *SLC7A8* and LAT2 expression affects disease progression and survival. The presence of metastasis at diagnosis is the best clinical predictor of poor treatment outcome in patients with osteosarcoma, and therefore, this was also taken into account and analyses were stratified for metastases. *Figure 3A and S5* shows poorest OS and DFS, respectively, in patients with metastases at diagnosis and without LAT2 expression. Best DFS and OS was observed patients without metastases at diagnosis and with LAT2 expression ($p_{DFS}=0.025$, $p_{OS}=0.017$). This indicates that LAT2 expression may enhance survival, especially in patients without metastases at diagnosis. Similarly, *SLC7A8* mRNA expression is significantly associated with overall survival in patients without metastases ($p = 0.0099$, *Figure 3*).

p-mTOR was stained in IHC as mTOR phosphorylation was previously implicated to be a downstream process of LAT2-mediated amino-acid uptake (18-20). A representable example of LAT2 expression in tissue is displayed in *Figure S3. Table S4* describes that LAT2 expression was present in 25.5% of patients and p-mTOR in 27.9% of patients. Expression of LAT2 or p-mTOR was not significantly associated to the *SLC7A8* rs1884545

T allele or EPD (*Table S5*). In addition, LAT2 expression was not significantly associated to p-mTOR expression (OR (95%CI) = 3.5 (0.8-15.947), p=0.117). Although not significant, p-mTOR expression showed a trend towards poorer DF disease-free survival (DFS) (p_{DFS}=0.08, p_{OS}=0.92, *Figure S4*) and LAT2 expression may improve DFS and overall survival (OS), however this was neither significant (p_{DFS}=0.15, p_{OS}=0.081, *Figure S4*).

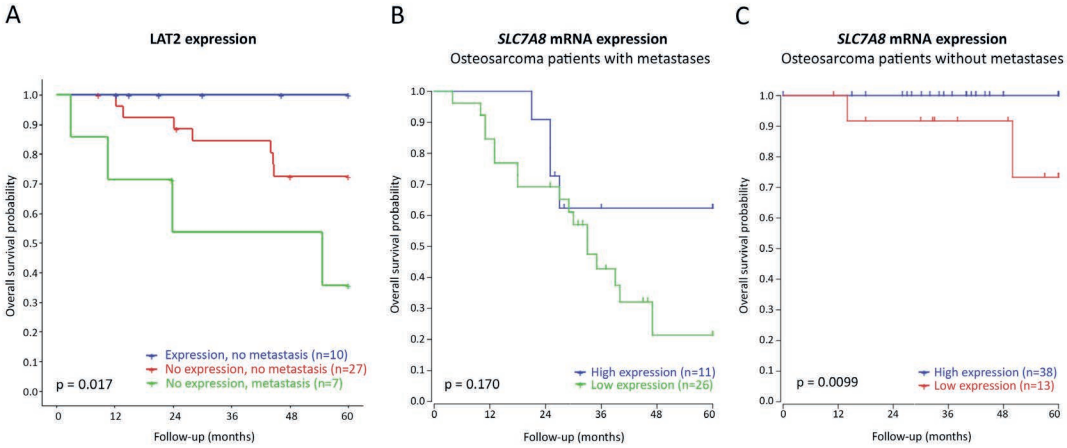


Figure 3. Overall survival of osteosarcoma patients according to or LAT2 protein expression (A), stratified for the presence of metastasis, or *SLC7A8* mRNA expression (B and C). Protein expression was determined by immunohistochemistry in the discovery cohort of this study and no patients with LAT2 expression and metastases at diagnosis were identified. mRNA expression was analyzed in an independent patient cohort (available from Kuijjer *et al*. (15, 16)).

Discussion

In our study we provided evidence that five genetic variants are associated with EPD in osteosarcoma, of which a locus in *SLC7A8* was confirmed in an independent validation cohort. In addition, three variants in other genes showed a similar direction of effect in the discovery and validation cohorts and were significantly associated in meta-analyses. Functional analysis showed that L-type amino acid transporter 2 (LAT2, gene *SLC7A8*) is involved in the transport of doxorubicin. Our results may have novel implications for personalized treatment of patients with osteosarcoma, because we suggest that LAT2-mediated doxorubicin uptake in osteosarcoma tumor cells could play an important role in treatment resistance and eventually treatment response.

In this study, we identified a novel locus in the *SLC7A8* gene to be associated with EPD in patients with osteosarcoma. The *SLC7A8* gene encodes the LAT2 transporter present in the basolateral membrane of the proximal tubule in the kidney, as well as

in the colon and intestine (21). LAT2 (light chain) is active when it forms a heterodimer with 4F2 (heavy chain) via a disulphide bond and is involved in the uptake and efflux of large and small neutral amino acids. At our current state of knowledge, little is known on the role and function of LAT2 in osteosarcoma or osteosarcoma treatment response, although it has been assessed in pharmacogenetic studies in other cancer types. Other germline variants and in this gene, that are not in linkage disequilibrium (LD) with the locus we identified, have been linked to outcome after platinum-based therapy in esophageal cancer (22). Acquired mutations in *SLC7A8* in the same patient group caused resistance to cisplatin-verapamil combination therapy and these results were reinforced by functional experiments (23). When looking at expression of LAT2 in tumor tissues, LAT2 expression was associated with improved survival in estrogen receptor positive (ER+) breast cancer (24, 25) and in lung cancer (26). El Ansari *et al.* describes that *SLC7A8* mRNA expression and LAT2 protein expression in ER+ breast cancer were strongly associated with good prognostic features. Conversely, LAT2 expression was associated with poorer survival in pancreatic cancer and was indicated to play a role in the pathogenesis of glomerulonephritis, both through LAT2-mediated mTOR activation (18, 20).

Our results show that patients with expression of LAT2 in osteosarcoma tissue may have better survival compared to patients without expression of LAT2, especially in patients that present without metastases. Previous literature showed that amino acid uptake by LAT2 could enhance mTOR phosphorylation leading to mTORC1 activation and thereby a less favourable prognosis (18-20). Therefore, p-mTOR expression was assessed by immunohistochemistry. Based on this hypothesis it would be expected that high LAT2 expression is associated with p-mTOR and both would result in poorer overall survival (OS) and disease-free survival (DFS). However, our non-significant results are in contrast with those expected if LAT2 led to mTORC1 phosphorylation suggesting no LAT2-mediated mTOR phosphorylation in tissue of osteosarcoma patients.

Apart from large and small neutral amino acids, other studies have shown that LAT2 also contributes to the transport of various medicines e.g. L-DOPA, melphalan, baclofen, gabapentin, and thyroid hormones (27). However, a potential interaction between LAT2 and osteosarcoma chemotherapeutics has never been investigated. In this study, we found that doxorubicin is a Na^+-independent substrate of LAT2. Doxorubicin was not an inhibitor of L-alanine in our assay. This could indicate that the LAT2 affinity for L-alanine is greater than that to doxorubicin. This may lead to L-alanine to be the superior substrate in competition with doxorubicin, resulting in the absence of an inhibitory effect of doxorubicin. In combination with the association of LAT2 expression in tumor tissue with improved survival in patients without metastasis, LAT2-mediated doxorubicin transport could mechanistically explain the association of genetic variation in *SLC7A8* that is associated with EPD in patients with osteosarcoma. A previous study in neuroendocrine tumors showed that LAT1 and LAT2 expression in tumor cells caused increased L-DOPA uptake (28). We hypothesize that increased LAT2 expression in osteosarcoma tissue could lead to increased uptake of doxorubicin, therefore higher intracellular exposure and improved cell death. Future research should further address this to translate the findings of this study to the clinical setting.

The genetic variants in the *SLC7A8* locus associated with EPD include a synonymous variant (rs1884545) and an intron variant (rs8013529). The exact effect of these variants on the protein function or on protein expression remains unknown. According to data from the ENCODE project, rs1884545 is more likely to have an effect on regulation of SLC7A8 expression than rs8013529. Regulation could be the result of histone modifications (H3K4me1 histone mark), transcription factor binding (POLR2A, CTCF, etc.) and DNase hypersensitivity peaks. However, no expression quantitative trait loci (eQTLs) are found to indicate an association of one of the variants to protein expression. The effect might as well be caused by linked variants, or even a combination of multiple linked rare variants. Additional studies, for example through fine-mapping or functional experiments are necessary to give additional insight.

Notably, two other genes (*SLC22A2,* Solute carrier family 22 member 2 and *SLC22A5,* Solute carrier family 22 member 5) of the four identified genes also encode transporters that function in the kidneys. *SLC22A2*, which codes for the organic cation transporter 2 (OCT2) is implicated in the transport of cisplatin in tubular cells, which was also confirmed in this study (*Figure S2*). A genetic variant in the gene (rs316019, not in LD with rs316003) has been linked to cisplatin induced nephrotoxicity (29-31). *SLC22A5* is involved in the reabsorption of carnitine by the proximal tubular cells. Studies have indicated that cisplatin inhibits *SLC22A5* functioning also leading to nephrotoxicity (32, 33). Unfortunately, we could not further investigate the relation between the identified variants in *SLC22A2* and *SLC22A5* and nephrotoxicity, as the availability of information on the adverse drug event in our study cohort was only limited. The fourth identified gene (*CYP8B1,* Cytochrome P450 8B1) is expressed in hepatocytes and is involved in bile acid production and glucose homeostasis (34). Thus far, no clear connection between this gene and chemotherapy treatment has been reported.

We have pharmacogenetically studied the largest cohort of osteosarcoma patients with EPD to date and are the first to include independent validation cohorts. Nevertheless, patient numbers are small for genetic association studies. This however reflects the rarity of the disease and even for these numbers, international collaboration was required. Therefore, we consider the current study as a first but important step into the pharmacogenetic background of a suboptimal drug response in patients with osteosarcoma. As we have retrospectively included patients diagnosed over the past decades, during which imaging techniques have improved, it is possible that we have missed some cases of EPD in patients diagnosed in the early years. In addition, the discovery cohort was heterogeneous regarding treatment protocols. A proportion of the patients received two drugs (doxorubicin and cisplatin), whereas others received drugs in addition to cisplatin and doxorubicin (mostly only MTX), which could give a more favorable outcome. However, there was no significant effect of the presence of MTX in the treatment regimen on EPD in our cohort, which makes it likely that the influence of the differences in treatment regimens on the results is limited. Furthermore, because we studied germline variants, we may have missed tumor-specific mutations in genes involved in the uptake of the chemotherapeutic drugs, although studies on genetic variants in genes involved in drug metabolism and transport showed high concordance between DNA derived from tumor and blood or saliva (35). In addition, the tumor genetic background is also likely to define the intrinsic response to chemotherapy (36).

The goal of identifying pharmacogenetic variants involved in EPD is to enable pre-treatment identification of patients who are at pharmacogenetic risk of a poor response to treatment with conventional chemotherapy. The genetic loci identified in the present study are not yet discriminative for implementation in the clinical setting, but the evidence that is presented here does present novel opportunities for the future. Upfront identification of such patients could provide an opportunity to personalize therapy and help achieve a better balance of treatment outcome and toxicity burden. For example, other second-line treatments could be considered as first-line treatment for this subgroup of patients (37-42). In addition to such a clinical implementation, these genetic association studies are important to gain more insight into the mechanisms of action of the drugs investigated. This is illustrated by the outcome of the functional studies where doxorubicin is identified as a novel substrate of the LAT2 transporter in this study and cisplatin is linked to both *SLC22A2* and *SLC22A5* in literature (32, 33). In addition, these results may provide new leads for development of agents to modulate the response to chemotherapy.

From the patient's perspective, it is important to identify those at risk of a suboptimal response to chemotherapy and possible EPD. This study illustrates that it is indeed possible to distinguish this patient group as we identified pharmacogenetic variants specifically associated with EPD in osteosarcoma. Ultimately, clinical implementation of a validated (genetic) risk profile may enable treatment strategies specifically targeting this subgroup of patients.

Acknowledgements

We thank all patients who participated in the study. We thank Christel van Riel for patient inclusion, Sita Vermeulen for genetic epidemiological advice, and Marco Tiller from Leiden University Medical Center (LUMC), Saskia van der Marel, Marlies Naber, Marina Hakobjan, Mascha Schijvenaars from Radboud university medical center for technical assistance. We also thank Judith Bovee, Lynne Ball, and Dorine Bresters from LUMC for providing samples or patients. From the Sydney Children's Hospitals Network Tumour Bank, we thank Aedan Roberts and Li Zhou for sample acquisition and acknowledge The Kids Cancer Project for its funding for the Tumour Bank. This work was supported by the Spanish Association against Cancer (AECC: Asociación Española contra el Cáncer).

References

1. Ries L, Smith M, Gurney J, Linet M, Tamra T, Young J, et al. Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975-1995. Bethesda, MD: National Cancer Institute, SEER Program; 1999. Contract No.: NIH Pub. No. 99-4649.
2. Yang LM, Li XH, Bao CF. Glutathione S-transferase P1 and DNA polymorphisms influence response to chemotherapy and prognosis of bone tumors. Asian Pacific journal of cancer prevention : APJCP. 2012;13(11):5883-6.
3. Liu ZF, Asila AL, Aikenmu K, Zhao J, Meng QC, Fang R. Influence of ERCC2 gene polymorphisms on the treatment outcome of osteosarcoma. Genetics and molecular research : GMR. 2015;14(4):12967-72.
4. Sun XH, Hou WG, Zhao HX, Zhao YL, Ma C, Liu Y. Single nucleotide polymorphisms in the NER pathway and clinical outcome of patients with bone malignant tumors. Asian Pacific journal of cancer prevention : APJCP. 2013;14(3):2049-52.
5. Bai SB, Chen HX, Bao YX, Luo X, Zhong JJ. Predictive impact of common variations in DNA repair genes on clinical outcome of osteosarcoma. Asian Pacific journal of cancer prevention : APJCP. 2013;14(6):3677-80.
6. Zhao YL, Yang LB, Geng XL, Zhou QL, Qin H, Yang L, et al. The association of XPG and MMS19L polymorphisms response to chemotherapy in osteosarcoma. Pakistan journal of medical sciences. 2013;29(5):1225-9.
7. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(2):407-14.
8. Caronia D, Patino-Garcia A, Perez-Martinez A, Pita G, Moreno LT, Zalacain-Diez M, et al. Effect of ABCB1 and ABCC3 polymorphisms on osteosarcoma survival after chemotherapy: a pharmacogenetic study. PLoS One. 2011;6(10):e26091.
9. Windsor RE, Strauss SJ, Kallis C, Wood NE, Whelan JS. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. Cancer. 2012;118(7):1856-67.
10. Hagleitner MM, Coenen MJ, Jeuken JW, Flucke U, Schreuder HW, te Loo DM, et al. Taqman genotyping assays can be used on decalcified and paraffin-embedded tissue from patients with osteosarcoma. Pediatr Blood Cancer. 2011;56(1):35-8.
11. Vos HI, van der Straaten T, Coenen MJ, Flucke U, Te Loo DM, Guchelaar HJ. High-quality genotyping data from formalin-fixed, paraffin-embedded tissue on the drug metabolizing enzymes and transporters plus array. The Journal of molecular diagnostics : JMD. 2015;17(1):4-9.
12. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81(3):559-75.
13. El-Sheikh AA, van den Heuvel JJ, Koenderink JB, Russel FG. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. J Pharmacol Exp Ther. 2007;320(1):229-35.
14. Khunweeraphong N, Nagamori S, Wiriyasermkul P, Nishinaka Y, Wongthai P, Ohgaki R, et al. Establishment of stable cell lines with high expression of heterodimers of human 4F2hc and human amino acid transporter LAT1 or LAT2 and delineation of their differential interaction with alpha-alkyl moieties. J Pharmacol Sci. 2012;119(4):368-80.
15. Kuijjer ML, Rydbeck H, Kresse SH, Buddingh EP, Lid AB, Roelofs H, et al. Identification of osteosarcoma driver genes by integrative analysis of copy number and gene expression data. Genes Chromosomes Cancer. 2012;51(7):696-706.

16. R2: Genomics Analysis and Visualization Platform [Available from: http://r2.amc.nl.
17. Hagleitner MM, Hoogerbrugge PM, van der Graaf WT, Flucke U, Schreuder HW, te Loo DM. Age as prognostic factor in patients with osteosarcoma. Bone. 2011;49(6):1173-7.
18. Feng M, Xiong G, Cao Z, Yang G, Zheng S, Qiu J, et al. LAT2 regulates glutamine-dependent mTOR activation to promote glycolysis and chemoresistance in pancreatic cancer. J Exp Clin Cancer Res. 2018;37(1):274.
19. Wang Q, Holst J. L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. Am J Cancer Res. 2015;5(4):1281-94.
20. Kurayama R, Ito N, Nishibori Y, Fukuhara D, Akimoto Y, Higashihara E, et al. Role of amino acid transporter LAT2 in the activation of mTORC1 pathway and the pathogenesis of crescentic glomerulonephritis. Lab Invest. 2011;91(7):992-1006.
21. Rossier G, Meier C, Bauch C, Summa V, Sordat B, Verrey F, et al. LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine. The Journal of biological chemistry. 1999;274(49):34948-54.
22. Rumiato E, Boldrin E, Malacrida S, Battaglia G, Bocus P, Castoro C, et al. A germline predictive signature of response to platinum chemotherapy in esophageal cancer. Translational research : the journal of laboratory and clinical medicine. 2016;171:29-37.e1.
23. Min Q, Wang Y, Wu Q, Li X, Teng H, Fan J, et al. Genomic and epigenomic evolution of acquired resistance to combination therapy in esophageal squamous cell carcinoma. JCI Insight. 2021;6(17).
24. El Ansari R, Alfarsi L, Craze ML, Masisi BK, Ellis IO, Rakha EA, et al. The solute carrier SLC7A8 is a marker of favourable prognosis in ER-positive low proliferative invasive breast cancer. Breast Cancer Res Treat. 2020;181(1):1-12.
25. Thakkar A, Raj H, Ravishankar, Muthuvelan B, Balakrishnan A, Padigaru M. High Expression of Three-Gene Signature Improves Prediction of Relapse-Free Survival in Estrogen Receptor-Positive and Node-Positive Breast Tumors. Biomark Insights. 2015;10:103-12.
26. Asada K, Kobayashi K, Joutard S, Tubaki M, Takahashi S, Takasawa K, et al. Uncovering Prognosis-Related Genes and Pathways by Multi-Omics Analysis in Lung Cancer. Biomolecules. 2020;10(4).
27. del Amo EM, Urtti A, Yliperttula M. Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. Eur J Pharm Sci. 2008;35(3):161-74.
28. Barollo S, Bertazza L, Watutantrige-Fernando S, Censi S, Cavedon E, Galuppini F, et al. Overexpression of L-Type Amino Acid Transporter 1 (LAT1) and 2 (LAT2): Novel Markers of Neuroendocrine Tumors. PLoS One. 2016;11(5):e0156044.
29. Zhang J, Zhou W. Ameliorative effects of SLC22A2 gene polymorphism 808 G/T and cimetidine on cisplatin-induced nephrotoxicity in Chinese cancer patients. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2012;50(7):2289-93.
30. Iwata K, Aizawa K, Kamitsu S, Jingami S, Fukunaga E, Yoshida M, et al. Effects of genetic variants in SLC22A2 organic cation transporter 2 and SLC47A1 multidrug and toxin extrusion 1 transporter on cisplatin-induced adverse events. Clinical and experimental nephrology. 2012;16(6):843-51.
31. Filipski KK, Mathijssen RH, Mikkelsen TS, Schinkel AH, Sparreboom A. Contribution of organic cation transporter 2 (OCT2) to cisplatin-induced nephrotoxicity. Clin Pharmacol Ther. 2009;86(4):396-402.
32. Haschke M, Vitins T, Lude S, Todesco L, Novakova K, Herrmann R, et al. Urinary excretion of carnitine as a marker of proximal tubular damage associated with platin-based antineoplastic drugs. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010;25(2):426-33.

33. Lancaster CS, Hu C, Franke RM, Filipski KK, Orwick SJ, Chen Z, et al. Cisplatin-induced downregulation of OCTN2 affects carnitine wasting. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(19):4789-99.

34. Kaur A, Patankar JV, de Haan W, Ruddle P, Wijesekara N, Groen AK, et al. Loss of Cyp8b1 improves glucose homeostasis by increasing GLP-1. Diabetes. 2015;64(4):1168-79.

35. van Huis-Tanja L, Kweekel D, Gelderblom H, Koopman M, Punt K, Guchelaar HJ, et al. Concordance of genotype for polymorphisms in DNA isolated from peripheral blood and colorectal cancer tumor samples. Pharmacogenomics. 2013;14(16):2005-12.

36. Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM. Genome-wide analyses on high-grade osteosarcoma: making sense of a genomically most unstable tumor. International journal of cancer Journal international du cancer. 2013;133(11):2512-21.

37. Harris MB, Cantor AB, Goorin AM, Shochat SJ, Ayala AG, Ferguson WS, et al. Treatment of osteosarcoma with ifosfamide: comparison of response in pediatric patients with recurrent disease versus patients previously untreated: a Pediatric Oncology Group study. Medical and pediatric oncology. 1995;24(2):87-92.

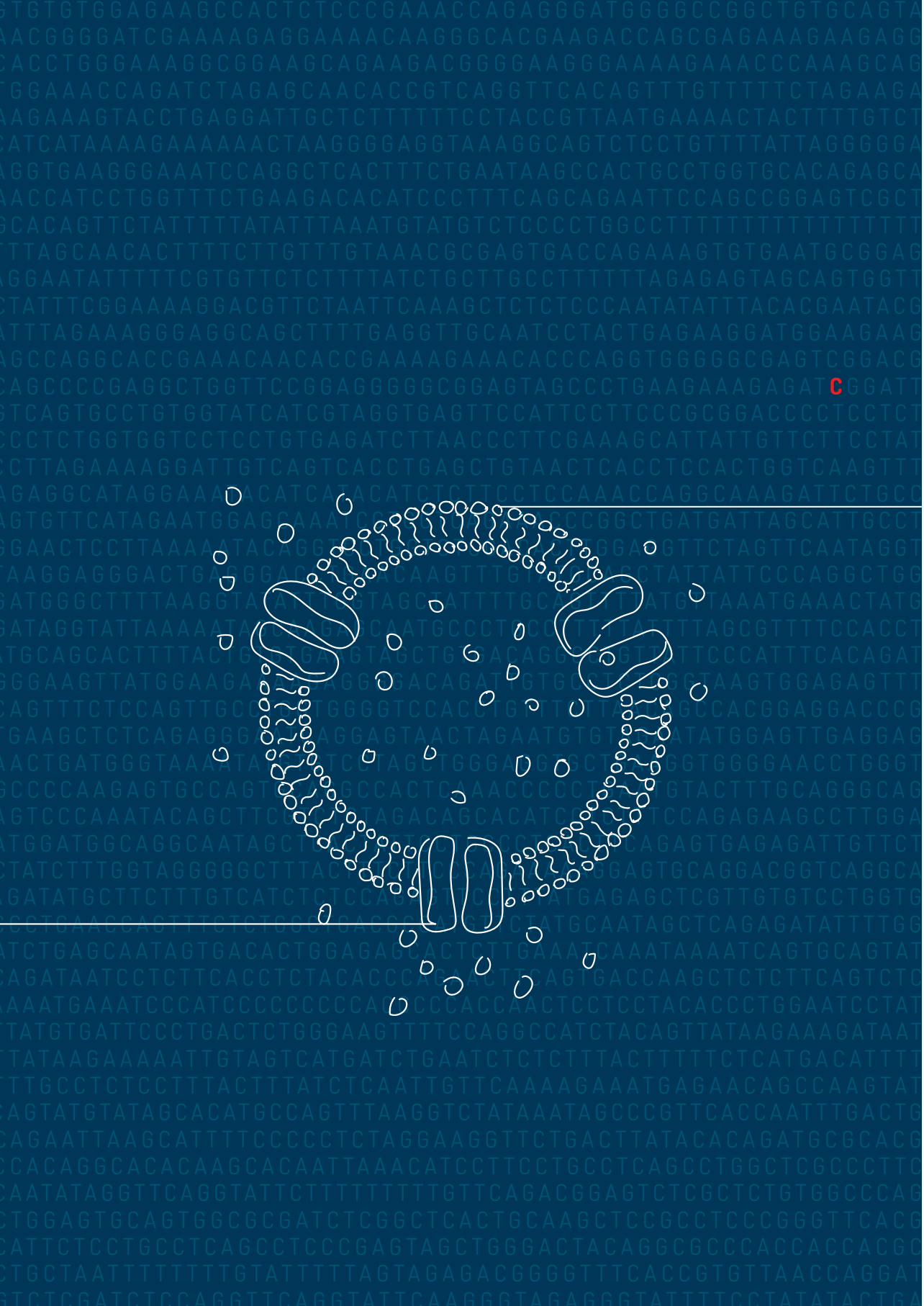
38. Rodriguez-Galindo C, Daw NC, Kaste SC, Meyer WH, Dome JS, Pappo AS, et al. Treatment of refractory osteosarcoma with fractionated cyclophosphamide and etoposide. Journal of pediatric hematology/oncology. 2002;24(4):250-5.

39. Berger M, Grignani G, Ferrari S, Biasin E, Brach del Prever A, Aliberti S, et al. Phase 2 trial of two courses of cyclophosphamide and etoposide for relapsed high-risk osteosarcoma patients. Cancer. 2009;115(13):2980-7.

40. Navid F, Willert JR, McCarville MB, Furman W, Watkins A, Roberts W, et al. Combination of gemcitabine and docetaxel in the treatment of children and young adults with refractory bone sarcoma. Cancer. 2008;113(2):419-25.

41. Song BS, Seo J, Kim DH, Lim JS, Yoo JY, Lee JA. Gemcitabine and docetaxel for the treatment of children and adolescents with recurrent or refractory osteosarcoma: Korea Cancer Center Hospital experience. Pediatr Blood Cancer. 2014;61(8):1376-81.

42. Grignani G, Palmerini E, Ferraresi V, D'Ambrosio L, Bertulli R, Asaftei SD, et al. Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: a non-randomised phase 2 clinical trial. Lancet Oncol. 2015;16(1):98-107.



CHAPTER 4

Supplementary materials

Novel pharmacogenetic association of SLC7A8 locus with early disease progression in osteosarcoma leads to discovery of doxorubicin as a LAT2 substrate.

Supplementary methods

Inductively coupled plasma mass spectrometry to measure platinum concentration

Cisplatin uptake concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS). For this, a Thermo Scientific (Waltham, Massachusetts, USA) iCAP TQ mass spectrometer was used (in SQ modus). The system operated under the standard conditions, briefly: Rf power 1550 W, argon gas flow rates: cooling 14 L/min, auxiliary 0.8 L/min, nebulizer 1.03 L/min, sampler, skimmer: nickel, spray chamber: cyclone, 3 °C, torch: quartz, nebulizer: concentric, data acquisition: masses 89, 195, sweeps 10, dwell time 100 ms. The calibration standards were prepared from commercially available stock solutions. The platinum standard (1000 mg/L) was supplied by VWR (Leicestershire, England). Yttrium, used as the internal standard, was prepared from a 1000 mg/L solution obtained from Merck (Darmstadt, Germany). Measurements were performed in a matrix of 0.1 M NaOH, which is consistent with the lysis buffer of cells in the transport experiment (Merck - Darmstadt, Germany).

Immunohistochemistry

Immunohistochemistry (IHC) staining on 4 µm thick slides from eight osteosarcoma tissue micro arrays (TMAs) (one from the Australian cohort and seven from the Dutch cohort), with one or two 2.0 mm cores per tumor samples from representative tumor areas, was performed to assess LAT2 protein expression and p-mTOR expression to assess possible LAT2 mediated mTOR phosphorylation. Pancreas and kidney served as positive controls for LAT2 and p-mTOR, respectively. Sections were deparaffinized in xylene and rehydrated through a graded ethanol into water series. Antigen retrieval was performed by heating the slides in citrate buffer, pH6 for 10 min (p-mTOR) at 100 °C or heating the slides in the 2100-Retriever (Diagnostic Technology, Belrose, Australia) (LAT2). Endogenous peroxidase activity was blocked with 3% H2O2 in distilled water for 10 min at room temperature. Subsequently, sections were incubated with monoclonal mouse anti-LAT2 antibody (1:50, UMAB70 antibody, OriGene Technologies, Inc., Rockville, USA) or monoclonal rabbit anti-phospho-mTOR (Ser2448) (1:50, #2976, Cell Signaling Technology, Danvers, USA) in antibody diluent in a humidified chamber overnight at 4 °C. Next, tissue sections were incubated with Poly-HRP-GAMs/Rb IgG (ImmunoLogic, Duiven, The Netherlands) in EnVision™ FLEX Wash Buffer (Dako, Agilent Technologies, Santa Clara, CA, USA) (1:1) for 30 min at room temperature. Antibody binding was visualized using the EnVision™ FLEX Substrate Working Solution (Dako) for 10 min at room temperature. Finally, slides were counterstained with haematoxylin, dehydrated and coverslipped. Only tissue samples from diagnostic biopsies, that were not exposed to chemotherapy, were analyzed. TMAs were scored by two independent observers on percentage of cells with expression (none, <10%, 10-50% or >50%) and intensity of expression (0, + or ++). Differences between observers were resolved by consensus. Eventually, expression data were dichotomized and tissues with expression ≥10% of cells were considered positive for LAT2 or p-mTOR expression.

Table S1. Clinical characteristics of osteosarcoma patients of the discovery cohort, including patients without sufficient DNA for DMET array genotyping, but enough for manual genotyping of genetic variants significantly associated to progressive disease in the original patient cohort. As this mostly concerns deceased patients, it is critical to involve this group that was not randomly missing.

	Original discovery cohort		**Additional patients with limited DNA**		**Total cohort**	
	Progression (*N*=39)	**Controls (*N*=168)**	**Progression (*N*=11)**	**Controls (*N*=5)**	**Progression (*N*=50)**	**Controls (*N*=173)**
Age at diagnosis, median (range)	17.2 (6.8-44.7)	15.2 (3.4-45.8)	17.7 (2.8-34.1)	19.9 (14.6-44.5)	17.2 (2.8-44.7)	15.3 (3.4-45.8)
Male sex, *n* (%)	29 (74.4%)	81 (48.2%)	8 (72.7%)	3 (60%)	37 (74%)	84 (48.6%)
Axial tumor, *n* (%)	4 (10.3%)	5 (2.98%)	2 (18.2%)	2 (40%)	6 (12%)	7 (4%)
Primary metastases, *n* (%)	16 (41.0%)	16 (9.52%)	4 (36.4%)	2 (40%)	20 (40%)	18 (10.4%)
Cumulative dose (mg/m^2) Cisplatin[a], median (range) Doxorubicin, median (range)	 400 (100-600) 450 (150-450)	 480 (200-720) 450 (150-455)	 300 (200-600) 450 (420-550)	 540 (260-600) 450 (405-450)	 400 (100-600) 450 (150-550)	 480 (200-720) 450 (150-455)
MTX treatment, *n* (%)	27 (69.2%)	90 (53.6%)	3 (27.3%)	1 (20%)	40 (60%)	91 (52.6%)
Poor histologic response[b], *n* (%)	28 (82.4%)	78 (48.8%)	6 (100%)	1 (50%)	34 (85%)	79 (48.5%)
5-year overall survival	28.2%	100%	0%	60%	22%	98.9%

MTX, methotrexate

Patients with recurrent disease are not included in the table: discovery cohort *N*=76; additional cohort *N*=9.

[a]Number of patients with cumulative dose of cisplatin data available; discovery cohort: progression *N*=39, controls *N*=165; additional cohort: all patients

[b]Number of patients with histologic response data available; discovery cohort: progression *N*=34, controls *N*=160; additional cohort: progression *N*=6, controls *N*=2

Table S2. Association analysis results for the discovery cohort, including 16 additional non-randomly excluded patients due to limited availability of DNA.

SNP	Gene	Chromosome	Minor allele	MAF	OR	95% CI	*P*-value
rs1884545	*SLC7A8*	14	T	0.12	0.27	0.1-0.77	0.014
rs8013529	*SLC7A8*	14	C	0.12	0.29	0.1-0.79	0.016
rs6771233	*CYP8B1*	3	C	0.34	1.51	0.89-2.56	0.129
rs316003	*SLC22A2*	6	G	0.21	0.52	0.26-1.06	0.073
rs274548	*SLC22A5*	5	T	0.14	1.62	0.81-3.25	0.170

MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval.
OR and 95% CI are reported for the minor allele, an OR<1 indicates a protective effect of the minor allele and risk of progression for the major allele.

Table S3. Association analysis results validation cohort and meta-analyses, including 16 patients with limited DNA available in discovery cohort.

				Validation						Second validation		
				Validation cohort			Meta-analysis			Meta-analysis overall		
SNP	**Gene**	**Chromosome**	**Minor allele**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
rs8013529	*SLC7A8*	14	C	0.05	0.005-0.63	0.02	0.21	0.08-0.55	0.001	0.24	0.09-0.59	0.002
rs1884545	*SLC7A8*	14	T	0.05	0.005-0.63	0.02	0.22	0.09-0.57	0.002	0.26	0.1-0.65	0.004
rs6771233	*CYP8B1*	3	A	1.7	0.78-3.72	0.184	1.57	1.01-2.43	0.045	1.61	1.05-2.49	0.03
rs316003	*SLC22A2*	6	G	0.58	0.20-1.69	0.318	0.54	0.3-0.97	0.041	0.54	0.31-0.96	0.035
rs274548	*SLC22A5*	5	T	1.75	0.59-5.18	0.311	1.66	0.93-2.98	0.089	1.64	0.93-2.89	0.086

OR, odds ratio; 95% CI, 95% confidence interval; NA, not analyzed.
OR and 95% CI are reported for the minor allele, i.e. an OR<1 indicates risk of progression for the major allele.
[a] I^2>50.

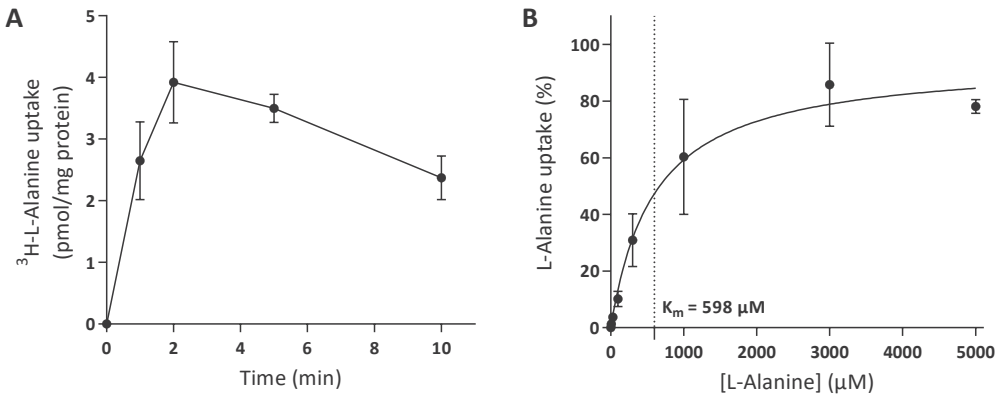


Figure S1. Time- and concentration dependent uptake of L-Alanine in LAT2-4F2-HEK293 cells. Figure A shows ^{3}H-L-Alanine uptake in LAT2-4F2-HEK293 cells after exposure of 0.017 µM ^{3}H-L-Alanine at 37˚C. The uptake rate is linear until 2 minutes, and therefore, the Michaelis-Menten curve was performed with 1 minute incubation time. For B, cells were exposed to increasing concentrations of L-Alanine and 0.017 µM ^{3}H-L-Alanine. The K_m for LAT2-4F2 mediated L-alanine uptake was estimated at 598 µM (95% CI = 304 µM – 892 µM). All signals were subtracted by the background signal that was measured in EYFP-HEK293 cells under the same conditions. Data in figure A is expressed as mean ± SD (N=3). Figure B is expressed as a percentage ± SD of V_{max}, which was fixed at 100% (N=3).

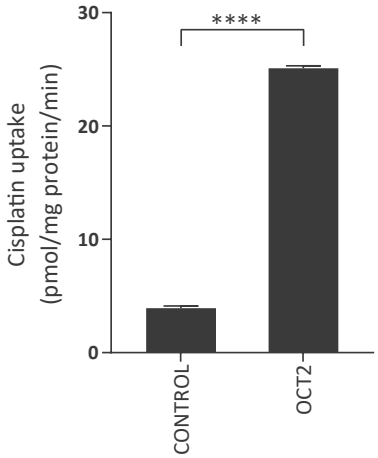


Figure S2. Transport assay to validate that platinum concentration measurements by ICP-MS are appropriate to measure uptake in this cell model. The *SLC22A2* gene codes for the organic cation transporter 2 (OCT2), which was previously indicated in the transport of cisplatin. 50 µM cisplatin was exposed for 10 minutes to EYFP-HEK293 cells and LAT2-4F2-HEK293 cells at 37˚C. The cisplatin uptake was significantly higher in LAT2-2F4-HEK293 cells ($p < 0.0001$). Altogether, it was concluded that cisplatin uptake was measured accordingly by ICP-MS. All data is expressed as mean ± SD (N=3).

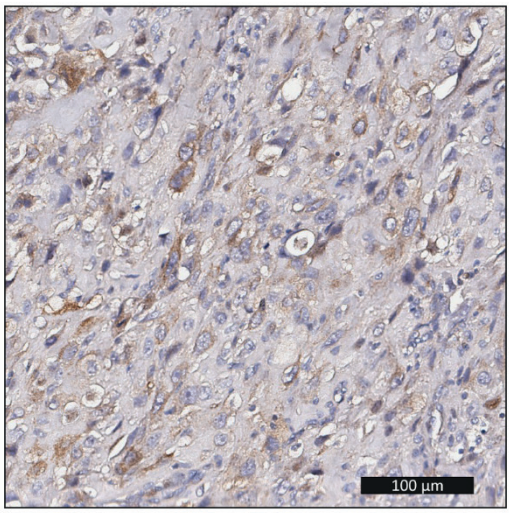


Figure S3. Representative example of LAT2 protein expression in osteosarcoma tissue at diagnosis. Brown staining indicates LAT2.

Table S4. Immunohistochemistry staining of LAT2 and p-mTOR in osteosarcoma tissue at diagnosis. Patients with <10% expression are considered controls and patients with ≥10% expression are considered cases.

	LAT2 expression			p-mTOR expression		
	All patients (N=47)	Progressive disease[a] (N=7)	No progressive disease (N=26)	All patients (N=43)	Progressive disease[a] (N=7)	No progressive disease (N=23)
Controls	35 (74.5%)	7 (100%)	17 (65.4%)	31 (72.1%)	4 (57.1%)	20 (87%)
Cases	12 (25.5%)	0 (0%)	9 (34.6%)	12 (27.9%)	3 (42.9%)	3 (13%)

[a] Progressive disease was defined as: (1) growth of the primary tumor (>20%) and/or metastases (>20%), or development of new lesions, in the time from start of primary treatment until 3 months after end of adjuvant chemotherapy or end of first-line treatment in case of primary metastatic disease, and/or (2) inadequacy to reach complete remission at the end of (surgical and chemotherapeutic) therapy for primary localized or primary metastatic osteosarcoma. The opposite extremes, patients showing an adequate drug response with no signs of relapse were considered controls. Thus patients with recurrent disease, defined as local or distant relapse from 3 months after end of primary treatment to end of follow-up, were excluded from the comparison for progressive disease.

Table S5. Association of LAT2 or p-mTOR protein expression with genetic variant or disease progression, calculated with X^2 test.

	LAT2 expression			p-mTOR expression		
	N	OR (95%CI)	*p*	N	OR (95%CI)	*p*
SLC7A8 rs1884545 T-allele	47	0.26 (0.03-2.33)	0.414	43	1.71 (0.40-7.43)	0.467
Progressive disease	33	0.65 (0.49-0.87)	0.149	30	5 (0.73-34.35)	0.361

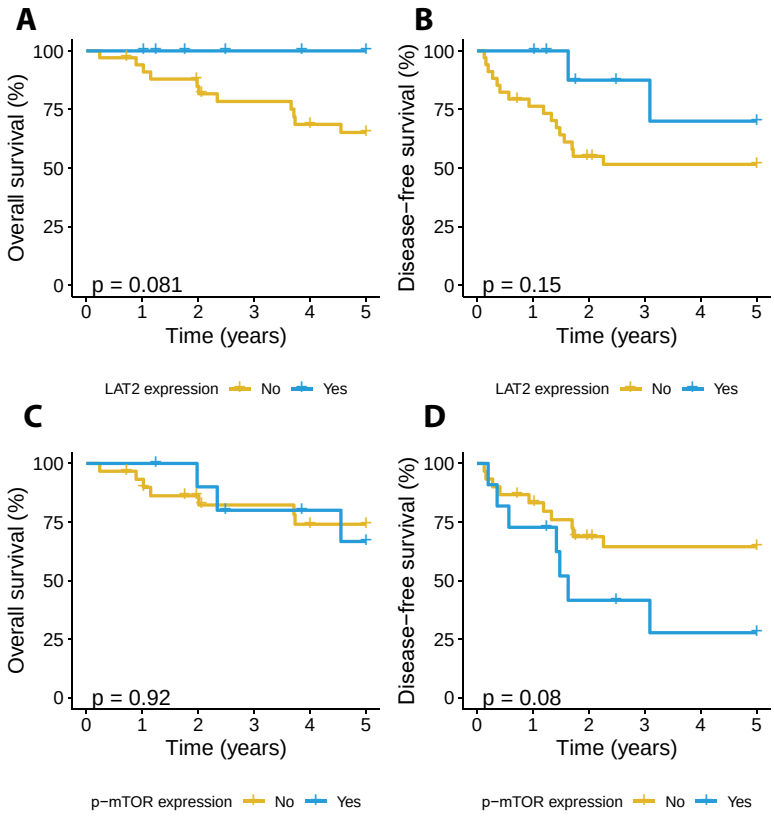


Figure S4. Kaplan Meier survival curves of overall survival (A and C) and disease-free survival (B and D) in osteosarcoma patients with and without LAT2 (A and B) or p-mTOR (C and D) protein expression in tumor tissue at diagnosis. P-values are the result of a log-rank test.

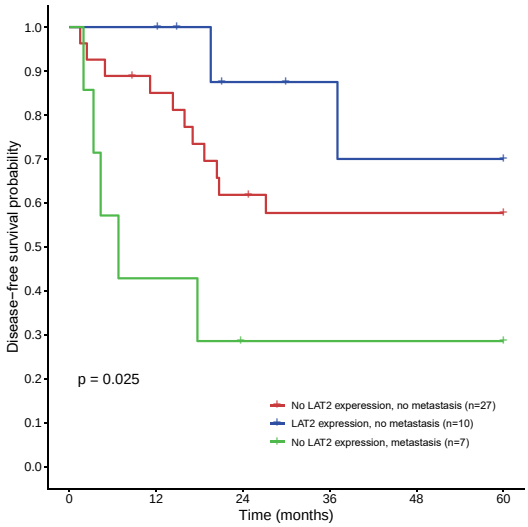
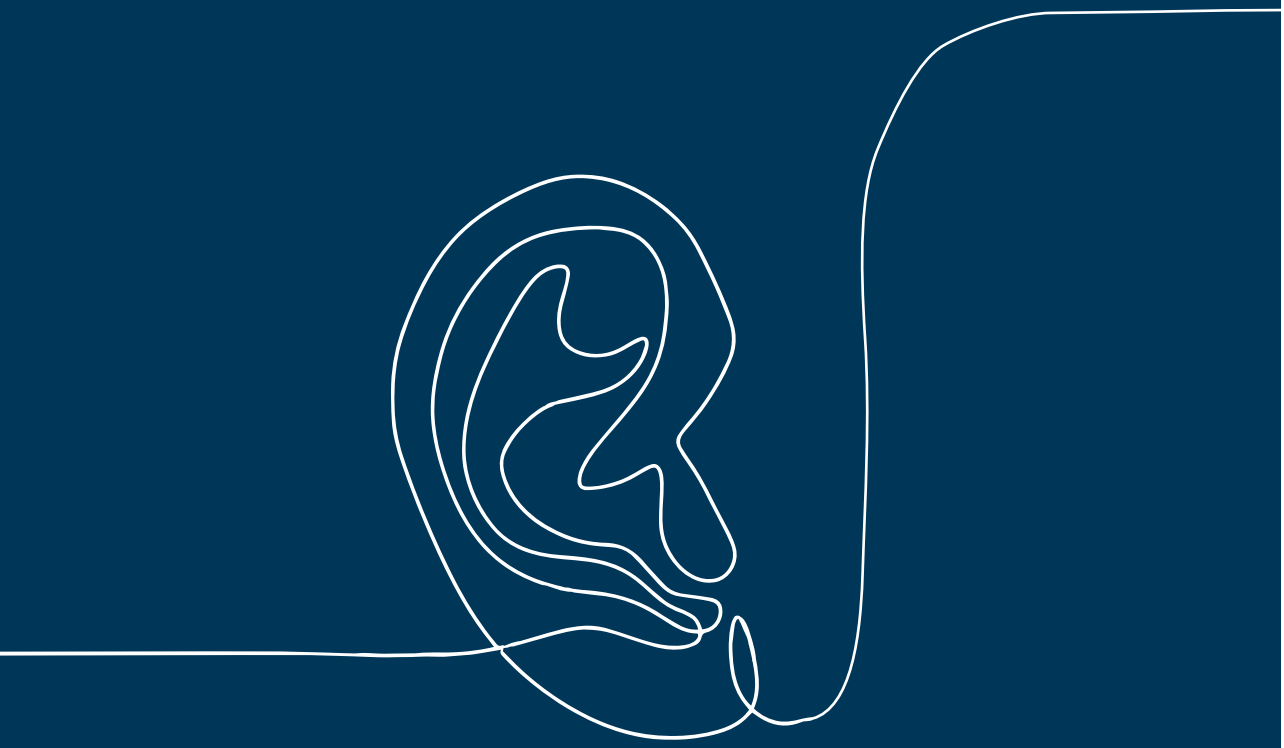


Figure S5. Kaplan Meier survival curve of disease-free survival of osteosarcoma patients stratified for LAT2 expression in osteosarcoma tissue and metastasis at diagnosis. In this cohort, no patients with LAT2 expression and metastases at diagnosis were identified.



CHAPTER 5

TSPAN5 locus associated with platinum-induced hearing loss in childhood cancer patients: results of GO-CAT and UK MAGIC consortia

Evelien G.E. Hurkmans[1#], Marije J. Klumpers[2#], Cinzia Dello Russo[3], Ward de Witte[1], Henk-Jan Guchelaar[4], Hans Gelderblom[5], Anne-Marie Cleton-Jansen[6], Sita Vermeulen[7], Suzanne Kaal[8], Winette T. A. van der Graaf[8,9], Uta Flucke[10], Corrie E.M. Gidding[11], Hendrik W. B. Schreuder[12], Eveline S. J. M. de Bont[13], Leontien C. M. Kremer[14], Huib Caron[14], Giovanna Gattuso[15], Elisabetta Schiavello[15], Monica Terenziani[15], Maura Massimino[15], Geoff McCowage[16], Sumanth Nagabushan[16,17], Daniel Catchpoole[18], Andrea L. Jorgensen[19], Daniel B. Hawcutt[20,21], Barry Pizer[22], Marieke J.H. Coenen[1]*, D. Maroeska W.M. te Loo[2]*

[1] Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands
[2] Department of Pediatrics, Radboud university medical center, Nijmegen, The Netherlands
[3] Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, United Kingdom
[4] Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, The Netherlands
[5] Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands
[6] Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[7] Department for Health Evidence, Radboud university medical center, Nijmegen, The Netherlands
[8] Department of Medical Oncology, Radboud university medical center, Nijmegen, The Netherlands
[9] Department of Medical Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
[10] Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands
[11] Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands
[12] Department of Orthopedics, Radboud university medical center, Nijmegen, The Netherlands
[13] Department of Pediatrics, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, The Netherlands
[14] Department of Pediatrics, Amsterdam University Medical Centers, Emma Children's Hospital, Amsterdam, Netherlands
[15] Pediatric Oncology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[16] Cancer Centre for Children, The Children's Hospital at Westmead, Sydney NSW, Australia
[17] Discipline of Child and adolescent health, University of Sydney, Sydney NSW, Australia
[18] Children's Cancer Research Unit, The Children's Hospital at Westmead, Sydney NSW, Australia
[19] Department of Biostatistics, University of Liverpool, Liverpool, United Kingdom
[20] Department of Women's and Children's Health, University of Liverpool, Liverpool, United Kingdom
[21] NIHR Alder Hey Clinical Research Facility, Alder Hey Children's Hospital, Liverpool, United Kingdom
[22] Department of Pediatric Oncology, Alder Hey Children's Hospital, Liverpool, United Kingdom
[#] Authors contributed equally
* Authors contributed equally

Submitted in adapted version

Abstract

Cisplatin and carboplatin are cornerstone anti-neoplastic medicines in many malignancies. Despite their beneficiary effects on survival rates, hearing loss is a major side effect. Platinum-induced hearing loss can affect quality-of-life, especially in childhood cancer patients, and when it occurs during treatment it may lead to dose reduction, risking reduced anti-tumor effect of platinum treatment. Pharmacogenetics has the ability to not only predict platinum-induced hearing loss but also elucidate the biological mechanism. Therefore, the aim of this study is to identify novel genetic variants that play a role in platinum-induced hearing loss.

In the present study, a genome-wide association analysis (GWAS) was performed using two childhood cancer patient cohorts: the Genetics of Childhood Cancer Treatment (GO-CAT) cohort (n=261) and the United Kingdom Molecular Genetics of Adverse Drug Reactions in Children Study (UK MAGIC) study cohort (n=248). In primary analysis, patients with SIOP Boston grade ≥1 were considered cases. Results of both cohorts were combined in a meta-analysis and suggestively significant results were replicated in the PanCareLIFE cohort, consisting of 390 platinum-treated childhood cancer patients.

No genome-wide significant associations are found, but variants in *TSPAN5, RBBP4P5, AC010090.1 and RNU6-38P* were suggestively associated to platinum-induced hearing loss. The lowest *p*-value was found for the T-allele of rs7671702 in *TSPAN5* (OR (95%CI) = 2.04 (1.54 – 2.69), $p = 5.01 \times 10^{-7}$). None of the associations were significant in the replication cohort, although effect directions were consistent among all cohorts.

The functional role of *TSPAN5* in the development of platinum-induced ototoxicity is hypothesized by influencing regulation of ADAM10 and thereby influencing cisplatin sensitivity in cells. Additional research is necessary to further explore this association. These insights in the development of platinum-induced hearing loss can ultimately contribute to a more personalized treatment plan without hampering treatment efficacy.

Introduction

Cisplatin and carboplatin are used as cornerstone anti-neoplastic treatments in many malignancies. Despite their desirable anti-tumor effects, ototoxicity (or hearing loss) is a major side effect. Platinum-induced ototoxicity is generally irreversible and occurs in 42-67% of patients treated with cisplatin (1-4) and in up to 20% of patients after carboplatin treatment (5, 6). Severe hearing loss during chemotherapy treatment may lead to a dose reduction of the platinum compound to prevent further hearing loss, risking its anti-tumor effect. In addition, it can have a negative effect on quality of life, especially in childhood patients treated for cancer. It was shown that these children are at increased risk for learning and reading problems and psychosocial difficulties (7, 8).

Clinical risk factors for platinum-induced ototoxicity include co-treatment with other ototoxic drugs such as aminoglycosides or furosemide, cumulative dose and administration duration of platinum treatment, cranial irradiation, young age and male sex (9). These risk factors can partly explain the interindividual differences in the development of ototoxicity, although genetic risk factors are hypothesized to play a role. For example, Xu *et al*. found a common variant in the *ACYP2* gene (rs1872328) to be associated with cisplatin-induced hearing loss (10). This finding was replicated multiple times and meta-analysis showed an almost 4-times increased risk for patients carrying the G-allele to develop ototoxicity (11, 12). Also, genetic variants in *TCERG1L*, *SLC22A2*, *WFS1*, *OTOS*, *ABCC3* and others were indicated in the risk of cisplatin-induced ototoxicity. These studies show that genetic variation plays a role in the development of platinum-induced ototoxicity. Despite the existing evidence, no widely accepted treatment protocols are in place for pharmacogenetic testing in clinical practice, nor to guide treatment (13, 14).

Larger patient cohorts are required to establish and replicate potential pharmacogenomic signals with certainty. As pediatric cancer is relatively rare, an international collaboration between the Genetics of Childhood Cancer Treatment (GO-CAT) consortium and UK Molecular Genetics of Adverse Drug Reactions in Children (MAGIC) study was initiated to establish such an international cohort with enough patients to perform statistically meaningful analyses. A genome-wide association study (GWAS) was performed in a childhood cancer cohort who were treated with platinum chemotherapy. The recently published GWAS by the PanCareLIFE consortium served as a replication cohort (14). The aim of this study was to identify novel genetic markers that can predict the occurrence of platinum-induced ototoxicity and possibly elucidate more about its biological mechanisms.

Materials and methods

Patients and treatment

The discovery study was a meta-analysis of GWASs in two patient cohorts; the GO-CAT cohort and the UK MAGIC study cohort. The GO-CAT cohort is a multinational retrospective cohort of pediatric cancer patients, treated between 1975 and 2020. Participating centers included Radboud university medical center (Nijmegen, the Netherlands), University Medical Center of Groningen (Groningen, the Netherlands),

Leiden University Medical Center (Leiden, the Netherlands), Academic Medical Center (Amsterdam, the Netherlands), Fondazione IRCCS Istituto Nazionale Tumori (Milan, Italy) and The Children's Hospital at Westmead (Sydney, Australia). The majority of this cohort was platinum-treated, and of a subset, genetic material was available sufficient for genome-wide genotyping. The UK MAGIC study cohort was a retrospective cohort of platinum-treated pediatric cancer patients recruited between January 2012 and March 2018 at eight UK sites, being Alder Hey Children's Hospital (Liverpool, UK), Leeds General Infirmary (Leeds, UK), Royal Manchester Children's Hospital (Manchester UK), Great Ormond Street Hospital NHS Trust (London, UK), Nottingham University Hospitals NHS Trust (Nottingham, UK), Leicester Royal Infirmary NHS Trust (Leicester, UK), Newcastle Hospitals NHS Trust (Newcastle, UK), York Hill Hospital (Glasgow, UK)(12). These studies were approved by all local ethical committees. Written informed consent was obtained of all included patients that were alive at the moment of inclusion and/or their parents or legal guardians if applicable.

Patients were eligible for inclusion if they were diagnosed with a tumor (with or without metastases), mainly patients with medulloblastoma or osteosarcoma, which was histological proven or, if no tumor material has been investigated, confirmed by imaging, and received primary chemotherapeutic treatment including a platinum agent. Patients from the GO-CAT cohort were treated with cisplatin or carboplatin, whereas patients from the UK MAGIC cohort were primarily treated with cisplatin. Treatment regimens depended on tumor type and local treatment protocols. Full inclusion and exclusion criteria for MAGIC have been published previously (12). Generally, patients were included if material for DNA isolation or genotyping data with genome-wide coverage was available, and passed genotyping quality control, and well-documented patient data was available concerning baseline characteristics and received treatment to establish clinical factors with potential impact on hearing loss (e.g., age, platinum dose and concomitant use of other ototoxic drugs). If applicable, data regarding cranial or craniospinal irradiation was a necessity, since its known impact on hearing. Audiograms were collected at baseline (and should not have clinically relevant hearing loss at baseline, being >20 dB hearing loss at any frequency), during chemotherapy (if available) and (at least one) during follow-up for ototoxicity assessment.

Ototoxicity assessment

For case-control assignment in childhood patient cohorts, the SIOP Boston ototoxicity scale was used (*Table S1*). This scale is based on sensorineural hearing thresholds in dB hearing level (2). Sensorineural hearing loss was established by examining unaided audiograms showing bone conduction, or air conduction with a normal tympanogram to rule out a conductive hearing loss component. From all patient's available audiograms during chemotherapy and follow-up, the worst audiogram and the worst ear were scored. Scoring of audiograms was performed by trained audiologists or experienced clinicians. For the primary analysis, patients with grade 0 were considered controls and patients with grade 1 or higher were assigned as cases. In secondary analyses, patients with grade 0 and 1 were considered controls and patients with grade 2-4 were cases.

Genotyping and quality control

Germline DNA was extracted from blood or saliva using the QIAamp DNA Blood Midi kit (Qiagen, Venlo, The Netherlands) and GeneFiX collection kits (GeneFiX DNA Saliva Collector GFX-02, Isohelix, UK), respectively. DNA isolation took place using ChemagicStar (Hamilton Robotics, Reno, NV, USA), using Chemagic STAR DNA Saliva 4k Kit, according to the manufacturer protocol. From patients who had passed away before inclusion, germline DNA was isolated from paraffin-embedded tissue samples as described previously (15). All samples were genotyped using genotyping arrays with genome-wide coverage. Samples from the GO-CAT cohort were genotyped using the Illumina Infinium Global Screening Array-24 version 2.0 and version 3.0, performed by Human Genomics Facility at Erasmus MC, Rotterdam, The Netherlands. Samples from the UK MAGIC study cohort were genotyped using the Illumina Infinium OmniExpressExome-8 version 1.4, performed by Illumina Cambridge Ltd, Cambridge, UK. To increase number of genetic variants and achieve harmonization across different genotyping arrays, genotyping data from all sites were phased and imputed using software Eagle (v2.3.5) and Minimac3, respectively, with the 1000 Genomes European dataset as a reference panel (16, 17).

Quality control (QC) of genotyped data started with exclusion of samples with individual call rates below 90%. On the marker level, genetic variants were removed if they showed a call rate below 98%, when minor allele frequency (MAF) was below 0.5% or when they deviated from the Hardy Weinberg equilibrium (HWE), with a *p*-value below $1x10^{-6}$. To retain a homogenous cohort, principal component analysis was performed, using a PCA cut-off of -0.01 in GO-CAT, and 2 standard deviations of two principal components in UK MAGIC. Samples were excluded if they showed sex discrepancies between genotyping and phenotyping data, and in cases of relatedness between samples within the cohort (proportion inherited by descent (PI-HAT above 0.2). All steps in the process of phasing, imputation and QC in the GO-CAT cohort were performed using the Rapid Imputation and Computational Pipeline for Genome-Wide Association Studies (18). In the UK MAGIC study cohort, identical analyses were performed using multiple command line programs (PLINK was used for QC, Eaglev2.4 for phasing (EUR population) and imputation was performed using the Michigan Server using Minimac4 1.5.7 with the 1000G Phase 3 v5 Reference panel).

Statistical analysis

A power calculation was performed to explore the power of detecting a statistical association with the number of available subjects, using Quanto (version 1.2.4, Los Angeles, CA). It was shown that a GWAS analysis in a cohort with sample size of 500, a case-control ratio of 1, a log additive inheritance mode and an alpha of $5x10^{-8}$, would result in a power of 0.06 – 0.97 for a variant with an odds ratio of 2 and an allele frequency of 0.05 – 0.3. Under these conditions, a power of 0.8 was reached at minor allele frequencies of 0.16 or higher.

To test for potential associations between clinical characteristics and platinum-induced hearing loss, variables were analyzed using Pearson's Chi-square, Fisher's Exact, independent samples T or Mann-Whitney U, depending on the type of data and the Gaussian distribution, using SPSS Statistics (version 25.0, IBM Corp.). A two-sided

p-value of less than 0.05 was considered to be significant, which resulted in inclusion of this clinical variable in as a covariate in further GWAS analysis. Multiple GWAS analyses were performed to study the effect of cranial irradiation, cisplatin/carboplatin treatment and phenotype definition (as explained under *Ototoxicity assessment*) on the results of the analysis. Altogether, this article will focus on the primary analysis, in which patients with SIOP grade 0 are controls and patients with SIOP grade 1-4 are cases, a subset of patients received cranial irradiation and patients are treated with cisplatin or carboplatin. Furthermore, secondary analyses were performed in subgroup cohorts with only cisplatin treated patients, non-irradiated patients and different case-control designation (SIOP grade 0-1 compared to grade 2-4), which will be included in the supplemental material. It is hypothesized that cohorts are more homogenous with more stringent inclusion criteria, leading to lower overall variance. However, in the secondary analyses power will be lower due to lower patient numbers, but power may be restored by decreased variance.

The GWAS was performed as a logistic regression analysis, under the assumption of an additive model, using the software PLINK (v2.0, Cambridge, MA) (19). Based on each cohorts' eigenvalues, the first four (GO-CAT) and two (UK MAGIC) principle components were included as covariates to account for potential population stratification bias. Age at diagnosis, or age at first cisplatin treatment, and concomitant vincristine treatment were included as a covariate as a result of the covariate analysis. The *p*-value threshold for genome-wide statistical significance was set to 5×10^{-8}, and the threshold for suggestive significance is *p*-value of 1×10^{-5}. Summary statistics were shared among GO-CAT and the UK MAGIC study and a random effect meta-analysis was performed using METAL (v2007-2009 Goncalo Abecasis, released on 2020-05-05) (20). Effect size estimates and standard errors from the summary statistics were used to perform the meta-analysis (SCHEME STDERR). In addition, heterogeneity across samples (I^2) was calculated. Meta-analysis results were filtered for variants that were present in both cohorts (leaving results of 7,272,050 variants in the primary analysis) and were subsequently annotated and visualized using FUMA (v1.3.6b, Amsterdam, The Netherlands)(21). FUMA was used to identify risk loci and their lead SNPs with a *p*-value below the suggestive significance threshold of 1×10^{-5}. In addition, a gene-wide analysis was performed using MAGMA, which is integrated in FUMA.

Replication

Variants suggestively associated to platinum-induced ototoxicity in the primary analysis (*p*-value $< 1 \times 10^{-5}$) were eligible for replication. The recently published GWAS in the PanCareLIFE cohort was also performed in a childhood cancer cohort, and therefore, results of suggestively associated variants from this study were extracted for publicly available summary statistics (14). The PanCareLIFE data of these variants was combined with the GO-CAT and UK MAGIC study cohort in a meta-analysis under the same conditions as described under *Statistical analysis*.

Results

Patient population

The process of patient inclusion is depicted in *Figure 1*. In summary, of the 971 subjects in the clinical dataset of GO-CAT, a total of 591 subjects met the clinical inclusion criteria, of whom 360 subjects had genetic material available for genotyping. In the UK-MAGIC study cohort, of 435 subjects who were considered eligible, a total of 248 met the inclusion criteria and had genetic material available for genotyping. After quality control of genetic data (*Figure 1*), a total of 509 patients remained for GWAS and meta-analysis in the discovery phase of this study, of which 261 were analyzed in the GO-CAT cohort and 248 in the UK MAGIC study cohort (with originating center of inclusion presented in *Table S2*).

The clinical characteristics of the GO-CAT and UK MAGIC study cohort are represented in *Table 1*. Despite variety in diagnoses, the common denominator among these patients was the platinum treatment. The GO-CAT cohort consists of 136 patients with osteosarcoma (52.1%), 79 with medulloblastoma (30.3%) and 46 with low-grade glioma (17.6%). Of these, 212 patients were primarily treated with cisplatin of which the median cumulative dose was 480 mg/m^2, ranging from 120-900 mg/m^2. 49 patients were primarily carboplatin treated, and in 21 patients, primary chemotherapeutic regimen contained both platinum agents, with a median cumulative carboplatin dose of 1,300 mg/m^2 (range 640-16,047). The majority of patients in the UK MAGIC study cohort are also patients with osteosarcoma (25.5%) or medulloblastoma (25.1%), the rest of these patients being diagnosed with hepatoblastoma (10.9%), neuroblastoma (10.9%), low-grade glioma (5.3%), Hodgkin's lymphoma (2.0%), ependymoma (4.6%), intracranial germ cell tumor (2.8%), nasopharyngeal carcinoma (2.8%), or even rarer types of childhood cancer. All patients in the UK MAGIC study cohort received treatment with cisplatin, with a median cumulative dose of 350 mg/m^2, ranging from 60-720 mg/m^2. A total of 40.2% of patients in the UK MAGIC study cohort received concomitant carboplatin, mainly including medulloblastoma (16.7%), neuroblastoma (8.9%), hepatoblastoma (4.0%) and ependymoma (4.0%). The use of concomitant vincristine treatment was significantly higher in ototoxicity cases than in controls in the UK MAGIC cohort (p = 0.001). Despite that this difference was not significant in the GO-CAT cohort (p = 0.092), concomitant vincristine use was included as a covariate in all GWAS analyses. Males were overrepresented in both cohorts, with 150 males (57.5%) in the GO-CAT cohort and 151 (60.9%) in the UK MAGIC study cohort, nevertheless the proportion of males was not different between cases and controls. The median age ranged from 10.1 to 10.8 years among all groups, except for ototoxicity cases in the UK MAGIC study cohort where the median age was 7.9 years. As age is significantly lower in these cases than in controls (p = 0.048), it was included as a covariate in all GWAS analyses.

Grade 1-4 platinum-induced ototoxicity occurred in 59.3% (302 cases and 207 controls) of the total discovery cohort. The percentage of cases was higher in the UK MAGIC cohort (66.5%) than in the GO-CAT cohort (52.5%). In the both the GO-CAT cohort and the UK MAGIC study cohort, the percentage of patients with cranial irradiation was significantly higher in cases compared to controls (p < 0.0001 in both cohorts). The significant difference is related to significant differences in diagnosis, intracranial tumor

site, receiving cranial surgery and total dose of radiotherapy received between cases and controls (*Table 1*). Including all these factors as covariates would represent the same variation multiple times and lead to overcorrection. Therefore, secondary analyses were performed where patients who received cranial irradiation are excluded to assess how the presence of patients with cranial irradiation influences the main results.

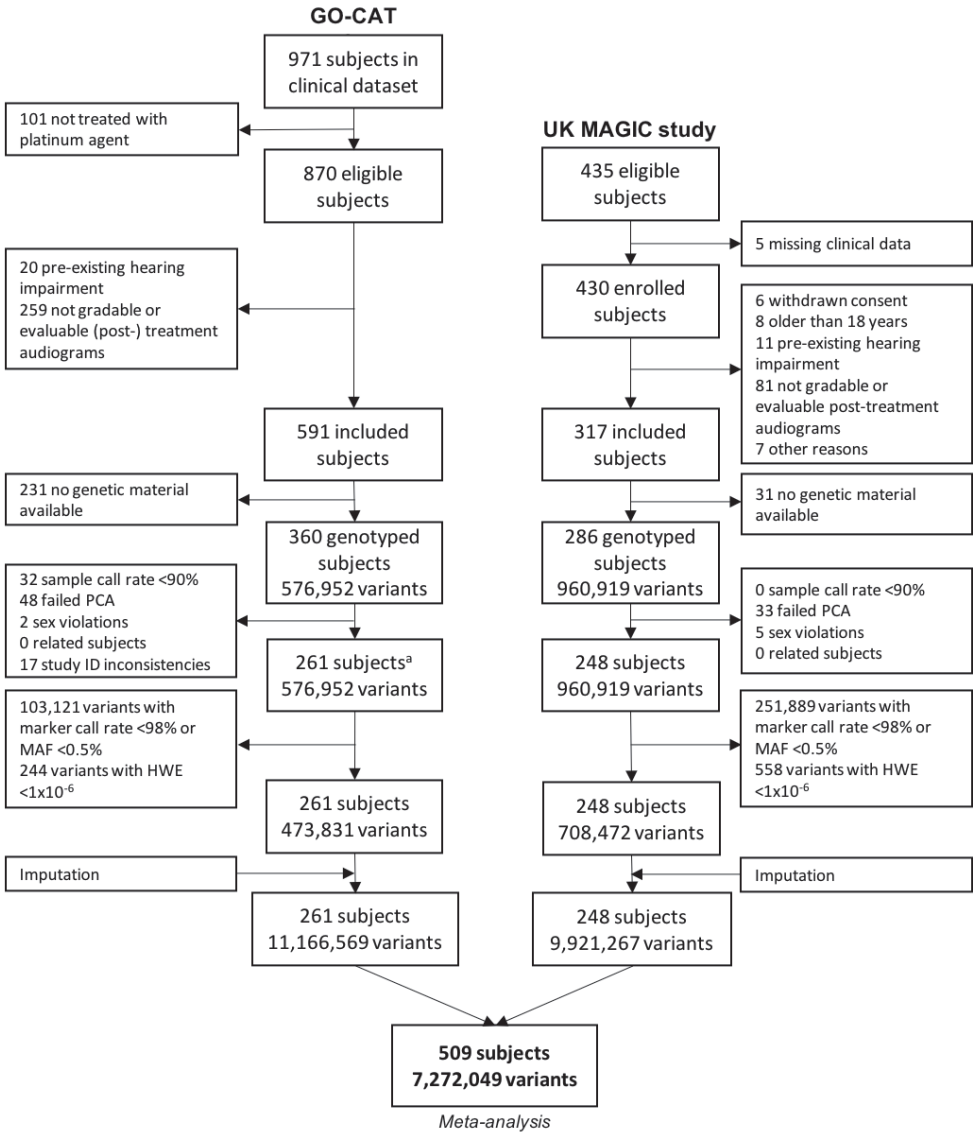


Figure 1. Genetic variant and sample selection flowchart. Process of quality control of clinical and genetic data for GO-CAT cohort and UK MAGIC study cohort.

[a] quality control of genetic data of GO-CAT cohort was performed in a larger dataset of 848 pediatric oncology subjects, of which a subset of subjects included in this study were extracted after imputation

Genotyping

The process of genotyping, QC and imputation for the GO-CAT cohort took place in larger cohort of pediatric cancer patients (848 subjects), of which a subset of subjects included in this study was extracted after imputation. Genotyping in the UK-MAGIC cohort was performed in 248 subjects. The process of QC for both cohorts is depicted in *Figure 1*. After QC, imputation and matching clinical and genetic data, a total of 261 subjects and 11,166,569 variants were included from the GO-CAT cohort, and a total of 248 subjects and 9,921,267 variants in the UK-MAGIC study cohort. Combining both cohort in meta-analysis, this resulted in a total of 509 subjects and 7,272,049 genetic variants.

Genome-wide association analyses and meta-analysis

In the primary meta-analysis, four variants were suggestively associated to platinum-induced ototoxicity (*p*-value <1 x 10^{-5}, *Table 2*). Of these, the result of analysis of rs7671702 in *TSPAN5* showed the lowest *p*-value. The T-allele of this variant was shown to increase risk of ototoxicity 2.04 (95%CI 1.54 - 2.69) times in childhood cancer patients with cisplatin or carboplatin as primary platinum treatment (p = 5.01×10^{-7}) (*Table 2*). When looking only at patients with cisplatin as their primary platinum treatment, the effects sizes are similar, but the *p*-value is higher (p = 7.08×10^{-6}).

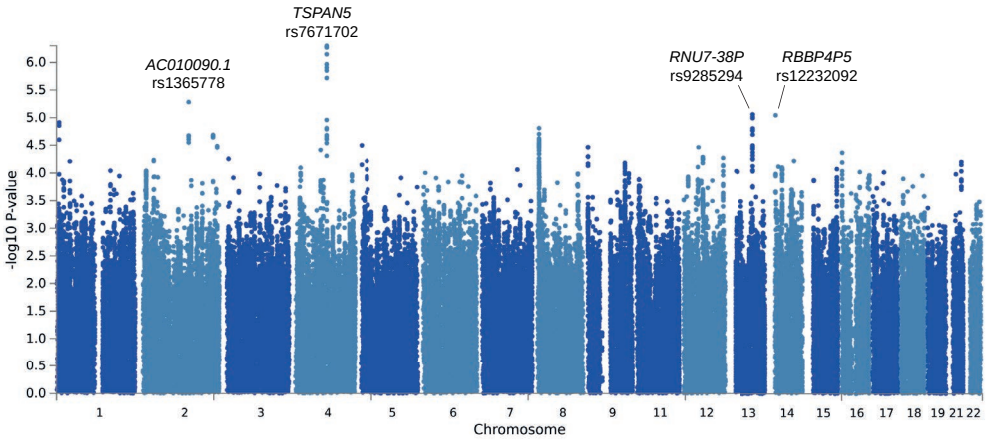


Figure 2. Manhattan plot of the primary GWAS meta-analysis, including patients treated with cisplatin or carboplatin and patients with and without cranial irradiation. In this analysis, patients with SIOP grade 0 are considered controls and patients with SIOP grade ≥ 1 are cases.

Table 1. Demographic data of patients in the discovery cohort of pediatric cancer patients. Median and range are reported for continuous variables.

	GO-CAT cohort				UK MAGIC study cohort			
	N *	**Controls SIOP grade 0 (n=124)**	**Cases SIOP grade 1-4 (n=137)**	***p*-value**	**N ***	**Controls SIOP grade 0 (n=83)**	**Cases SIOP grade 1-4 (n=165)**	***p*-value**
Demographics								
Male sex (%)	261	73 (53.3%)	77 (62.1%)	0.169	248	49 (59.04%)	102 (61.82%)	0.681
Age (years)	261	10.8 (0 – 38.9)	10.1 (1 – 41.1)	0.4	247	10.2 (1-18)	7.85 (0 - 18)	0.048
Self-reported Caucasian ethnicity (%)	261	124 (100%)	133 (97.1%)	0.124	245	83 (100%)	162 (100%)	NA
Disease and treatment								
Diagnosis	261			<0.0001	247			<0.0001
Osteosarcoma		72 (52.6%)	64 (51.6%)			27 (32.53%)	36 (21.95%)	
Medulloblastoma		27 (19.7%)	52 (41.9%)			3 (3.61%)	59 (35.98%)	
Other tumors		38 (27.7%)	8 (6.5%)			53 (63.86%)	69 (42.07%)	
Intracranial tumor site (%)	261	65 (47.4%)	60 (48.4%)	0.902	247	23 (27.71%)	88 (53.66%)	<0.0001
Received cranial surgery (%)	261	50 (36.5%)	59 (47.6%)	0.079	NA	NA	NA	NA
Received cranial radiotherapy (%)	261	26 (19.0%)	50 (40.3%)	<0.0001	248	8 (9.6%)	70 (42.42%)	<0.0001
Total dose on tumor bed (Gy)	74	54.0 (39.0 – 60.0)	55.8 (54.0 – 69.4)	0.307	73	55.80 (54-154)	54.40 (32.0 -91.8)	<0.0001
Primarily cisplatin treated	261	101 (73.7%)	111 (89.5%)	0.0014	248	83 (100%)	165 (100%)	NA
Cisplatin cumulative dose (mg/m²)	208	480 (120 – 900)	480 (120 – 766)	<0.0001	248	350 (60 -600)	350 (60-720)	0.779
Primarily carboplatin treated	241	41 (33.9%)	29 (24.2%)	0.119	NA	NA	NA	NA
Carboplatin cumulative dose (mg/m²)	65	1800 (640 – 16047)	1050 (800 – 4400)	0.012	NA	NA	NA	NA
Concomitant ototoxic medication (%)								
Vincristine	241	46 (38.0%)	59 (49.2%)	0.092	245	29 (35.80%)	97 (52.72%)	0.001
Aminoglycosides	97	6 (11.3%)	9 (20.5%)	0.411	NA	NA	NA	NA
Vancomycin	97	3 (5.7%)	7 (15.9)	0.178	NA	NA	NA	NA
Furosemide	107	9 (15.3%)	2 (4.2%)	0.107	NA	NA	NA	NA

	GO-CAT cohort				UK MAGIC study cohort			
	N *	Controls SIOP grade 0 (n=124)	Cases SIOP grade 1-4 (n=137)	*p*-value	N *	Controls SIOP grade 0 (n=83)	Cases SIOP grade 1-4 (n=165)	*p*-value
Carboplatin as co-medication (in primarily cisplatin treated patients)	190	3 (3.6%)	16 (15.0%)	0.013	246	21 (25.61%)	78 (47.56%)	0.001
Concomitant otoprotective medication (%)								
Amifostine	128	3 (4.2%)	9 (16.1%)	0.031	NA	NA	NA	NA
Sodium Thiosulfate	128	0 (0%)	0 (0%)	-	NA	NA	NA	NA

* Number of patients with data available for this variable.
NA; not available

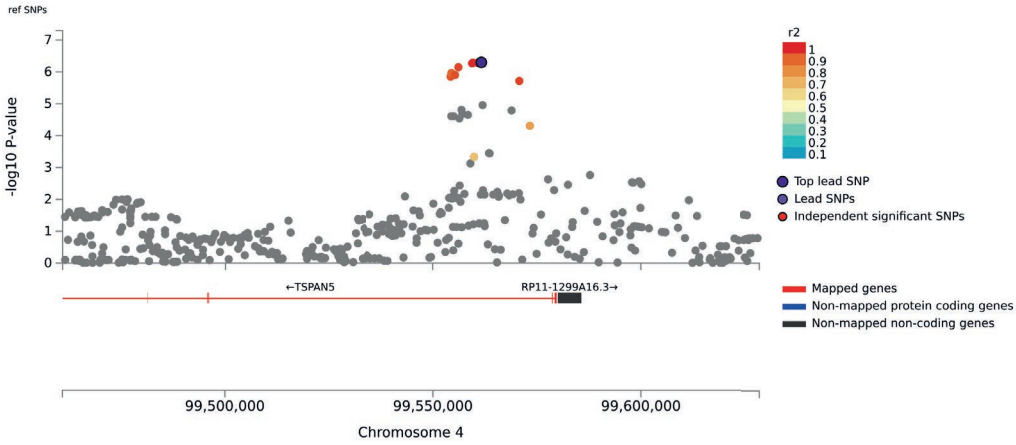


Figure 3. Zoom plot of *TSPAN5* locus in chromosome 4. Locus suggestively associated to platinum-induced hearing loss in the primary GWAS meta-analysis

The *TSPAN5* locus can be recognized as the hit in chromosome 4 on the Manhattan plot in *Figure 2*. In addition, *Figure 3* shows that this concerns an intronic locus, where multiple other genetic variants, which are in high linkage disequilibrium, are also associated with similar significance and effect sizes. The other variants that were suggestively associated to platinum-induced ototoxicity were located in *RBBP4P5, AC010090.1 and RNU6-38P*. The quantile-quantile plot of this analysis (*Figure S1*) shows that the GWAS meta-analysis was underpowered.

In total, eight genome-wide association analyses were performed according to the same protocols, with varying inclusion criteria and phenotype definitions. It is depicted in *Figure 4* (and *Table S3*) that excluding patients from this cohort often caused decreased power, which can be interpreted from the larger 95% confidence intervals. Also, excluding not irradiated patients had a larger effect on the results of the analysis than excluding patients treated with carboplatin primarily. Above all, when patients with SIOP grade 1 were considered controls instead of cases, the effects size decreased for the associations of all four variants. This emphasizes the importance of homogenous patient cohorts, especially in terms of cranial irradiation and phenotype designation.

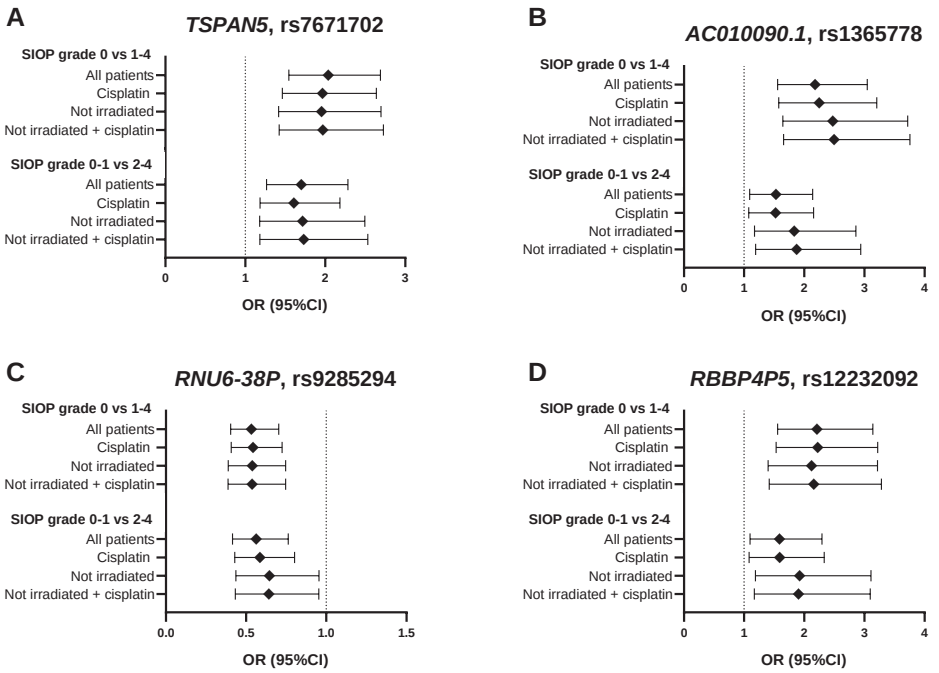


Figure 4. Results in primary and secondary analyses of four variants (A-D) that were suggestively associated with platinum-induced ototoxicity in the primary GWAS meta-analysis. The top bar represents the primary analysis (SIOP grade 0 vs 1-4, 'all patients'). In the 'cisplatin' subgroup, patients treated with carboplatin were excluded and in the 'not irradiated' subgroup, patients that received cranial irradiation were excluded.

Table 2. Results of the genetic variants that are suggestively associated to platinum-induced hearing loss in the primary GWAS meta-analysis. The GO-CAT and UK MAGIC study cohorts form together the discovery cohort. Results are specified per cohort and the combined results are determined with a meta-analysis.

	GWAS GO-CAT consortium N=261		**GWAS UK MAGIC study cohort N=245**		**Meta-analysis (GO-CAT + UK MAGIC)**			**Replication PanCareLIFE cohort N=390**		**Meta-analysis (GO-CAT + UK MAGIC + PanCareLIFE)**		
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**	**I^2 P_{het}**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**	**I^2 P_{het}**
***TSPAN5*, rs7671702, A1=T**	1.83	1.84×10^{-3}	2.31	2.43×10^{-5}	2.04	**5.01×10^{-7}**	0	1.19	0.242	1.58	**8.88×10^{-6}**	73.6
	(1.25 - 2.67)		(1.56 - 3.41)		(1.54 - 2.69)		0.411	(0.89 - 1.59)		(1.29 - 1.93)		0.0233
***RBBP4P5*, rs12232092, A1=A**	2.53	1.18×10^{-3}	2.03	2.14×10^{-3}	2.21	**9.11×10^{-6}**	0	1.23	0.275	1.68	**7.09×10^{-5}**	63.4
	(1.44 - 4.44)		(1.3 - 3.17)		(1.56 - 3.14)		0.545	(0.85 - 1.78)		(1.3 - 2.16)		0.0652
***AC010090.1*, rs1365778, A1=A**	2.38	6.42×10^{-4}	2.03	2.40×10^{-3}	2.18	**5.24×10^{-6}**	0	1.18	0.328	1.61	**8.92×10^{-5}**	69.5
	(1.45 - 3.91)		(1.32 - 3.11)		(1.56 - 3.05)		0.644	(0.84 - 1.66)		(1.27 - 2.04)		0.0375
***RNU6-38P*, rs9285294, A1=T**	0.48	2.11×10^{-4}	0.6	1.02×10^{-2}	0.53	**8.76×10^{-6}**	0	1	0.999	0.71	**1.10×10^{-3}**	79.4
	(0.32 - 0.71)		(0.41 - 0.88)		(0.4 - 0.7)		0.441	(0.74 - 1.35)		(0.58 - 0.87)		0.00782

A1, effect allele; OR, odds ratio; 95%CI, 95% confidence interval; I^2, heterogeneity value

In the primary gene-wide and gene-enrichment analysis, no statistically significant associations were found after Bonferroni or FDR multiple testing correction (*data not shown*). Despite the non-significant results, the *GBP1* gene in chromosome 1 is in the top 5 in all gene-wide analyses. This suggests that this robust finding is not largely dependent on the exact inclusion criteria of the patient cohorts.

Replication

Four variants were eligible for replication in the PanCareLIFE cohort. This cohort consists of 390 cisplatin-treated, non-cranially irradiated childhood cancer patients. Forty-three percent (n=168) of these patients suffered from cisplatin-induced hearing loss (Muenster ≥ grade 2b) (14). None of the associations were significant in the replication cohort alone, however the effect goes in the same direction in all groups (*Table 2*). In a meta-analysis of all pediatric cancer cohorts (GO-CAT, UK MAGIC and PanCareLIFE), the effect of the *TSPAN5* variant remains suggestively significant (OR (95%CI) = 1.58 (1.29 – 1.93), $p = 8.88 \times 10^{-6}$).

Discussion

In this study, the main finding was the suggestive association of a genetic variant in the *TSPAN5* gene with platinum-induced hearing loss in pediatric cancer patient cohorts. In addition, suggestively significant associations were found for variants in *RBBP4P5, AC010090.1 and RNU6-38P*. These findings confirm that germline genetic variation may play a role in the development of hearing loss after platinum treatment.

The T-allele of rs7671702 in the *TSPAN5* gene was suggestively associated to a two-times increased risk of platinum-induced hearing loss in childhood cancer patients. rs7671702 is an intronic variant in the first intron of the *TSPAN5* gene. This variant was previously found to be an expression quantitative trait locus (eQTL) for *TSPAN5* in skeletal muscle and tibial nerves, meaning that the variant is associated to expression of *TSPAN5* (22). *TSPAN5* codes for tetraspanin 5, which is an ubiquitously expressed protein that is responsible for the translocation of ADAM10 to the cell membrane (23). It was previously shown that ADAM10 is involved in cisplatin-induced renal toxicity through cleaving C-X-C ligand 16 (CXCL16) into its soluble form, causing recruitment of T-cells and subsequent inflammation-mediated apoptosis (24). The drug Enoxaparin could relieve this form of platinum-induced nephrotoxicity *in vitro* (24). Also, regulation of ADAM10 by PAX2 or miR-320a influences cisplatin sensitivity of melanoma cells and gastric cancer cells, respectively (25, 26). This indicates that deviated ADAM10 translocation by TSPAN5 may also affect sensitivity to platinum compounds. In addition, ADAM10 regulates sensory regeneration in the avian vestibular organs (27). In previous gene-wide analyses, it was shown that *TSPAN5* is associated to tinnitus ($p_{uncorrected} = 0.00187$ (28)) and that there is an association of *ADAM10* with cisplatin-induced ototoxicity ($p_{uncorrected} = 0.0466$ (29)). Altogether, this indicates that there may be a role for both *TSPAN5* and *ADAM10* in platinum-induced hearing loss.

Despite that there could be a mechanical explanation for the association between *TSPAN5* variant and platinum-induced ototoxicity, this association was not found in the study in the PanCareLIFE cohort published by Meijer *et al*. The patient cohorts are

relatively comparable, as they both consist of pediatric cancer patients, but the definition of ototoxicity is different. In the current study's primary analysis, patients with SIOP grade ≥ 1 are considered cases (>20 dB hearing loss at >4 kHz) and in the PanCareLIFE study hearing loss was considered deleterious at Muenster level 2b (>40 dB hearing loss at ≥4 kHz), which are more stringent criteria. In the secondary analyses of this study, SIOP grade ≥ 2 are considered cases (>20 dB hearing loss at ≥4 kHz), which would be more comparable to the PanCareLIFE study. Secondary analyses are also more comparable to the PanCareLIFE study in terms of inclusion criteria, because PanCareLIFE only included cisplatin-treated, non-irradiated patients. Different hearing thresholds in the case-control designation can have a large effect on the results of a genetic association study, as was previously described in a study focusing on cisplatin induced nephrotoxicity (30). They used four grading tools to represent acute kidney injury, including CTCAE grading, adjusted CTCAE grading, serum creatinine and estimated glomerular filtration rate. It was found that these different case designations lead to variability in risk ascertainment of the phenotype. This effect was also illustrated by the differences in results in the analyses with cases defined as SIOP grade ≥ 1 compared to cases defined as SIOP grade ≥ 2, respectively (*Figure 4*). Altogether, this highlights the importance of homogenous clinically relevant outcome definitions.

In the primary association analysis of this study, both cis- and carboplatin treated patients are analyzed together. Despite known differences in ototoxic potency of these agents, cisplatin being more ototoxic than carboplatin, the mechanism of ototoxicity is carried out through the same biological mechanism, being death of sensory hair cells in the cochlea (2). Since the aim of this genetic association study is to unraveling biological mechanisms via statistical methods in order to increase knowledge of interindividual differences, patients treated with either or both agents were included in primary analyses, also to enhance power by increasing cohort size. Contrary to that, excluding patients treated primarily with carboplatin, and/or patients who received cranial irradiation, was hypothesized to decrease variance in the data and thereby increase power. However, the resulting decrease in patient number and subsequent decrease in power is detrimental for a small study, leading to minimal (suggestively) significantly associated findings in secondary analyses (*Table S4*). For the top-hit variants in the primary analysis, the *p*-values were higher and confidence intervals were larger in secondary analyses (*Figure 4, Table S3*), which further confirms that the patient number was more important than stringent selection criteria in this study.

Despite the investment in collaboration with multiple research groups, no genome-wide significant associations are found and the results were not replicated in both the PanCareLIFE cohort. Fifteen genetic variants that were previously associated to platinum-induced ototoxicity were not significantly associated to platinum-induced ototoxicity in this study (*Table S5*). The lack of replication could be due to heterogeneity between studies, differences in outcome definitions, different analysis methods or false-positive findings in discovery studies. Poor reproducibility remains an issue in genetic association studies, and specifically when studying platinum-induced ototoxicity (31). In the quest for meaningful associations with an impact on patient care, homogenous and powerful analyses are necessary.

To conclude, an association of a variant in the *TSPAN5* gene with platinum-induced hearing loss was identified in this study. Moreover, there may be a functional role for *TSPAN5* in the development of platinum-induced ototoxicity by regulation of ADAM10 and thereby influencing cisplatin sensitivity in cells. This study once again emphasizes the importance of standardized outcome definitions, homogenized analyses and collaboration among research groups to optimize power in GWAS studies. Therefore, the Genetics of Childhood Cancer Treatment (GO-CAT) consortium will continue to invest in collaborations to perform larger analyses in the future. This exploratory study is a step in the identification of genetic variants involved in platinum-induced hearing loss. This will ultimately lead to prediction models to identify patients at risk for hearing loss before treatment. On top of that, it may contribute to an improved understanding of the mechanism behind platinum-induced hearing loss and the development of interventions to improve the quality-of-life of cancer survivors.

Acknowledgments

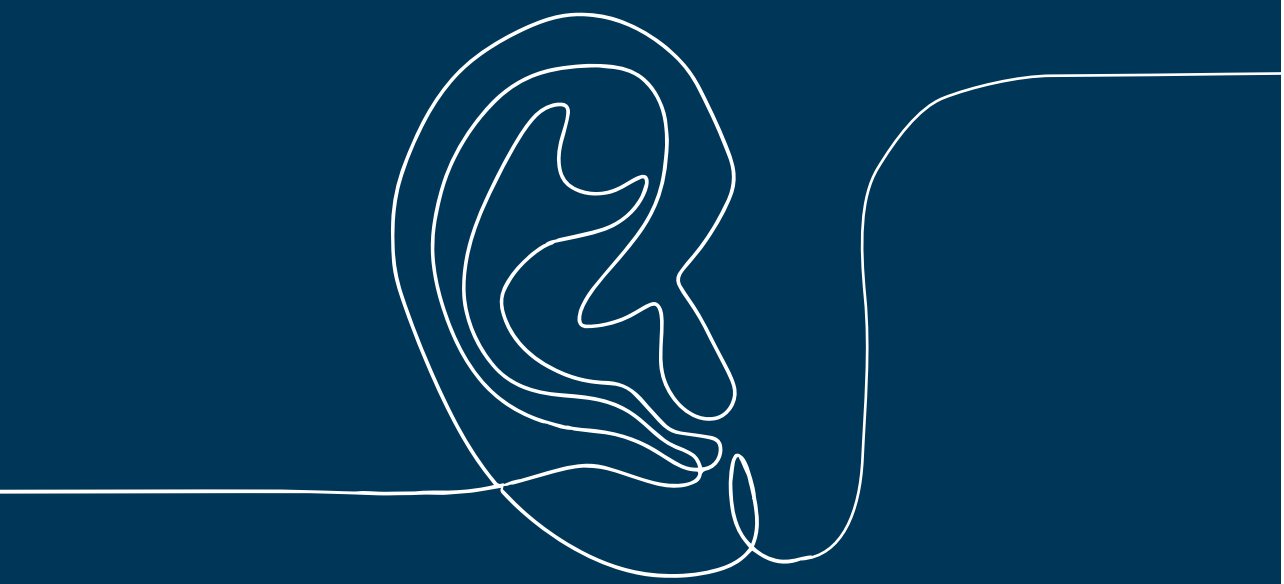
We would like to acknowledge all patients and parents who participated in this study. We acknowledge the contribution of Johanne M. Groothuismink (Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands) for her guidance and assistance on genotyping. We acknowledge Regine Ketting (Department of Pediatrics, Radboud univseritiy medical center, Nijmegen, The Netherlands) for her guidance in data management. We acknowledge Li Zhou (Tumour Bank at the Children's Hospital at Westmead, Sydney NSW, Australia) for her work on sample processing.

This work is part of the research program Computing Time National Computing Facilities Processing Round pilots 2018 with project number 17666, which is (partly) financed by the Dutch Research Council (NWO). This work was carried out on the Dutch national e-infrastructure with the support of SURF Cooperative.

References

1. Berg AL, Spitzer JB, Garvin JH, Jr. Ototoxic impact of cisplatin in pediatric oncology patients. Laryngoscope. 1999;109(11):1806-14.
2. Brock PR, Knight KR, Freyer DR, Campbell KC, Steyger PS, Blakley BW, et al. Platinum-induced ototoxicity in children: a consensus review on mechanisms, predisposition, and protection, including a new International Society of Pediatric Oncology Boston ototoxicity scale. J Clin Oncol. 2012;30(19):2408-17.
3. Yancey A, Harris MS, Egbelakin A, Gilbert J, Pisoni DB, Renbarger J. Risk factors for cisplatin-associated ototoxicity in pediatric oncology patients. Pediatr Blood Cancer. 2012;59(1):144-8.
4. Stohr W, Langer T, Kremers A, Bielack S, Lamprecht-Dinnesen A, Frey E, et al. Cisplatin-induced ototoxicity in osteosarcoma patients: a report from the late effects surveillance system. Cancer Invest. 2005;23(3):201-7.
5. Clemens E, de Vries AC, Am Zehnhoff-Dinnesen A, Tissing WJ, Loonen JJ, Pluijm SF, et al. Hearing loss after platinum treatment is irreversible in noncranial irradiated childhood cancer survivors. Pediatr Hematol Oncol. 2017;34(2):120-9.
6. Qaddoumi I, Bass JK, Wu J, Billups CA, Wozniak AW, Merchant TE, et al. Carboplatin-associated ototoxicity in children with retinoblastoma. J Clin Oncol. 2012;30(10):1034-41.
7. Gurney JG, Tersak JM, Ness KK, Landier W, Matthay KK, Schmidt ML. Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group. Pediatrics. 2007;120(5):e1229-36.
8. Olivier TW, Bass JK, Ashford JM, Beaulieu R, Scott SM, Schreiber JE, et al. Cognitive Implications of Ototoxicity in Pediatric Patients With Embryonal Brain Tumors. J Clin Oncol. 2019;37(18):1566-75.
9. Langer T, am Zehnhoff-Dinnesen A, Radtke S, Meitert J, Zolk O. Understanding platinum-induced ototoxicity. Trends Pharmacol Sci. 2013;34(8):458-69.
10. Xu H, Robinson GW, Huang J, Lim JY, Zhang H, Bass JK, et al. Common variants in ACYP2 influence susceptibility to cisplatin-induced hearing loss. Nat Genet. 2015;47(3):263-6.
11. Clemens E, Broer L, Langer T, Uitterlinden AG, de Vries ACH, van Grotel M, et al. Genetic variation of cisplatin-induced ototoxicity in non-cranial-irradiated pediatric patients using a candidate gene approach: The International PanCareLIFE Study. Pharmacogenomics J. 2020;20(2):294-305.
12. Thiesen S, Yin P, Jorgensen AL, Zhang JE, Manzo V, McEvoy L, et al. TPMT, COMT and ACYP2 genetic variants in paediatric cancer patients with cisplatin-induced ototoxicity. Pharmacogenet Genomics. 2017;27(6):213-22.
13. Langer T, Clemens E, Broer L, Maier L, Uitterlinden AG, de Vries ACH, et al. Usefulness of current candidate genetic markers to identify childhood cancer patients at risk for platinum-induced ototoxicity: Results of the European PanCareLIFE cohort study. Eur J Cancer. 2020;138:212-24.
14. Meijer AJM, Diepstraten FA, Langer T, Broer L, Domingo IK, Clemens E, et al. TCERG1L allelic variation is associated with cisplatin-induced hearing loss in childhood cancer, a PanCareLIFE study. NPJ Precis Oncol. 2021;5(1):64.
15. Hagleitner MM, Coenen MJ, Jeuken JW, Flucke U, Schreuder HW, te Loo DM, et al. Taqman genotyping assays can be used on decalcified and paraffin-embedded tissue from patients with osteosarcoma. Pediatr Blood Cancer. 2011;56(1):35-8.
16. Loh PR, Danecek P, Palamara PF, Fuchsberger C, Y AR, H KF, et al. Reference-based phasing using the Haplotype Reference Consortium panel. Nat Genet. 2016;48(11):1443-8.
17. Das S, Forer L, Schonherr S, Sidore C, Locke AE, Kwong A, et al. Next-generation genotype imputation service and methods. Nat Genet. 2016;48(10):1284-7.

18. Lam M, Awasthi S, Watson HJ, Goldstein J, Panagiotaropoulou G, Trubetskoy V, et al. RICOPILI: Rapid Imputation for COnsortias PIpeLIne. Bioinformatics. 2020;36(3):930-3.
19. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience. 2015;4:7.
20. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26(17):2190-1.
21. Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. Nat Commun. 2017;8(1):1826.
22. Consortium GT. The GTEx Consortium atlas of genetic regulatory effects across human tissues. Science. 2020;369(6509):1318-30.
23. Matthews AL, Szyroka J, Collier R, Noy PJ, Tomlinson MG. Scissor sisters: regulation of ADAM10 by the TspanC8 tetraspanins. Biochem Soc Trans. 2017;45(3):719-30.
24. Aboyoussef AM, Abdel-Sattar AR, Abdel-Bakky MS, Messiha BAS. Enoxaparin prevents CXCL16/ADAM10-mediated cisplatin renal toxicity: Role of the coagulation system and the transcriptional factor NF-kappaB. Life Sci. 2021;270:119120.
25. Lee SB, Doberstein K, Baumgarten P, Wieland A, Ungerer C, Burger C, et al. PAX2 regulates ADAM10 expression and mediates anchorage-independent cell growth of melanoma cells. PLoS One. 2011;6(8):e22312.
26. Ge X, Cui H, Zhou Y, Yin D, Feng Y, Xin Q, et al. miR-320a modulates cell growth and chemosensitivity via regulating ADAM10 in gastric cancer. Mol Med Rep. 2017;16(6):9664-70.
27. Warchol ME, Stone J, Barton M, Ku J, Veile R, Daudet N, et al. ADAM10 and gamma-secretase regulate sensory regeneration in the avian vestibular organs. Dev Biol. 2017;428(1):39-51.
28. Watanabe K, Stringer S, Frei O, Umicevic Mirkov M, de Leeuw C, Polderman TJC, et al. A global overview of pleiotropy and genetic architecture in complex traits. Nat Genet. 2019;51(9):1339-48.
29. Wheeler HE, Gamazon ER, Frisina RD, Perez-Cervantes C, El Charif O, Mapes B, et al. Variants in WFS1 and Other Mendelian Deafness Genes Are Associated with Cisplatin-Associated Ototoxicity. Clin Cancer Res. 2017;23(13):3325-33.
30. Zazuli Z, Otten LS, Drogemoller BI, Medeiros M, Monzon JG, Wright GEB, et al. Outcome Definition Influences the Relationship Between Genetic Polymorphisms of ERCC1, ERCC2, SLC22A2 and Cisplatin Nephrotoxicity in Adult Testicular Cancer Patients. Genes (Basel). 2019;10(5).
31. Drogemoller BI, Wright GEB, Lo C, Le T, Brooks B, Bhavsar AP, et al. Pharmacogenomics of Cisplatin-Induced Ototoxicity: Successes, Shortcomings, and Future Avenues of Research. Clin Pharmacol Ther. 2019;106(2):350-9.



CHAPTER 5

Supplementary materials

TSPAN5 locus associated with platinum-induced hearing loss in childhood cancer patients: results of GO-CAT and UK MAGIC consortia

Table S1. Hearing levels that are designated to toxicity grades according to SIOP Boston ototoxicity scale for pediatric patients (2).

	SIOP Boston
Grade 0	≤ 20 dB all
Grade 1	>20 dB at >4 kHz
Grade 2	>20 dB at ≥4 kHz
Grade 3	>20 dB at 2 or 3 kHz
Grade 4	>40 dB at ≥2 kHz

Table S2. Hospital of inclusion of the patients in the discovery and replication cohorts.

	Controls	Cases
Genetics of Childhood Cancer Treatment (GO-CAT) consortium		
Radboudumc Nijmegen, The Netherlands	45	27
Leiden UMC, The Netherlands	4	12
AMC, Amsterdam, The Netherlands	6	11
UMC Groningen, The Netherlands	4	12
The Children's Hospital at Westmead, Australia	27	16
Fondazione IRCCS Istituto Nazionale Tumori Milan, Italy	37	31
UK MAGIC study cohort		
AHC, Alder Hey Children's Hospital, Liverpool, England	16	30
GOS, Great Ormond Street Hospital NHS Trust, London, UK	5	13
LGI, Leeds General Infirmary, Leeds, UK	21	43
LRI, Leicester Royal Infirmary NHS Trust, Leicester, UK	1	3
NUT, Nottingham University Hospitals NHS Trust, Nottingham, UK	28	50
QMC, Newcastle Hospitals NHS Trust, Newcastle, UK	4	12
RMC, Royal Manchester Children's Hospital, Manchester, UK	8	11
YHH, York Hill Hospital, Glasgow, UK	0	3
PanCareLIFE		
Available at: Meijer AJM, Diepstraten FA, Langer T, Broer L, Domingo IK, Clemens E, et al. TCERG1L allelic variation is associated with cisplatin-induced hearing loss in childhood cancer, a PanCareLIFE study. NPJ Precis Oncol. 2021;5(1):64.		

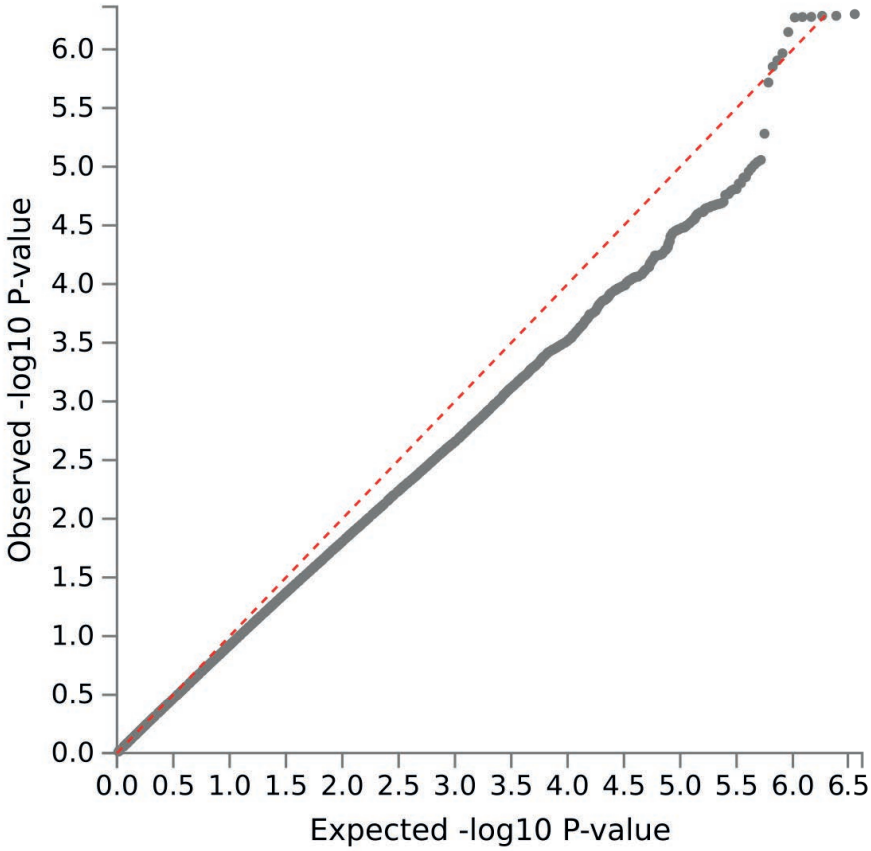


Figure S1. QQ-plot of the primary GWAS meta-analysis to identify genetic variants associated to platinum-induced hearing loss.

Table S3. Four variants were suggestively associated with platinum-induced ototoxicity in primary analysis and this table shows the results of these associations in the secondary analyses. Selection criteria of patients in primary (1A) and secondary analyses (1B-4B) are specified in this table. In the 'cisplatin' subgroup, patients treated with carboplatin were excluded and in the 'not irradiated' subgroup, patients that received cranial irradiation were excluded.

***Gene*, rsID**	**#**	**Inclusion criteria**	**Control SIOP grade**	**Case SIOP grade**	**N**	**OR (95% CI)**	**Uncorrected *p*-value**
***TSPAN5*, rs7671702**	1A	All patients	0	1-4	506	2.21 (1.56 - 3.14)	9.112E-06
	1B	Cisplatin	0	1-4	456	2.22 (1.53 - 3.22)	2.491E-05
	2A	Not irradiated	0	1-4	352	2.12 (1.4 - 3.22)	0.0004074
	2B	Not irradiated + Cisplatin	0	1-4	331	2.16 (1.42 - 3.28)	0.0003275
	3A	All patients	0-1	2-4	506	1.59 (1.1 - 2.29)	0.01363
	3B	Cisplatin	0-1	2-4	456	1.59 (1.08 - 2.33)	0.01757
	4A	Not irradiated	0-1	2-4	352	1.92 (1.19 - 3.11)	0.007856
	4B	Not irradiated + Cisplatin	0-1	2-4	331	1.9 (1.17 - 3.1)	0.009534
***AC010090.1*, rs1365778**	1A	All patients	0	1-4	506	2.18 (1.56 - 3.05)	5.235E-06
	1B	Cisplatin	0	1-4	456	2.25 (1.58 - 3.2)	7.569E-06
	2A	Not irradiated	0	1-4	352	2.47 (1.64 - 3.72)	1.363E-05
	2B	Not irradiated + Cisplatin	0	1-4	331	2.49 (1.66 - 3.76)	1.239E-05
	3A	All patients	0-1	2-4	506	1.53 (1.09 - 2.14)	0.01302
	3B	Cisplatin	0-1	2-4	456	1.52 (1.08 - 2.15)	0.0172
	4A	Not irradiated	0-1	2-4	352	1.83 (1.17 - 2.86)	0.007635
	4B	Not irradiated + Cisplatin	0-1	2-4	331	1.87 (1.19 - 2.94)	0.006409
***RNU6-38P*, rs9285294**	1A	All patients	0	1-4	506	2.04 (1.54 - 2.69)	5.013E-07
	1B	Cisplatin	0	1-4	456	1.96 (1.46 - 2.64)	7.081E-06
	2A	Not irradiated	0	1-4	352	1.95 (1.42 - 2.7)	4.562E-05
	2B	Not irradiated + Cisplatin	0	1-4	331	1.97 (1.42 - 2.73)	4.361E-05
	3A	All patients	0-1	2-4	506	1.7 (1.27 - 2.28)	0.0004241
	3B	Cisplatin	0-1	2-4	456	1.61 (1.18 - 2.18)	0.002354
	4A	Not irradiated	0-1	2-4	352	1.72 (1.18 - 2.49)	0.00468
	4B	Not irradiated + Cisplatin	0-1	2-4	331	1.73 (1.18 - 2.53)	0.004764
***RBBP4P5*, rs12232092**	1A	All patients	0	1-4	506	0.53 (0.4 - 0.7)	8.758E-06
	1B	Cisplatin	0	1-4	456	0.54 (0.41 - 0.72)	3.358E-05
	2A	Not irradiated	0	1-4	352	0.54 (0.39 - 0.75)	0.0002025
	2B	Not irradiated + Cisplatin	0	1-4	331	0.54 (0.39 - 0.75)	0.0002073
	3A	All patients	0-1	2-4	506	0.56 (0.41 - 0.76)	0.0002158
	3B	Cisplatin	0-1	2-4	456	0.59 (0.43 - 0.8)	0.0008312
	4A	Not irradiated	0-1	2-4	352	0.65 (0.44 - 0.95)	0.02829
	4B	Not irradiated + Cisplatin	0-1	2-4	331	0.64 (0.43 - 0.95)	0.02796

OR, odds ratio; CI, confidence interval.

Table S4. Results of the suggestive associations ($p<1\times10^{-5}$) of primary (1A) and secondary (1B-4B) GWAS meta-analyses in the discovery cohort.

#*	rsID	chr	Position	Nearest Gene	Distance	Non effect allele	Effect allele	MAF	Effect direction[1]	N	OR	95 CI (lower)	95 CI (upper)	Uncorrected *p*-value
1A	rs7671702	4	99561695	***TSPAN5***	0	C	T	0.491	++	506	2.04	1.76	2.32	**5.01E-07**
	rs1365778	2	145305277	***AC010090.1***	18724	G	A	0.327	++	506	2.18	1.84	2.51	**5.24E-06**
	rs9285294	13	75672116	***RNU6-38P***	11607	C	T	0.453	--	506	0.53	0.26	0.81	**8.76E-06**
	rs12232092	14	22028781	***RBBP4P5***	3168	G	A	0.275	++	506	2.21	1.86	2.56	**9.11E-06**
1B	rs7671702	4	99561695	***TSPAN5***	0	C	T	0.491	++	456	1.96	1.67	2.26	**7.08E-06**
	rs1365778	2	145305277	***AC010090.1***	18724	G	A	0.327	++	456	2.25	1.89	2.60	**7.57E-06**
2B	rs11173964	12	61795352	***RP11-471N19.1***	168633	C	T	0.392	++	331	2.20	1.86	2.54	**5.16E-06**
3A	rs2800881	1	112133910	***RAP1A***	0	C	T	0.358	++	506	2.07	1.75	2.39	**8.68E-06**
3B	rs2800881	1	112133910	***RAP1A***	0	C	T	0.358	++	456	2.14	1.81	2.48	**6.75E-06**
	rs11058553	12	126635113	***RP1-116K23.1***	17667	G	A	0.255	--	456	0.43	0.05	0.80	**9.48E-06**
4A	rs9973644	2	21648283	***AC067959.1:AC011752.1***	0	C	T	0.320	--	352	0.38	-0.03	0.80	**5.45E-06**
4B	rs13403903	2	21609157	***AC067959.1:AC011752.1***	0	C	T	0.342	++	331	2.58	2.16	3.00	**9.57E-06**

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

* case-control designation and inclusion criteria of each analysis are specified in *Table S3*.

[1] Effect direction represents the effect direction in the GO-CAT cohort and the UK MAGIC cohort, respectively, where '+' means that the effect allele is linked to increased risk to develop the phenotype (OR > 1) and '–' to decreased risk (OR < 1).

Table S5. Results of genetic variants that were associated to platinum-induced ototoxicity in previous studies, in the GWAS meta-analysis of this study.

#*	Gene	SNP	Allele 1	Allele 2	Effect direction[1]	OR (95CI)	Uncorrected *p*-value
1B	*ABCC3*	rs1051640	a	g	--	0.87 (0.6 - 1.27)	0.476
1A	*ABCC3*	rs1051640	a	g	+-	0.91 (0.64 - 1.3)	0.603
4B	*ABCC3*	rs1051640	a	g	++	1.12 (0.67 - 1.88)	0.666
4A	*ABCC3*	rs1051640	a	g	-+	1.1 (0.66 - 1.82)	0.714
2A	*ABCC3*	rs1051640	a	g	-+	0.95 (0.62 - 1.45)	0.802
3B	*ABCC3*	rs1051640	a	g	-+	0.95 (0.64 - 1.42)	0.811
2B	*ABCC3*	rs1051640	a	g	-+	0.96 (0.62 - 1.48)	0.858
3A	*ABCC3*	rs1051640	a	g	-+	1.03 (0.7 - 1.51)	0.89
3B	*ACYP2*	rs1872328	a	g	--	0.58 (0.19 - 1.78)	0.339
3A	*ACYP2*	rs1872328	a	g	--	0.63 (0.23 - 1.72)	0.364
4A	*ACYP2*	rs1872328	a	g	--	0.77 (0.23 - 2.62)	0.68
4B	*ACYP2*	rs1872328	a	g	+-	0.82 (0.24 - 2.79)	0.748
1B	*ACYP2*	rs1872328	a	g	++	1.17 (0.44 - 3.08)	0.757
2B	*ACYP2*	rs1872328	a	g	++	1.1 (0.38 - 3.13)	0.865
2A	*ACYP2*	rs1872328	a	g	++	1.07 (0.38 - 3.01)	0.903
1A	*ACYP2*	rs1872328	a	g	-+	1 (0.42 - 2.37)	0.999
3A	*GSTP1*	rs1695	a	g	+-	0.77 (0.56 - 1.06)	0.112
3B	*GSTP1*	rs1695	a	g	+-	0.79 (0.57 - 1.1)	0.16
2B	*GSTP1*	rs1695	a	g	+-	0.8 (0.56 - 1.14)	0.214
1A	*GSTP1*	rs1695	a	g	+-	0.84 (0.62 - 1.13)	0.245
1B	*GSTP1*	rs1695	a	g	+-	0.83 (0.61 - 1.14)	0.251
4B	*GSTP1*	rs1695	a	g	+-	0.8 (0.53 - 1.21)	0.294
2A	*GSTP1*	rs1695	a	g	+-	0.83 (0.58 - 1.18)	0.302
4A	*GSTP1*	rs1695	a	g	+-	0.82 (0.54 - 1.23)	0.336
1B	*LRP2*	rs2075252	t	c	++	1.16 (0.82 - 1.64)	0.402
1A	*LRP2*	rs2075252	t	c	++	1.06 (0.76 - 1.47)	0.726
2A	*LRP2*	rs2075252	t	c	+-	0.96 (0.66 - 1.39)	0.817
3B	*LRP2*	rs2075252	t	c	-+	1.04 (0.72 - 1.52)	0.823
3A	*LRP2*	rs2075252	t	c	-+	0.97 (0.67 - 1.4)	0.87
4A	*LRP2*	rs2075252	t	c	+-	0.98 (0.63 - 1.54)	0.937
4B	*LRP2*	rs2075252	t	c	+-	1.02 (0.65 - 1.59)	0.948
2B	*LRP2*	rs2075252	t	c	+-	0.99 (0.68 - 1.45)	0.976
2B	***NFE2L2***	**rs6721961**	**t**	**g**	**--**	**0.57 (0.32 - 1)**	**0.049**
2A	*NFE2L2*	rs6721961	t	g	--	0.59 (0.34 - 1.03)	0.063
1B	*NFE2L2*	rs6721961	t	g	--	0.69 (0.43 - 1.08)	0.106
1A	*NFE2L2*	rs6721961	t	g	--	0.73 (0.48 - 1.13)	0.162

#*	Gene	SNP	Allele 1	Allele 2	Effect direction[1]	OR (95CI)	Uncorrected *p*-value
4B	*NFE2L2*	rs6721961	t	g	--	0.68 (0.35 - 1.33)	0.261
4A	*NFE2L2*	rs6721961	t	g	--	0.7 (0.37 - 1.35)	0.287
3A	*NFE2L2*	rs6721961	t	g	--	0.89 (0.57 - 1.4)	0.624
3B	*NFE2L2*	rs6721961	t	g	+-	0.93 (0.59 - 1.47)	0.758
4A	*OTOS*	rs2291767	t	c	-+	2.05 (0.7 - 6.06)	0.193
4B	*OTOS*	rs2291767	t	c	-+	1.97 (0.67 - 5.84)	0.22
2A	*OTOS*	rs2291767	t	c	++	1.63 (0.61 - 4.4)	0.332
2B	*OTOS*	rs2291767	t	c	++	1.63 (0.6 - 4.41)	0.337
1B	*OTOS*	rs2291767	t	c	++	1.45 (0.58 - 3.65)	0.431
1A	*OTOS*	rs2291767	t	c	++	1.34 (0.58 - 3.1)	0.49
3A	*OTOS*	rs2291767	t	c	-+	1.07 (0.46 - 2.5)	0.88
3B	*OTOS*	rs2291767	t	c	-+	1.04 (0.42 - 2.6)	0.935
3A	*SLC16A5*	rs4788863	t	c	++	1.32 (0.95 - 1.83)	0.096
4B	*SLC16A5*	rs4788863	t	c	++	1.42 (0.92 - 2.2)	0.115
4A	*SLC16A5*	rs4788863	t	c	++	1.4 (0.91 - 2.15)	0.131
3B	*SLC16A5*	rs4788863	t	c	++	1.22 (0.87 - 1.71)	0.26
1A	*SLC16A5*	rs4788863	t	c	+-	1.17 (0.86 - 1.6)	0.319
2B	*SLC16A5*	rs4788863	t	c	+-	1.15 (0.8 - 1.66)	0.445
1B	*SLC16A5*	rs4788863	t	c	+-	1.12 (0.81 - 1.55)	0.482
2A	*SLC16A5*	rs4788863	t	c	+-	1.13 (0.79 - 1.63)	0.494
4A	*SLC22A2*	rs316019	a	c	++	1.14 (0.61 - 2.12)	0.69
2A	*SLC22A2*	rs316019	a	c	+-	1.05 (0.62 - 1.8)	0.849
3A	*SLC22A2*	rs316019	a	c	--	0.96 (0.59 - 1.54)	0.853
3B	*SLC22A2*	rs316019	a	c	--	0.95 (0.58 - 1.57)	0.855
4B	*SLC22A2*	rs316019	a	c	++	1.05 (0.55 - 2.01)	0.874
1B	*SLC22A2*	rs316019	a	c	+-	1.02 (0.63 - 1.63)	0.948
1A	*SLC22A2*	rs316019	a	c	+-	1.01 (0.65 - 1.58)	0.952
2B	*SLC22A2*	rs316019	a	c	+-	0.99 (0.58 - 1.71)	0.981
2A	*SOD2*	rs4880	a	g	--	0.76 (0.55 - 1.07)	0.114
2B	*SOD2*	rs4880	a	g	--	0.77 (0.55 - 1.08)	0.129
1A	*SOD2*	rs4880	a	g	--	0.83 (0.62 - 1.1)	0.199
1B	*SOD2*	rs4880	a	g	--	0.85 (0.63 - 1.14)	0.268
3B	*SOD2*	rs4880	a	g	--	0.85 (0.63 - 1.15)	0.296
3A	*SOD2*	rs4880	a	g	--	0.87 (0.64 - 1.17)	0.344
4B	*SOD2*	rs4880	a	g	+-	0.85 (0.57 - 1.25)	0.403
4A	*SOD2*	rs4880	a	g	+-	0.86 (0.58 - 1.27)	0.445

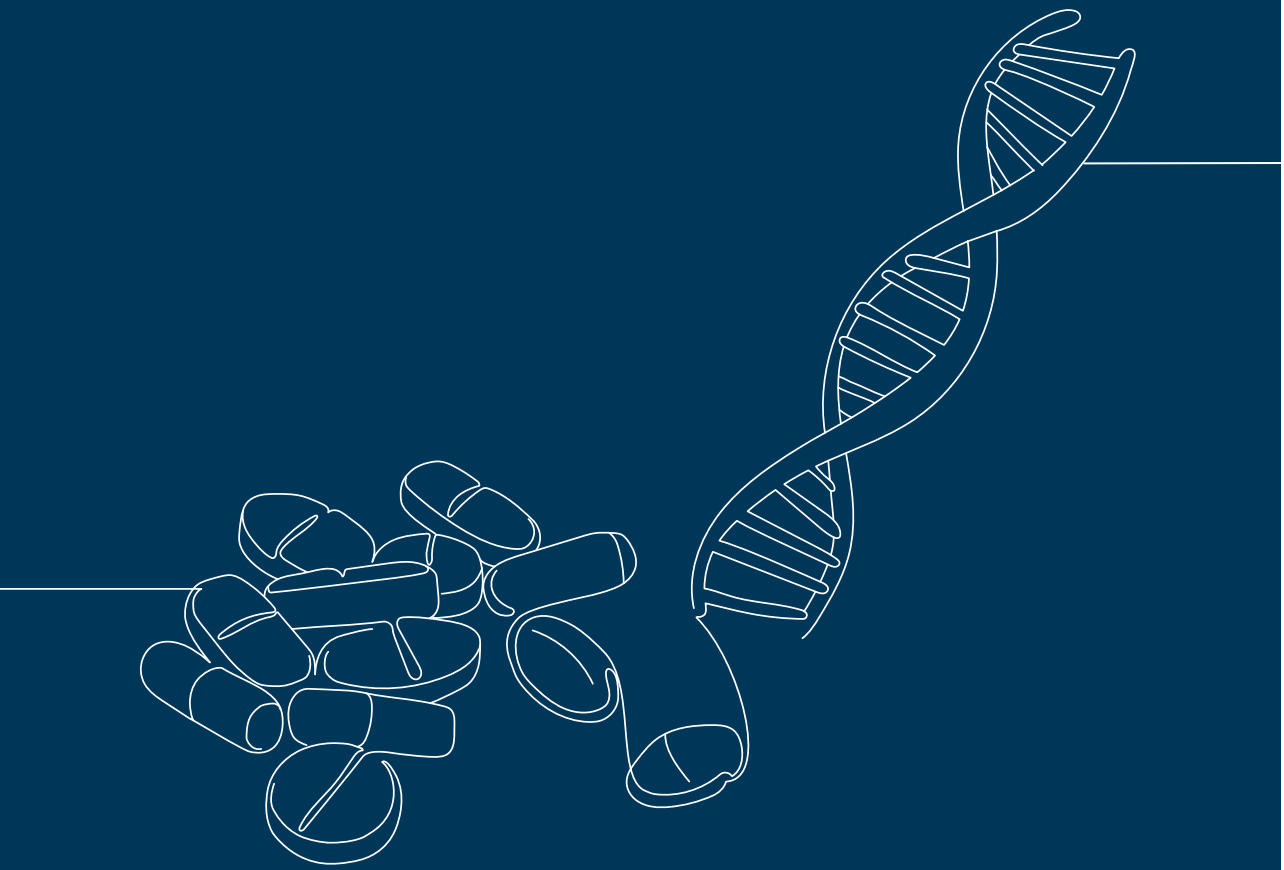
#*	Gene	SNP	Allele 1	Allele 2	Effect direction[1]	OR (95CI)	Uncorrected *p*-value
1B	*TCERG1L*	rs893507	t	c	+?	1.28 (0.72 - 2.31)	0.402
1A	*TCERG1L*	rs893507	t	c	+?	1.15 (0.68 - 1.93)	0.607
2A	*TCERG1L*	rs893507	t	c	+?	1.14 (0.61 - 2.12)	0.675
2B	*TCERG1L*	rs893507	t	c	+?	1.14 (0.61 - 2.13)	0.675
3A	*TCERG1L*	rs893507	t	c	-?	0.88 (0.47 - 1.65)	0.7
4B	*TCERG1L*	rs893507	t	c	-?	0.88 (0.39 - 1.96)	0.747
4A	*TCERG1L*	rs893507	t	c	-?	0.92 (0.41 - 2.04)	0.833
3B	*TCERG1L*	rs893507	t	c	+?	1.06 (0.52 - 2.15)	0.872
4A	*TPMT*	rs1142345	t	c	++	2.23 (0.92 - 5.39)	0.076
4B	*TPMT*	rs1142345	t	c	++	2.22 (0.92 - 5.38)	0.076
3B	*TPMT*	rs1142345	t	c	++	1.54 (0.75 - 3.14)	0.24
3A	*TPMT*	rs1142345	t	c	++	1.52 (0.75 - 3.09)	0.245
1B	*TPMT*	rs1142345	t	c	-+	1.1 (0.56 - 2.18)	0.783
2B	*TPMT*	rs1142345	t	c	-+	0.95 (0.45 - 2.01)	0.887
2A	*TPMT*	rs1142345	t	c	-+	0.95 (0.45 - 2)	0.889
1A	*TPMT*	rs1142345	t	c	-+	1.04 (0.54 - 2)	0.907
4A	*TPMT*	rs12201199	a	t	++	1.8 (0.84 - 3.88)	0.132
4B	*TPMT*	rs12201199	a	t	++	1.79 (0.83 - 3.86)	0.136
3A	*TPMT*	rs12201199	a	t	++	1.51 (0.82 - 2.76)	0.182
3B	*TPMT*	rs12201199	a	t	++	1.52 (0.82 - 2.8)	0.183
2A	*TPMT*	rs12201199	a	t	-+	0.77 (0.4 - 1.48)	0.431
2B	*TPMT*	rs12201199	a	t	-+	0.77 (0.4 - 1.49)	0.435
1A	*TPMT*	rs12201199	a	t	-+	0.93 (0.53 - 1.61)	0.786
1B	*TPMT*	rs12201199	a	t	-+	0.94 (0.53 - 1.67)	0.825
4A	***TPMT***	**rs1800460**	**t**	**c**	**--**	**0.37 (0.14 - 0.94)**	**0.036**
4B	***TPMT***	**rs1800460**	**t**	**c**	**--**	**0.37 (0.15 - 0.94)**	**0.038**
3B	*TPMT*	rs1800460	t	c	--	0.52 (0.24 - 1.12)	0.094
3A	*TPMT*	rs1800460	t	c	--	0.53 (0.25 - 1.12)	0.096
1B	*TPMT*	rs1800460	t	c	+-	0.8 (0.38 - 1.7)	0.569
1A	*TPMT*	rs1800460	t	c	+-	0.86 (0.42 - 1.75)	0.681
2A	*TPMT*	rs1800460	t	c	+-	0.85 (0.38 - 1.91)	0.687
2B	*TPMT*	rs1800460	t	c	+-	0.85 (0.37 - 1.94)	0.701
1A	*TPMT*	rs1800462	Not available		??		
1B	*TPMT*	rs1800462	Not available		??		
2A	*TPMT*	rs1800462	Not available		??		
2B	*TPMT*	rs1800462	Not available		??		
3A	*TPMT*	rs1800462	Not available		??		

#*	Gene	SNP	Allele 1	Allele 2	Effect direction[1]	OR (95CI)	Uncorrected *p*-value
3B	*TPMT*	rs1800462	Not available		??		
4A	*TPMT*	rs1800462	Not available		??		
4B	*TPMT*	rs1800462	Not available		??		
2A	*WFS1*	rs62283056	c	g	++	1.29 (0.85 - 1.95)	0.23
2B	*WFS1*	rs62283056	c	g	++	1.28 (0.85 - 1.95)	0.24
1B	*WFS1*	rs62283056	c	g	++	1.14 (0.79 - 1.66)	0.474
3A	*WFS1*	rs62283056	c	g	+-	0.89 (0.61 - 1.3)	0.552
4A	*WFS1*	rs62283056	c	g	+-	1.14 (0.7 - 1.83)	0.604
1A	*WFS1*	rs62283056	c	g	-+	1.08 (0.76 - 1.54)	0.655
4B	*WFS1*	rs62283056	c	g	+-	1.11 (0.68 - 1.8)	0.683
3B	*WFS1*	rs62283056	c	g	+-	0.93 (0.63 - 1.38)	0.726

OR, odds ratio; CI, confidence interval.

* case-control designation and inclusion criteria of each analysis are specified in *Table S3*.

[1] Effect direction represents the effect direction in the GO-CAT cohort and the UK MAGIC cohort, respectively, where '+' means that allele 1 is linked to increased risk to develop the phenotype (OR > 1) and '–' to decreased risk (OR < 1). A question mark means this variant was absent in this cohort.



CHAPTER 6

General discussion

Children and young adults with osteosarcoma do not always benefit enough from the chemotherapy treatment that is given to eradicate their disease. A proportion of patients develop severe toxicities due to the drugs given or tumor growth can be observed despite treatment. In order to predict who is at risk for an inadequate response to treatment, or to further understand why these interindividual differences exist, the main aim of this thesis was to identify genetic variants that are associated with treatment response or toxicities in patients with osteosarcoma. In this general discussion, I will first reflect on the main results including the genetic associations that are found in this thesis. Subsequently, the next steps towards clinical implementation of personalized treatments will be discussed.

Discovery of pharmacogenetic associations

The main challenge in a genome-wide association study (GWAS) is to obtain sufficiently large patient cohorts, which is even harder in a relatively rare disease as osteosarcoma. In **Chapter 2**, a systematic literature search showed that many genetic variants were previously associated with multiple phenotypes related to osteosarcoma treatment response, however these often were poorly reproducible. Reasons for the lack of reproducibility may be small patient cohorts in the discovery study, leading to false-positive findings, or fundamental differences between discovery and replications studies. Despite that we were aware of these challenges, we were not able in this thesis to fully overcome them. In this thesis, a retrospectively recruited cohort of patients with osteosarcoma was used to identify genetic variants associated with treatment-related phenotypes. All patients were treated with a cisplatin and doxorubicin-based chemotherapy regimen, which was supplemented with methotrexate in the majority of patients. In addition to that, it was decided to join forces among researchers with similar interests from around the world and form the Genetics of Childhood Cancer Treatment (GO-CAT) consortium in the final chapters of the thesis.

Statistical power can be increased by performing fewer tests than in a GWAS by making informed decisions about the focus of the study. In **Chapter 3 and 4**, an array with variants in drug-metabolizing enzymes and transporter genes was used. As these genes play a crucial role in pharmacokinetics, these are more likely to affect treatment response and were therefore prioritized. In **Chapter 3**, the aim was to identify variants associated with methotrexate-induced toxicities. To that end, laboratory markers for multiple toxicities were collected after every course of methotrexate and a generalized estimated equation (GEE) analysis was performed. GEE is an analysis that uses multiple measures per patients, taking into account that the variance within patients is lower than between patients and thereby increases power compared to a conventional linear regression analysis. A genetic variant (rs3736599) in *SULT1E1* was found to be statistically significantly associated with lower methotrexate plasma levels. Furthermore, two intronic variants in *CYP2B6* (rs4803418 and rs4803419) were associated to methotrexate-induced decreased thrombocyte counts and an association with increased thrombocyte counts was identified for the intronic variant rs4808326 in *CYP4F8*. In this study, thrombocyte counts were one of the proxies used to reflect methotrexate-induced bone marrow

suppression and therefore, a role for these genes is indicated in the development of this adverse event. These genes were not previously indicated in this phenotype or another phenotype related to methotrexate metabolism. This, together with the challenge of predicting the effect of intronic variants on gene function, emphasize that additional research is required before these results can be translated to clinical practice.

Chapter 4 focusses on patients with early progressive disease. This subcategory is extremely hard to treat in clinical practice, interestingly however, this study led to the identification of a subcategory of patients with improved survival rates. In the initial genetic association study, it was attempted to identify genetic variants associated to early progressive disease to study if these patients have a distinct pharmacogenetic profile. In total, seven variants were identified to be associated with early progressive disease (rs8013529 and rs1884545 in *SLC7A8*, rs6771233 in *CYP8B1*, rs316003 in *SLC22A2*, rs274548 in *SLC22A5*, rs7886938 in *FMO6* and rs688755 in *CYP4F12*). The two variants in *SLC7A8* were validated in independent cohorts, samples and/or data were provided by collaborators form Spain, Australia and the United Kingdom within the GO-CAT consortium. *SLC7A8* codes for the L-type amino acid transporter 2 (LAT2) and, it was found that doxorubicin is a substrate for this transporter. Moreover, it was shown that expression of LAT2 in osteosarcoma tumor tissue at diagnosis was associated to improved survival in patients without metastases. This was also confirmed in mRNA expression data in the non-metastatic subgroup of an independent patient cohort. The fact that this association refrains itself to non-metastatic patients can be explained by the detrimental effect that metastases have on treatment success. For now, it was proposed that increased expression of LAT2 in tumor tissue, causes higher chemotherapy exposure and therefore decreased risk of progression and improved survival rates. Future research will have to prove this functional hypothesis but despite that, LAT2 expression has proven to be a novel prognostic factor in patients with osteosarcoma without metastases.

In **Chapter 5**, a genome-wide association study was performed to identify genetic variants that play a role in platinum-induced ototoxicity. To increase power, the osteosarcoma patient cohort was combined with other cohorts of platinum-treated childhood cancer patients within the framework of the GO-CAT consortium. Despite that, no genome-wide significant associations were found, but a variant in *TSPAN5* (rs7671702) was suggestively associated with platinum-induced ototoxicity. Interestingly, there may be a functional role for *TSPAN5* in the development of platinum-induced ototoxicity by regulation of ADAM10 and thereby influencing cisplatin sensitivity in cells. Therefore, this finding may contribute to the elucidation of the functional mechanism behind platinum-induced ototoxicity. Larger patient cohort will be necessary to identify reliable prediction models to identify patients at high risk for platinum-induced ototoxicity, using standardized outcome definitions and homogenized analyses. The GO-CAT consortium aims to contribute to these challenges. In **Chapter 4**, the data of the consortium was used to replicate results in independent patient cohorts and in **Chapter 5** the data was included in the discovery phase of the GWAS using a meta-analysis approach to greatly increase power. Unfortunately, power was still insufficient to reach genome-wide significant results. Notwithstanding, this is only the beginning of the GO-CAT consortium and larger studies with additional collaborators from the United States and Canada are in the pipeline. For more information, please visit www.go-consortium.eu.

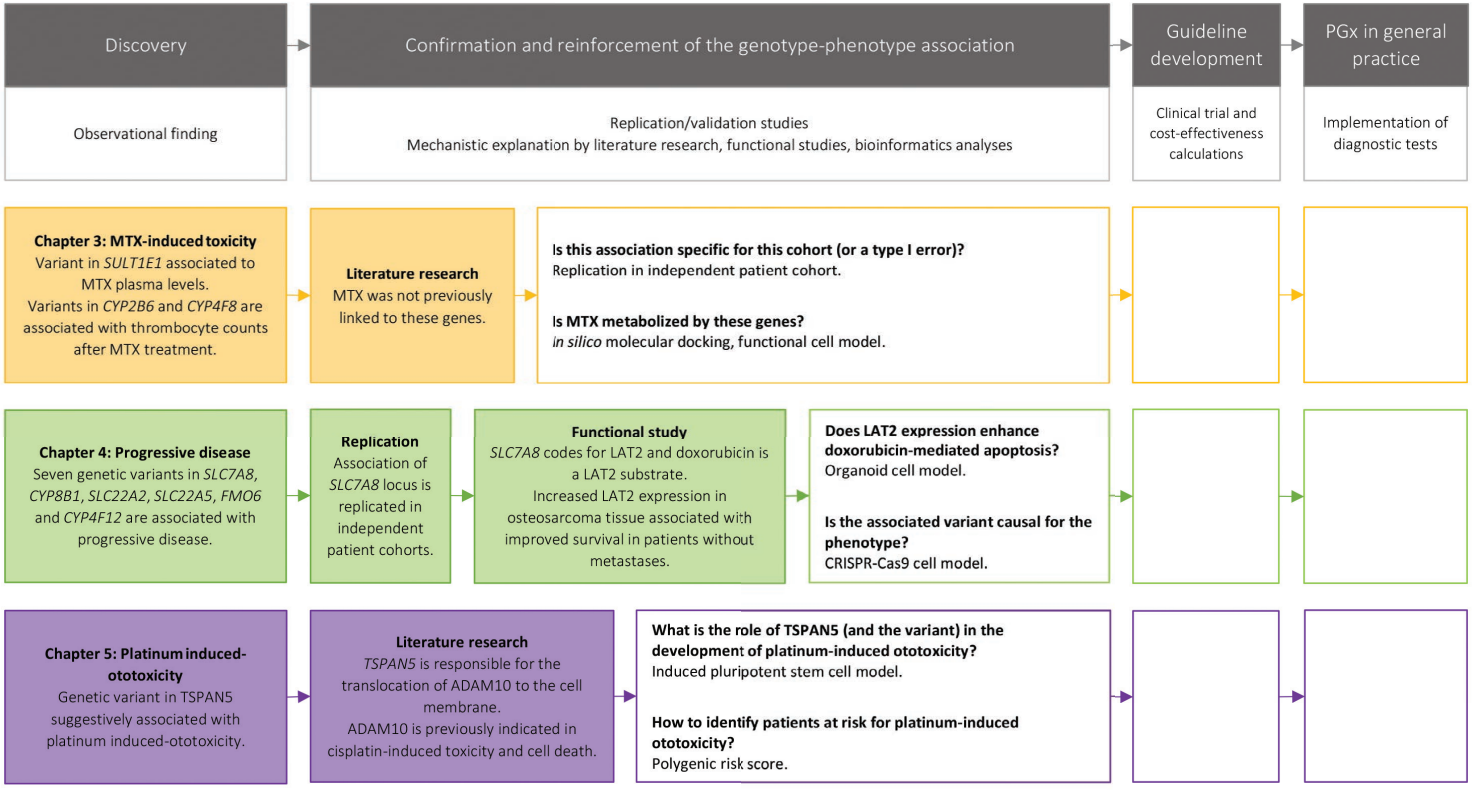


Figure 1. The road towards clinical implementation of pharmacogenetic studies in personalized treatments is described in the gray boxes (adapted from Thorn et al. 2019 (1)). The filled colored boxes show the main results of this thesis and where this research fits on the road towards implementation. In addition, suggestions of follow-up research questions are given with proposed research models. PGx; pharmacogenetics, MTX; methotrexate, LAT2; L-type amino acid transporter 2.

The road towards clinical implementation

The discovery of genetic variants associated to treatment-related phenotypes through observational studies is a necessary start, but after that there is still a long road to go until patients can benefit from this knowledge. *Figure 1* shows what research is needed preparatory to the implementation of novel pharmacogenetic treatment strategies in clinical practice (adapted from Thorn *et al*. (1)). In general, pharmacogenetic associations should first be replicated in multiple patient cohorts and/or one should mechanistically understand why there is an association, e.g. through functional assays. Thereafter, it should be studied which diagnostic test and subsequent intervention can improve treatment response or reduce toxicities in (subgroups of) patients. Finally, it is important to show cost-effectiveness and relevance of the novel intervention in clinical trials to ultimately convince clinicians to implement new treatment regimens.

For the confirmation and reinforcement of the evidence, literature search, database searches and bioinformatics tools could be applied to find out what existing knowledge can help in giving a functional explanation for the association. This appeared to be a fruitful approach to determine possible functional relevance of the *TSPAN5* gene in the development of platinum-induced ototoxicity. In the literature search, it was found that *TSPAN5* codes for tetraspanin 5, which is responsible for the translocation of ADAM10 to the cell membrane (2). ADAM10 is a desintegrin and metalloprotease that cleaves proteins at the cell membrane, involved in cisplatin-induced renal toxicity and cisplatin sensitivity of melanoma cells and gastric cancer cells (3-5). In addition, ADAM10 regulates sensory regeneration in the avian vestibular organs (6). An expression quantitative trait locus (eQTL) of the discovered variant for *TSPAN5* expression in skeletal muscle and tibial nerves was found in the GTEx database, meaning that the variant is associated to expression of *TSPAN5* in these tissues (7). In previous gene-wide analyses, which were identified using the Atlas of GWAS Summary Statistics (8), it was found that TSPAN5 is associated to tinnitus (8) and that ADAM10 with cisplatin-induced ototoxicity (9). Altogether, this indicates that there may be a role for both *TSPAN5* and ADAM10 in platinum-induced hearing loss and this reinforced the relevance of the suggestive significant finding.

Additional bioinformatics analyses could further exploit GWAS results. FUMA is an online interface that analyzes and visualizes GWAS results in gene-wide and pathway-based analyses. This could give more insight into genes and pathways that may be involved in the development of the phenotype (10). A polygenic risk score (PRS) could enhance the predictive potential of GWAS results by combining many small effects of single variants to create a genetic risk score (11). To calculate a PRS, two independent patient samples are required. In the first sample (base data) the GWAS is performed and in the second sample (target data), PRSs based on effects form the base data, are calculated in individual-level genotype and phenotype data. These scores can ultimately be used to provide an estimate of genetic liability to a trait at the individual level. When different, but related, traits are studied in the base and target data, shared etiology among traits can be assessed. For example, if a GWAS on cisplatin-induced tinnitus (12) was used as base sample and our GWAS on platinum-induced ototoxicity as target sample, it could be studied if platinum induced tinnitus and hearing loss develop following similar

mechanisms. Similarly, a significant PRS with serum platinum levels (13) as base sample would suggest that the development of platinum-induced ototoxicity may be a result of increased serum platinum levels. Furthermore, GWASs on age-related hearing impairment (14) or cisplatin-induced peripheral neuropathy (15) would be relevant to include in such analyses. Other bioinformatics approached to combine different GWASs are calculations of genetic correlation through LD score regression (16). However, the advantage of PRSs over LD score regression is that the target sample in PRSs can be small. For LD score regression, also two patient samples are required and large power is necessary in both cohorts. On contrary, LD score regression does not require individual genotype data of patients so when only summary statistics are available this may be more suitable.

Sequencing pharmacogenes can allow for fine-mapping the rare genetic variation of a locus of interest. It was previously shown that rare variants account for a large proportion of variance in gene function of transporters (17). Especially fine-mapping in ethnically diverse populations will give thorough understanding of common and rare genetic variation within a gene and its effect on phenotypes. Similar to 78% of all other genetic association studies, this thesis only focuses on patients from European descent (18). This means that these data cannot be extrapolated to other populations because allele frequencies differ among ethnic groups which compromises their predictive value, leading to unequal availability of personalized treatments. In previous studies, sequencing the *CYP2D6* gene in sub-Saharan African, American Indian and Alaska Native populations lead to the identification of novel high-frequency missense variants (19, 20). The translation of *CYP2D6* guidelines to other populations is very valuable, yet future pharmacogenetic studies should make efforts to represent more diverse populations in the identification of novel pharmacogenes (21, 22).

Cell models can be used to functionally explain the biological mechanism behind associations. In chapter 4 we described that doxorubicin is a substrate of the LAT2 transporter, giving new implications for further research. We hypothesize that increased LAT2 expression in osteosarcoma tumor tissue leads to increased uptake of doxorubicin and more cell death, which ultimately decreases risk on progression and increases survival rates. A previous study in neuroendocrine tumors showed that LAT1 and LAT2 expression in the tumor caused increased DOPA uptake according to a similar mechanism (23). Another study also revealed that progesterone significantly upregulates SLC7A8 mRNA and LAT2 protein expression in uterine leiomyoma tissues, and knockdown of SLC7A8 markedly increased leiomyoma cell proliferation (24). An osteosarcoma cell model, e.g. using FOB cells which express LAT2, would be suitable to test if LAT2 expression enhances doxorubicin-mediated apoptosis and how progesterone could induce this further (25). A 3D cell culture system, for example using organoids, would even better mimic the tumor microenvironment *in vivo* (26, 27). Future functional studies would have to show if LAT2 expression can be modulated to optimize intracellular doxorubicin exposure and eventually improve patient survival.

When genetic variants are found to be associated with toxicities functional studies should be performed in the relevant tissues. An elegant functional model that may be applied are patient-derived induced pluripotent stem cells (iPSC). One could isolate cells

from patients with and without the genetic variants of interest. Thereafter, use CRISPR-Cas9 systems to introduce the variant in a cell line without the specific variants and to remove the variant in a line with a variant. The four cell lines should then be differentiated into a relevant cell type, for example hairy cells in case of an association with cisplatin-induced ototoxicity or cardiomyocytes when there was an association with anthracycline-induced cardiomyopathy (28, 29). The exposure of different concentrations of the relevant chemotherapeutic compound to all four cell lines will show if there are differences in cell death among cell lines and if these are caused by the variant of interest. Eventually, the effect may be rescued with other medicines that can be a candidate as an intervention in patients. This gives novel evidence as to the mechanism behind the toxicity, the model can confirm the functional role of the genetic variant in the risk of developing the toxicity and interventions to relieve the toxicity can be tested. Combined with the possibility of applying this model to the different adverse events, it gives major opportunities to bring pharmacogenetic findings closer to patient care.

Once it is proven with different methods that a genetic variant is predictive of a phenotype and a suitable intervention, for example a genotype-guided dose reduction, is established, prospective clinical trials can be performed. These clinical trials will show clinical utility and cost-effectiveness of these pharmacogenetic tests. Several factors must be taken into account when looking at cost-effectiveness, for example the variant allele frequencies, the test sensitivity, specificity and costs, the prevalence, outcomes and economic impacts of the disease and its treatment (30). Nevertheless, cost-effectiveness studies remain scarce in the field of pediatric oncology, but they are inevitably necessary to reach implementation of novel diagnostic tests.

Concluding remarks

Since the 1970s, treatment regimens of patients with osteosarcoma have remained the same. Survival rates have not improved substantially and patients suffer from long-term side effects as a result of the harsh treatment. In this thesis, we identified genetic variants that are associated with treatment outcome and toxicities. These studies show that there is a genetic basis of treatment response and support the potential of pharmacogenetics in the treatment of osteosarcoma. However, large collaborative efforts are required to further validate these findings and ultimately develop pharmacogenetic guidelines. Eventually, this may lead to refinements in treatment regimens, decreased toxicities and improved quality-of-life and survival rates of patients with osteosarcoma.

References

1. Thorn CF, Whirl-Carrillo M, Hachad H, Johnson JA, McDonagh EM, Ratain MJ, et al. Essential Characteristics of Pharmacogenomics Study Publications. Clin Pharmacol Ther. 2019;105(1):86-91.
2. Matthews AL, Szyroka J, Collier R, Noy PJ, Tomlinson MG. Scissor sisters: regulation of ADAM10 by the TspanC8 tetraspanins. Biochem Soc Trans. 2017;45(3):719-30.
3. Aboyoussef AM, Abdel-Sattar AR, Abdel-Bakky MS, Messiha BAS. Enoxaparin prevents CXCL16/ADAM10-mediated cisplatin renal toxicity: Role of the coagulation system and the transcriptional factor NF-kappaB. Life Sci. 2021;270:119120.
4. Lee SB, Doberstein K, Baumgarten P, Wieland A, Ungerer C, Burger C, et al. PAX2 regulates ADAM10 expression and mediates anchorage-independent cell growth of melanoma cells. PLoS One. 2011;6(8):e22312.
5. Ge X, Cui H, Zhou Y, Yin D, Feng Y, Xin Q, et al. miR-320a modulates cell growth and chemosensitivity via regulating ADAM10 in gastric cancer. Mol Med Rep. 2017;16(6):9664-70.
6. Warchol ME, Stone J, Barton M, Ku J, Veile R, Daudet N, et al. ADAM10 and gamma-secretase regulate sensory regeneration in the avian vestibular organs. Dev Biol. 2017;428(1):39-51.
7. Consortium GT. The GTEx Consortium atlas of genetic regulatory effects across human tissues. Science. 2020;369(6509):1318-30.
8. Watanabe K, Stringer S, Frei O, Umicevic Mirkov M, de Leeuw C, Polderman TJC, et al. A global overview of pleiotropy and genetic architecture in complex traits. Nat Genet. 2019;51(9):1339-48.
9. Wheeler HE, Gamazon ER, Frisina RD, Perez-Cervantes C, El Charif O, Mapes B, et al. Variants in WFS1 and Other Mendelian Deafness Genes Are Associated with Cisplatin-Associated Ototoxicity. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(13):3325-33.
10. Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. Nat Commun. 2017;8(1):1826.
11. Choi SW, Mak TS, O'Reilly PF. Tutorial: a guide to performing polygenic risk score analyses. Nat Protoc. 2020;15(9):2759-72.
12. El Charif O, Mapes B, Trendowski MR, Wheeler HE, Wing C, Dinh PC, Jr., et al. Clinical and Genome-wide Analysis of Cisplatin-induced Tinnitus Implicates Novel Ototoxic Mechanisms. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019;25(13):4104-16.
13. Trendowski MR, El-Charif O, Ratain MJ, Monahan P, Mu Z, Wheeler HE, et al. Clinical and Genome-Wide Analysis of Serum Platinum Levels after Cisplatin-Based Chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019;25(19):5913-24.
14. Nagtegaal AP, Broer L, Zilhao NR, Jakobsdottir J, Bishop CE, Brumat M, et al. Genome-wide association meta-analysis identifies five novel loci for age-related hearing impairment. Sci Rep. 2019;9(1):15192.
15. Dolan ME, El Charif O, Wheeler HE, Gamazon ER, Ardeshir-Rouhani-Fard S, Monahan P, et al. Clinical and Genome-Wide Analysis of Cisplatin-Induced Peripheral Neuropathy in Survivors of Adult-Onset Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(19):5757-68.
16. Bulik-Sullivan B, Finucane HK, Anttila V, Gusev A, Day FR, Loh PR, et al. An atlas of genetic correlations across human diseases and traits. Nat Genet. 2015;47(11):1236-41.
17. Schaller L, Lauschke VM. The genetic landscape of the human solute carrier (SLC) transporter superfamily. Hum Genet. 2019;138(11-12):1359-77.
18. Sirugo G, Williams SM, Tishkoff SA. The Missing Diversity in Human Genetic Studies. Cell. 2019;177(1):26-31.

19. Wright GE, Niehaus DJ, Drogemoller BI, Koen L, Gaedigk A, Warnich L. Elucidation of CYP2D6 genetic diversity in a unique African population: implications for the future application of pharmacogenetics in the Xhosa population. Ann Hum Genet. 2010;74(4):340-50.
20. Fohner A, Muzquiz LI, Austin MA, Gaedigk A, Gordon A, Thornton T, et al. Pharmacogenetics in American Indian populations: analysis of CYP2D6, CYP3A4, CYP3A5, and CYP2C9 in the Confederated Salish and Kootenai Tribes. Pharmacogenet Genomics. 2013;23(8):403-14.
21. Luczak T, Stenehjem D, Brown J. Applying an equity lens to pharmacogenetic research and translation to under-represented populations. Clin Transl Sci. 2021;14(6):2117-23.
22. Carroll DM, Hernandez C, Braaten G, Meier E, Jacobson P, Begnaud A, et al. Recommendations to researchers for aiding in increasing American Indian representation in genetic research and personalized medicine. Per Med. 2021;18(1):67-74.
23. Barollo S, Bertazza L, Watutantrige-Fernando S, Censi S, Cavedon E, Galuppini F, et al. Overexpression of L-Type Amino Acid Transporter 1 (LAT1) and 2 (LAT2): Novel Markers of Neuroendocrine Tumors. PLoS One. 2016;11(5):e0156044.
24. Luo X, Yin P, Reierstad S, Ishikawa H, Lin Z, Pavone ME, et al. Progesterone and mifepristone regulate L-type amino acid transporter 2 and 4F2 heavy chain expression in uterine leiomyoma cells. J Clin Endocrinol Metab. 2009;94(11):4533-9.
25. Choi DW, Kim DK, Kanai Y, Wempe MF, Endou H, Kim JK. JPH203, a selective L-type amino acid transporter 1 inhibitor, induces mitochondria-dependent apoptosis in Saos2 human osteosarcoma cells. Korean J Physiol Pharmacol. 2017;21(6):599-607.
26. Akeda K, Nishimura A, Satonaka H, Shintani K, Kusuzaki K, Matsumine A, et al. Three-dimensional alginate spheroid culture system of murine osteosarcoma. Oncol Rep. 2009;22(5):997-1003.
27. He A, Huang Y, Cheng W, Zhang D, He W, Bai Y, et al. Organoid culture system for patient-derived lung metastatic osteosarcoma. Med Oncol. 2020;37(11):105.
28. Christidi E, Huang H, Shafaattalab S, Maillet A, Lin E, Huang K, et al. Variation in RARG increases susceptibility to doxorubicin-induced cardiotoxicity in patient specific induced pluripotent stem cell-derived cardiomyocytes. Sci Rep. 2020;10(1):10363.
29. Zine A, Messat Y, Fritzsch B. A human induced pluripotent stem cell-based modular platform to challenge sensorineural hearing loss. Stem Cells. 2021;39(6):697-706.
30. Flowers CR, Veenstra D. The role of cost-effectiveness analysis in the era of pharmacogenomics. Pharmacoeconomics. 2004;22(8):481-93.

Summary

Osteosarcoma is the most common primary bone tumor that occurs most often in children and adolescents. These patients are treated with a combination of surgery and intensive chemotherapy, based on a backbone of cisplatin and doxorubicin. This chemotherapy regimen was introduced in the 1970s and had a major impact on survival rates of the patients, but survival rates have not improved since then. In 2019, the 5-year overall survival (OS) was estimated 71%, along with 5-year event-free survival (EFS) of 54%. The presence of metastasis at diagnosis and an axial tumor site increase risk of poorer survival, however, it cannot explain all variation among patients. Moreover, the majority of patients encounters severe side effects that greatly reduce quality-of-life during and after treatment, for example cisplatin-induced ototoxicity, doxorubicin-induced cardiotoxicity, hepatotoxicity, nephrotoxicity and bone marrow toxicity. Whereas these toxicities can partly be predicted by young age, male sex, high anthracycline- and cisplatin dose and poor kidney function, these clinical factors do not explain all interindividual variation. Genetic variation has the potency to explain additional variation in treatment response and toxicity. The main goal of genetic association studies in the field of pharmacogenomics is to identify genetic variants which may explain interpatient variability in drug response to improve drug efficacy and reduce the risk of drug-induced toxicities. Eventually, it is intended to elucidate more of the functional mechanism behind the association when possible. This may give novel opportunities for the refinement of treatment.

In **Chapter 2**, an overview is provided of studies focusing on the discovery of genetic associations (germline polymorphisms) with treatment response and/or chemotherapy-induced toxicities in patient cohorts with osteosarcoma patients. For this, a systematic literature search in MEDLINE and Embase was performed. Fifteen articles met our inclusion criteria. Eight articles reported on doxorubicin-induced cardiomyopathy, four small studies reported on bone marrow, nephro- and/or hepatotoxicity and six studies included analysis for treatment efficacy. A structured literature search on all associations described in these articles was performed to estimate the complete body of evidence. Despite that the results are promising, the majority of associations were poorly reproducible due to heterogeneity among studies and small patient cohorts.

The aim of **Chapter 3** was to identify genetic variants associated to methotrexate-induced toxicities. To that end, methotrexate plasma levels and laboratory measurements during and after high-dose methotrexate treatment. The laboratory measurements concern renal function, liver damage and myelopoiesis were obtained to reflect respective toxicities. After data analysis, a variant in *SULT1E1* was associated with lower methotrexate plasma levels. Also, an association with methotrexate-induced decreased thrombocyte counts was found for two intronic variants in *CYP2B6* and association with increased thrombocyte counts was identified for a variant in *CYP4F8*. This is the first study to identify genetic variants in *SULT1E1*, *CYP2B6* and *CYP4F8* to be associated with methotrexate pharmacokinetics and toxicities.

Despite treatment regimen comprising intensive chemotherapy and surgery, a proportion of patients show on-treatment early disease progression (EDP). In **Chapter 4**,

we identified that the L-type amino acid transporter 2 (LAT2) may be important in governing treatment response to doxorubicin, a critical component of the treatment regimen. First, in a genetic association study of 287 patients with osteosarcoma, an association of EPD with a locus in *SLC7A8* was found. *SLC7A8* is the gene that codes for the LAT2 protein. This association was replicated in an independent patient cohort. Thereafter, LAT2 expression in osteosarcoma tissue was found to be a prognostic factor related to improved survival and doxorubicin was identified to be a substrate of LAT2. We postulate that the absence of LAT2 in osteosarcoma tissue may cause lower intracellular doxorubicin concentrations, contributing to EDP and poorer survival. Improved understanding of the mechanism may lead to potential for manipulation of treatment in those patients with osteosarcoma that are at risk for EPD.

In **Chapter 5** a genome-wide association study (GWAS) was performed to identify genetic variants associated to platinum-induced ototoxicity. Platinum-induced ototoxicity can affect quality-of-life, especially in childhood cancer patients, and when it occurs during treatment it may lead to dose reduction, risking reduced anti-tumor effect of platinum treatment. This study was performed in a cohort of 509 childhood cancer patients treated with cisplatin or carboplatin. No genome-wide significant associations were found, but the lowest p-value was found for the association of a variant in *TSPAN5* with platinum-induced ototoxicity. There may be a functional role for this gene in the development of platinum-induced ototoxicity by regulation of ADAM10 and thereby influencing cisplatin sensitivity in cells.

Altogether, additional research, e.g. through replication studies or functional studies, is necessary to further explore the association that are found in this thesis. Nevertheless, these results give novel insights in the development of methotrexate induced bone marrow suppression, platinum-induced ototoxicity, doxorubicin treatment response and early progressive disease. This can ultimately contribute to a more personalized treatment plan, giving new opportunities to improve survival rates and quality-of-life of osteosarcoma patients.

Samenvatting

Osteosarcoom is de meest voorkomende primaire bottumor en komt met name voor bij kinderen en adolescenten. Deze kinderen worden behandeld met een operatie en chemotherapie, gebaseerd op cisplatin, doxorubicine en methotrexaat. De combinatie van deze drie middelen wordt al gebruikt sinds de jaren '70 en had destijds een grote invloed op overlevingskansen, maar sindsdien zijn de protocollen nauwelijks verbeterd. De overlevingskansen zijn daarmee vergelijkbaar gebleven. In 2019 werd de 5-jaars overleving ('overall survival') geschat op 71% en de 5-jaars event-vrije overleving ('event-free survival') op 54%. De aanwezigheid van metastase bij diagnose en axiale lokalisatie van de tumor verslechteren de overlevingskansen, maar dit verklaart niet alle variatie in therapierespons tussen patiënten. Daarnaast ondervindt de meerderheid van de patiënten ernstige bijwerkingen, bijvoorbeeld gehoorschade (ototoxiciteit) als gevolg van cisplatin en cardiotoxiciteit door gebruik van doxorubicine. Patiënten met een jonge leeftijd, mannelijk geslacht, hoge doxorubicine- en cisplatindosering en een slechte nierfunctie hebben een groter risico deze bijwerkingen te ontwikkelen, maar deze klinische factoren verklaren wederom niet alle variatie tussen patiënten. Genetische variatie is mogelijk een aanvulling in het voorspellen van therapierespons en toxiciteit voor individuele patiënten. Het belangrijkste doel van genetische associatiestudies op het gebied van farmacogenetica is het identificeren van genetische varianten die geassocieerd zijn met de respons op geneesmiddelen. Uiteindelijk wordt nagestreefd om meer van de biologische mechanismen achter de klinische beelden te begrijpen en dit biedt kansen voor het personaliseren van de behandeling.

In **Hoofdstuk 2** wordt een overzicht gegeven van genetische associatiestudies die kijken naar therapierespons en/of bijwerkingen in cohorten met osteosarcoompatiënten. Voor dit overzicht is een systematisch literatuuronderzoek uitgevoerd in MEDLINE en Embase. Vijftien artikelen voldeden aan de inclusiecriteria. Daarvan rapporteerden acht artikelen over doxorubicine-geïnduceerde cardiotoxiciteit, vier studies over beenmerg-, nefro- en/of hepatotoxiciteit en zes studies bekeken de therapierespons. Van alle beschreven associaties in deze artikelen is vervolgens een gestructureerd literatuuronderzoek uitgevoerd om een vollediger beeld te krijgen van al het beschikbare bewijs. Ondanks dat de resultaten veelbelovend zijn, waren de meeste associaties slecht reproduceerbaar vanwege heterogeniteit tussen studies en kleine patiëntencohorten.

Het doel van **Hoofdstuk 3** was om genetische varianten te identificeren die geassocieerd zijn met methotrexaat-geïnduceerde toxiciteiten. Daartoe zijn methotrexaatplasmaspiegels en laboratoriummetingen verzameld van 114 patiënten met osteosarcoom, tijdens en na behandeling. De laboratoriummetingen hadden betrekking op nierfunctie, leverschade en bloedcellen en dienen als maat voor respectievelijke toxiciteiten. Na data-analyse is er een associatie gevonden van een variant in het *SULT1E1*-gen met lagere plasmaspiegels van methotrexaat. Daarnaast is er een verband gevonden van een intronische locus in het *CYP2B6*-gen met lagere trombocytenaantallen en is er een verband gevonden van een variant in het *CYP4F8*-gen met hogere trombocytenaantallen. Dit is de eerste studie waarin deze genen gerelateerd

worden aan de farmacokinetiek en toxiciteit van methotrexaat. In de toekomst draagt dit mogelijk bij aan een betere voorspelling van welke patiënten een groter risico hebben op bijwerkingen door methotrexaat.

Een deel van de patiënten met osteosarcoom ontwikkelt ondanks de intensieve behandeling alsnog ziekteprogressie. In **Hoofdstuk 4** is gevonden dat de L-type aminozuurtransporter 2 (LAT2) een belangrijke rol speelt in de therapierespons op doxorubicine. In een genetische associatiestudie van 287 patiënten met osteosarcoom is een associatie gevonden van progressieve ziekte met een locus in het *SLC7A8*-gen, het gen dat codeert voor LAT2. Deze associatie werd gerepliceerd in een onafhankelijk patiëntencohort. In de functionele studies die hierop volgden bleek LAT2-expressie in osteosarcoomweefsel een prognostische factor te zijn voor overleving én werd doxorubicine geïdentificeerd als een substraat van LAT2. Onze hypothese is dat de afwezigheid van LAT2 in osteosarcoomweefsel kan leiden tot lagere intracellulaire doxorubicineconcentraties, wat bijdraagt aan progressie en slechtere overleving. Nu we beter begrijpen waarom bij sommige patiënten ziekteprogressie optreedt, kan vervolgonderzoek bijdragen aan het personaliseren van de behandeling voor deze kwetsbare groep.

In **Hoofdstuk 5** is een genoomwijde associatiestudie (GWAS) uitgevoerd om genetische varianten te identificeren die geassocieerd zijn met platina-geïnduceerde ototoxiciteit, oftewel gehoorschade. Platina-geïnduceerde ototoxiciteit kan de kwaliteit van leven verslechteren, vooral bij kinderen. Daarnaast kan het leiden tot dosisverlaging, met het risico op een minder effectieve platinabehandeling. Deze GWAS is uitgevoerd in een cohort van 509 patiënten met kanker die behandeld werden met cisplatin of carboplatin. Er zijn geen genoomwijd significante associaties gevonden, maar de associatie met een variant in het *TSPAN5*-gen had de laagste p-waarde. Uit literatuuronderzoek blijkt dat dit gen mogelijk betrokken is bij de ontwikkeling van platina-geïnduceerde ototoxiciteit door regulatie van het ADAM10-eiwit, dat de gevoeligheid van platinum in cellen kan beïnvloeden. Toekomstig onderzoek moet uitwijzen welke rol dit gaat spelen in de klinische praktijk.

De resultaten die zijn beschreven in dit proefschrift geven nieuwe inzichten in de ontwikkeling van methotrexaat-geïnduceerde toxiciteiten, platina-geïnduceerde ototoxiciteit en progressieve ziekte. Aanvullend onderzoek is nodig om de bevindingen te bevestigen en om nieuwe diagnostische testen en interventies te ontwikkelen. Dit kan uiteindelijk bijdragen aan een meer gepersonaliseerd behandelplan voor patiënten met osteosarcoom, met betere overlevingskansen en een verbeterde kwaliteit van leven.

Data management

For this PhD thesis, data was obtained from patient files from the Radboudumc and other hospitals, DNA was genotyped and experimental data was collected. The data management of this study is described below.

The medical and ethical review board Committee on Research Involving Human Subjects Region Arnhem Nijmegen, Nijmegen, the Netherlands has reviewed the study protocol (2017-3978). They concluded that this study does not fall within the remit of the Medical Research Involving Human Subjects Act (WMO), and had passed a positive judgment on the study. Subsequently, all patients and/or their parents gave informed consent for this study. The signed informed consent forms are stored in the safe at Radboudumc Department of Human Genetics in room M320.05.072 and scans of these forms are uploaded in the patient files in Epic.

All data that was extracted from patient files is stored in a Castor EDC database which is accessible for all researchers involved. Castor EDC contains an audit trail, allowing safe storage of original data. For privacy, all participants are represented for by a study number instead of their names. The key to their name and date of birth are stored elsewhere and protected with a password. The data that was received from collaborators from Spain and Australia was also uploaded in Castor EDC to harmonize coding of the data and variable names. Eventually, data from Castor EDC was converted to SPSS files for data analysis and these are stored on the Radboudumc department server: *H:\GR Theme groups\09 PI Group Marieke Coenen\Kinderoncologie\OSTEO\Evelien\Pharmacogenetics*

Raw data will be saved on local Radboudumc servers and in Castor EDC for 15 years after termination of the study. The summary statistic data of all large-scale discovery studies will be made available at RIS (Research Information Services). RIS is the front-end of Metis, the current research information system of Radboud University.

The DNA samples of patients are stored at the Human Genetics department in a -20˚C freezer with Ultimo ID 96011 in room M320.05.048. Raw DMET genotyping data (chapter 3 and 4) is stored at *T:\PIgroup-Marieke-Coenen\Kinderoncologie\OSTEO\Hanneke.* GWAS data (chapter 5) is stored at the Radboudumc department server *T:\PIgroup-Marieke-Coenen\Kinderoncologie\GWAS data ototoxicity nephrotoxicity.*

All results from experimental data are stored in Labguru. Labguru is a digital lab book client which is centrally stored and daily backed-up on the local Radboudumc server. All data archives (view only) are stored on Labguru and accessible by the associated senior staff members.

About the author

Curriculum Vitae

Evelien Hurkmans was born on the 12th of October 1993 in Bakel, The Netherlands. After finishing her secondary school education in 2012 at the Peelland College in Deurne, she started with the bachelor Biomedical Sciences at the Radboud University Medical Center (Radboudumc). During her bachelor, she did an internship at the Department of Pharmacology and Toxicology of the Radboudumc studying uptake of anti-malarial drugs into HEK293 cells overexpressing transporter proteins to elucidate more about pharmacokinetic processes and possible drug-drug interactions of these drugs (Dr. Jan Koenderink, Maarten van der Velden). In addition, she gained additional experience in *in vitro* maturation, culture and fertilization of bovine oocytes at Hurkmans ET B.V. on Saturdays and during holidays. Evelien continued to do her master in Biomedical Sciences at the same university, majoring in Toxicology and Pathobiology. Her first master internship was at the Department of Product Safety of the National Institute of Public Health and the Environment (RIVM) where she studied the influence of nanoparticle shape on inflammasome activation in THP-1 cells (Dr. Rob Vandebriel). The final internship of her master was at the Department of Human Genetics at the Radboudumc (Dr. Marieke Coenen, Dr. Maroeska te Loo). In this final internship she investigated genetic variants associated with treatment toxicity and survival of children with acute lymphoblastic leukemia. Evelien graduated from her masters with honour (*Cum Laude*) in 2017. Directly after that, she started her PhD project at the Department of Human Genetics at the Radboudumc, under supervision of Dr. Marieke Coenen, Dr. Maroeska te Loo and Prof. Han Brunner. The PhD project focused on genetic variation involved in treatment outcome and toxicities in patients with osteosarcoma. In addition, she contributed to the formation international collaborations that would eventually form the Genetics of Osteosarcoma (GO)-consortium. As a part of that she spend three months at The Canadian Pharmacogenomics Network for Drug Safety (CPNDS) at the BC Children's Hospital in Vancouver, Canada (Prof. Bruce Carleton). The results of this PhD project can be found in this thesis. Next to her PhD, Evelien followed postgraduate education in toxicology (PET) courses to become a European Registered Toxicologist (ERT). The next step in Evelien's career will be a residency in clinical chemistry at the clinical laboratory of the Catharina Hospital in Eindhoven.

Awards

2019 - Poster award at the European Society of Pharmacogenomics and Personalised Therapy (ESPT) Congress in Seville.

2019 - Presentation award at the Netherlands Network of Precision Medicine (NNPM) symposium 'Value of Drug transporters in Precision Medicine'.

2019 - Simonsfonds travel grant for a research internship at the Canadian Pharmacogenomics Network for Drug Safety in Vancouver, Canada.

2018 - Gérard Siest award at the 4th ESPT summer school in Genève.

List of Publications

R.J. Vandebriel, S. Remy, J.P. Vermeulen, **E.G.E. Hurkmans**, K. Kevenaar, N.G. Bastús, B. Pelaz, M.G. Soliman, V.F. Puntes, W.J. Parak, J.L.A. Pennings, and I. Nelissen. "Pathways Related to NLRP3 Inflammasome Activation Induced by Gold Nanorods." *International Journal of Molecular Sciences* 23 (2022): 5763.

E.G.E. Hurkmans, M. J. Klumpers, S. H. Vermeulen, M. M. Hagleitner, U. Flucke, H. W. B. Schreuder, H. Gelderblom, J. Bras, H. J. Guchelaar, M. J. H. Coenen, and D.M.W.M Te Loo. "Analysis of Drug Metabolizing Gene Panel in Osteosarcoma Patients Identifies Association between Variants in *SULT1E1*, *CYP2B6* and *CYP4F8* and Methotrexate Levels and Toxicities." *Frontiers in Pharmacology* 11 (2020): 1241.

M. van der Velden, A. Bilos, J.J.M.W. van den Heuvel, S. R. Rijpma, **E. G. E. Hurkmans**, R. W. Sauerwein, F. G. M. Russel, and J. B. Koenderink. "Proguanil and Cycloguanil Are Organic Cation Transporter and Multidrug and Toxin Extrusion Substrates." *Malaria Journal* 16, no. 1 (2017): 422.

PhD portfolio Evelien Hurkmans

Department: **Human Genetics**
Graduate School: **Radboud Institute for Health Sciences**
PhD period: **1-9-2017 – 31-10-2021**
Promotor: **Prof. H.G. Brunner**
Copromotor(s): **Dr. M.J.H. Coenen, Dr. D.M.W.M. te Loo**

Training activities	Hours
Courses	
RIHS - Introduction course for PhD candidates (2017)	15.00
Radboudumc - Introduction day (2017)	6.00
Hand-on Genome data association analysis (2017)	80.00
Biometrics (2018)	102.00
Radboudumc - eBROK course (for Radboudumc researchers working with human subjects) (2018)	26.00
Management voor promovendi (2018)	40.00
Risk Assessment (2018)	40.00
RU - Mindfulness Based Stress Reduction (2019)	28.00
Radboudumc - Scientific integrity (2019)	20.00
Ecotoxicology (2021)	80.00
Seminars	
Figure making with Illustrator and Graphpad (2018)	1.00
CliniQuest (2018)	2.00
Cartesius computer (2018)	4.00
Radboud Research Rounds (2018) (incl. **laptop presentation**)	7.50
Neuro-oncology meeting, Princess Máxima Center (2019) (incl. **oral presentation**)	1.00
Klinische Farmacologiebespreking (2019) (incl. **oral presentation**)	18.00
Statistical Genetics (StatGen) meeting (2019) (incl. **oral presentation**)	6.00
Journal club (2019) (incl. **oral presentation**)	5.00
Genome Research Theme Discussion (2021) (incl. **oral presentation**)	60.00
Department wide Meeting Human Genetics (2021)	10.00

Conferences	
Genetics retreat – Nederlandse Vereniging voor Humane Genetica (NVHG) graduate meeting (2017) (incl. **oral presentation**)	16.00
National PhD day (2017)	8.00
Netherlands Network of Precision Medicine (NNPM) symposium 'Current and future applications of Personalized Medicine' (2017)	8.00
RIHS PhD retreat 2017 (2017)	16.00
NNPM symposium 'Value of Drug transporters in Precision Medicine' (2018) (incl. **oral presentation**)	8.00
Sardinian International Summer School 'From GWAS to function' in Geneva (2018)	50.00
Princess Máxima Center symposium on pediatric oncology (2018)	16.00
New Frontiers symposium (2018)	8.00
RIHS PhD retreat 2018 (2018) (incl. **4- slide oral presentation**)	16.00
Human genetics science infrastructure day 2019 (2019)	8.00
European Society of Pharmacogenomics and Personalised Therapy (ESPT) Congress in Seville (2019) (incl. **poster presentation** and poster prize)	24.00
RIHS PhD retreat 2019 (2019) (incl. **4- slide oral presentation**)	16.00
NNPM symposium "Functional work in precision medicine: from suspicion to confirmation" (2019) (incl. **oral presentation** and presentation prize)	8.00
European Society of Human Genetics (ESHG) online congress (2020)	32.00
Pharmacogenomics Global Research Network (PGRN) symposium (2020)	6.00
Personalized and Precision Medicine (PEMED) International Conference (2021)	24.00
Nederlandse Vereniging voor Toxicologie (NVT) annual meeting (2021)	16.00
ESHG online congress (2021) (incl. **e-poster presentation**)	32.00
BCF Career event (2021)	8.00
Pharmacogenomics Global Research Network (PGRN) symposium (2021)	6.00
Teaching activities	
Lecturing	
Medical Biology - Translational genomics - Wet practical (PCR) (2018)	4.00
Medical Biology - Translational genomics - Computer practical (2018)	5.00
MMD - Genomics & statistics (MMSTA) - Computer Practical (2019)	5.00
MMD - Genomics & statistics (MMSTA) - Interactive Lecture (2019)	5.00
Supervision of internships / other	
Supervision Master thesis (2018)	20.00
Supervision Bachelor internship (2018)	56.00
Supervision of 2nd year medicine student project (2019)	24.00
Supervision Master thesis (2020)	24.00
Supervision Master internship (2021)	40.00
Total	**1060.50**

A

Dankwoord

Allereerst wil ik mijn dank uitspreken naar alle patiënten die meededen aan dit onderzoek. Zij, en hun ouders, gaven ons toestemming hun gegevens te gebruiken zonder dat dit voordelen zou opleveren voor hun eigen behandelingstraject. Doordat zij dit belangeloos doen, is het mogelijk om onderzoek te doen dat hopelijk nieuwe inzichten oplevert ten behoeve van de behandeling van toekomstige patiënten.

Dit proefschrift zou niet geweest zijn wat het nu is zonder mijn promotor en co-promotoren. **Han Brunner**, ik heb veel geleerd van de manier waarop jij naar onderzoeksresultaten kijkt. Jouw adviezen waren ver vooruitgedacht en inspirerend. **Marieke Coenen**, ik wil je bedanken voor hoe jij mij met grote betrokkenheid hebt begeleid tijdens mijn promotietraject. Lockdown of niet; jouw (digitale) deur stond altijd open om even te sparren over moeilijke vragen of om te kletsen bij een kop koffie. Ik heb ook enorm genoten van ons tripje naar de ESPT in Sevilla, waar ik nog meer van jouw wetenschappelijke bevlogenheid heb leren kennen. **Maroeska te Loo**, jij hebt mij wegwijs gemaakt in de klinische aspecten van het wetenschappelijk onderzoek. Heel erg bedankt voor jouw kritische oog en aanstekelijk enthousiasme waarmee jij je werk doet. Marieke en Maroeska, jullie zijn een dynamisch duo dat elkaar aanvult en versterkt, bedankt dat ik de afgelopen vier jaar van jullie mocht leren.

Manuscriptcommissie, **prof. dr. Carla van Herpen**, **prof. dr. Frans Russel** en **prof. dr. Roos Masereeuw**, hartelijk dank voor het aandachtig lezen en beoordelen van dit proefschrift.

Co-authors and collaborators, thank you for showing me the team effort that science is. Special thanks to **Bruce Carleton** for letting me feel very welcome at the CPNDS in Vancouver, Canada. It was an honor to visit your lab and to learn from you and your team about pharmacogenetics and all its aspects. **Jan Koenderink** en **Jeroen van den Heuvel**, bedankt voor de gastvrijheid bij de afdeling Farmacologie en Toxicologie. Jullie expertise over transporters heeft ervoor gezorgd dat we een bevinding die enkel gebaseerd was op statistiek ook functioneel konden beschrijven. Mijns inziens is dat een van de wetenschappelijke hoogtepunten van dit proefschrift. **Melanie Hagleitner** en **Hanneke Vos**, bedankt voor het werk dat jullie gedaan hebben tijdens jullie promoties binnen dit project. Hier heb ik op kunnen voortbouwen.

Ik wil iedereen van de Multifacto-groep graag bedanken voor alles wat ik geleerd heb tijdens MF meetings en uitjes. In het bijzonder **Barbara**, **Alejandro**, **Janita** en **Nina** voor hun expertise in onder andere de statistische genetica. De MF groep was inhoudelijk een gevarieerde onderzoeksgroep en ik vond het inspirerend om na te denken over de toepassing van methoden uit de genetica van psychiatrische aandoeningen in farmacogenetica. Toen ik als masterstudent begon, voelde het als een warm bad om hier terecht te komen. Ik vond met name het lab een fijne plek met **Remco**, **Marina**, **Mascha**, **Marlies** en **Johanne**. Elk kopje koffie dat ik met jullie heb gedronken was een genietmomentje. In het bijzonder wil ik **Johanne** bedanken, omdat je zo veel wist over dit project en altijd wel een oplossing wist voor problemen. Jij hebt me veel geleerd over

DNA-technieken: PCR, primers ontwerpen, Taqman assays, KASP assays, Sanger sequencing en nog veel meer. Jij had beter overzicht over de DNA samples van osteosarcoom patiënten dan wie dan ook. Daarnaast had je altijd wel wat leuks te kletsen, ook tijdens de gezellige wandelingen gedurende de lockdowns. Het was voor mij dan ook vanzelfsprekend om jou te vragen mijn paranimf te zijn.

Ik wil de farmacogenetica-groep (**Marieke**, **Marije**, **Song**, **Niels, Sophie, Johanne en Mascha**) graag bedanken voor het tweewekelijkse momentje van inspiratie en onze gezamenlijke gedrevenheid om meer te leren over farmacogenetica. Met literatuurbesprekingen, koffiemomentjes en een komen en gaan van studenten was er altijd wel wat te beleven. Aan de studenten die ik heb begeleid tijdens mijn promotietraject: **Larissa**, **Hannes**, **Annouk** en **Grigor**, allereerst dankjewel voor jullie bijdrage aan dit proefschrift. Daarnaast wil ik jullie bedanken voor de interessante uitwisselingen, discussies en energie die jullie gaven. Ik vond het een eer om onderdeel te zijn van jullie studiecarrière. In het bijzonder wil ik **Marije** bedanken. Jij was mijn promotiebuddy, steun en toeverlaat en altijd in voor wat gezelligs. Ik ben enorm dankbaar voor de vriendschap die we hebben opgebouwd. Ik vond het fantastisch om met jou naar Canada te gaan. We hebben daar natuurlijk gewerkt, maar ook veel leuke dingen gedaan, zoals fietsen door de stad, skiën/snowboarden, uitgaan, borrelen en heel veel eten geproefd. Van alles wat we daar gegeten hebben was de brunch bij Jam Café toch wel onze favoriet. Ongelofelijk dat dat in Nederland nog niet bestaat. Dankjewel voor hoe jij altijd voor me klaarstond en een grote steun bent geweest. Ik had me geen betere promotiebuddy kunnen wensen en ben blij dat jij mij bij zal staan als paranimf.

Mijn kantoorgenoten van de Human Fanatics Office (**Marije**, **Judith**, **Iris**, **Siebren**, **Cenna**, **Nikoletta**, **Suzanne**, **Janine** en **Euginia**), bedankt voor de leuke tijd. Er was altijd wel een luisterend oor of iemand om mee te lachen. Daarnaast waren borrels in de Aesculaaf of bij iemand thuis ook een welkome afleiding van, het soms frustrerende, PhD-werk. Daarnaast wil ik ook alle andere collega's van de afdeling Genetica, die niet specifiek benoemd worden, bedanken voor de samenwerking.

Ik wil al mijn vrienden en familie graag bedanken voor de interesse in mijn werk en dit onderzoek en voor de steun die jullie tijdens dit traject hebben gegeven, ik heb jullie hard nodig gehad. In het bijzonder **Papa**, **Mama** en **Stan**, omdat jullie nooit ver weg waren en ik altijd bij jullie terecht kon. Schoonouders, **Bea** voor je enthousiasme en **Eddy** voor je trots en vertrouwen, jouw verlies blijft een groot gemis. **Alex** en **Lidia**, omdat ik geen seconde spijt heb gehad van de aanschaf van Gloomhaven en alle qualitytime die dat oplevert. **Moniek** en **Melvin**, omdat jullie zulke lieve, waardevolle mensen zijn en ik heel dankbaar ben dat we al zo lang vrienden zijn. **Fons** en **Lidewij**, voor alle kopjes koffie en gezelligheid, ik vond het fijn om jullie op slechts twee verdiepingen afstand te hebben. **Dian**, **Iris**, **Josien**, **Kelly**, **Megan**, **Michelle** en **Nienke**, voor de gezellige lunches, dinertjes en weekendjes. Ik vind het fijn dat we na de studie zo regelmatig contact hebben gehouden. **Fenna** voor het luisterende oor en de spelletjesmiddagen. **Dina**, for being a fantastic friend and a wonderful person. **Anke**, **Anke**, **Anne**, **Anouk**, **Carmen**, **Claudia**, **Daisy**, **Jolein**, **Linsey**, **Malou**, **Miranda**, **Rowan en Veerle**, voor de afleiding en gezelligheid wanneer ik dat nodig had.

Aafke, **Niels**, **Mette**, **Yves** en **Yvo**, ik wil jullie graag bedanken voor de ruimte die jullie gaven om uit te proberen en te leren. Jullie waren onmisbaar in de ontwikkeling die ik heb doorgemaakt. Die ontwikkeling was nodig om dit boekje te schrijven en zal ik nog lang met me meenemen. **Aafke,** jou wil ik in het bijzonder bedanken voor je expertise, steun en geduld, dat was heel erg waardevol voor mij.

Crossfit Waalfront coaches (**Hugo**, **Arjen**, **Reza**, **Roy**, **Gijs** en **Petra**), bedankt voor de ontspanning die jullie mij de afgelopen jaren hebben gebracht. De eindeloze variatie binnen crossfit, aangevuld met yoga, geeft steeds weer nieuwe energie om met sport en bewegen bezig te zijn. Daarnaast heb ik fantastische sportbuddies ontmoet: **Berber**, **Deniz** en **Siebolt**. Bedankt voor de gezelligheid, de afleiding en jullie enthousiasme. Niet alleen bij crossfit, maar ook tijdens het wandelweekendje in Groningen. **Berber**, onze wekelijkse sport- en koffiemomentjes hebben mij door dit promotietraject heen gesleept. Ik ben heel dankbaar dat ik jou heb leren kennen. Jij hebt mij geleerd om trots te zijn op elke stap, hoe klein ook.

Monique, jij bent mijn allergrootste steun en samen met jou red ik het wel. Jij hebt tijdens mijn promotietraject altijd vertrouwen gehouden en hebt nooit aan me getwijfeld. Jij en Pluk waren mijn favoriete thuiswerkcollega's. Dankjewel voor de energie die je geeft, je open blik en de veilige plek. Ik ben heel dankbaar voor onze momenten samen en ik kijk uit naar wat de toekomst ons gaat brengen.

Institute for Health Sciences

Radboudumc