Genetic variants associated with methotrexate-induced mucositis in cancer treatment: a systematic review and meta-analysis

Running title: Genetic variants and methotrexate-induced mucositis

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Highlights

- 57 studies were included in this systematic review; 26 in the meta-analysis
- A total of 34 SNPs were associated with (more severe) mucositis in ≥1 study
- MTHFRc.677C>T was associated with more severe mucositis (OR 2.53, 95%-CI 1.48-4.32)
- MTRRc.66A>G was associated with mucositis (OR 2.08, 95%-CI 1.16-3.73)
- Current studies are heterogeneous in design with different case-control definitions

Abstract

Methotrexate (MTX), an important chemotherapeutic agent, is often accompanied with mucositis. The occurrence and severity are unpredictable and show large interindividual variability. In this study, we review and meta-analyze previously studied genetic variants in relation to MTX-induced mucositis.

We conducted a systematic search in Medline and Embase. We included genetic association studies of MTX-induced mucositis in cancer patients. A meta-analysis was conducted for single nucleotide polymorphisms (SNPs) for which at least two studies found a statistically significant association.

A total of 34 SNPs were associated with mucositis in more than one study of the 57 included studies. Two of the seven SNPs included in our meta-analysis were statistically significantly associated with mucositis: *MTHFR* c.677C>T (recessive, grade ≥3 vs grade 0-2, OR 2.53, 95%CI [1.48-4.32], False Discovery Rate[FDR]-corrected p-value 0.011) and *MTRR* c.66A>G (overdominant, grade ≥1 vs grade 0, OR 2.08, 95%CI [1.16-3.73], FDR-corrected p-value 0.042).

Keywords

Methotrexate, mucositis, gastrointestinal toxicity, pharmacogenomics, pediatric, adult, cancer.

1. Introduction

Methotrexate (MTX), a folate antagonist, is a cornerstone of the treatment of childhood and adult cancers such as acute lymphoblastic leukemia (ALL), osteosarcoma and non-Hodgkin lymphoma. Although MTX has shown its value in the treatment of these cancer types, it is often accompanied with mucositis. Previous studies showed that 10-40% of patients develop oral or gastrointestinal mucositis after intravenous high-dose MTX therapy despite administration of folinic acid (leucovorin) rescue therapy [1–3]. Risk factors for the development of these toxicities remain uncertain and the severity is highly variable

among patients suggesting a strong interindividual susceptibility. Mucositis can impair oral intake of food and liquids and an impaired quality of life during therapy, often requiring dose reductions or cessations of treatment which can interfere with treatment efficacy [1,4,5].

Many studies have been conducted on the association between genetic variations in genes involved in MTX pharmacodynamics (e.g. *MTHFR*) or pharmacokinetics (e.g. *SLC19A1*) and the development of mucositis (**Figure 1**). In short, MTX is transported into the cell by the reduced folate carrier 1 (*RFC1/SLC19A1*) and proton-coupled folate transporter (*PCFT/SLC49A1*). *SLCO1B1* is an important MTX transporter in the liver. Inside the cell, MTX is polyglutamated (MTX-PG) by Folylpolyglutamate Synthase (*FPGS*) which augments cellular retention and pharmacological activity of MTX, and is afterwards de-polyglutamated by gamma-glutamyl hydrolase (*GGH*). MTX(-PG) inhibits Dihydrofolate Reductase (*DHFR*) and Thymidylate Synthase (*TS*). Often studied and relevant genes in the one-carbon and folate metabolism are Methylenetetrahydrofolate Reductase (*MTHFR*), Methylenetetrahydrofolate Dehydrogenase 1 (*MTHFD1*) and Methionine Synthase/Reductase (*MS/MTRR*) [6,7].

In addition, as coding regions only comprise about 1.5% of the entire genome, awareness was raised regarding the important regulatory functions of non-coding regions such as miRNAs [8]. Therefore, genetic variations in non-coding parts of the genome, such as in miRNA machinery genes and miRNAs have been studied in relation to developing MTX toxicity as well [8,9]. miRNAs play an important role in RNA-silencing and post-transcriptional regulation of gene expression [10]. Genetic variations in these genes and in miRNA products processed by these genes have been studied in relation to MTX-induced toxicity [8].

However, results are often inconsistent and not replicated by other studies. This is likely due to differences in patient characteristics, outcome definitions of toxicity, chemotherapy protocols, thoroughness of clinical phenotyping and often relatively small sample sizes. Several meta-analyses have been conducted for two of the most frequently studied *MTHFR* genetic variations c.677C>T (rs1801133) and c.1298A>C (rs1801131) [11–14]. However, these meta-analyses only included a small and variable subset of the available published studies. This resulted in different conclusions for c.677C>T from no association [11,12] to an increased risk of mucositis in patients with the TT-genotype compared to the CT- and CC-genotype [13,14]. For c.1298A>C three meta-analyses showed no association with an increased risk of developing mucositis [12–14], whereas one study showed an association (AC-genotype vs AA-genotype, Odds Ratio [OR] 1.690, 95%-CI[1.011 - 2.825] and CC-genotype vs AA-genotype, OR 1.889, 95%-CI [1.232-2.896]) between c.1298A>C and developing overall MTX toxicity [11].

To our knowledge no systematic review and meta-analysis of all the studied single nucleotide polymorphisms (SNPs) in relationship to MTX-induced mucositis has been performed. Consequently, it

remains unclear which SNPs have an increased likelihood of involvement in toxicity, and are thus relevant to be studied in more detail given their possible value in clinical prediction models. In this study, we review all previously studied SNPs and conduct a meta-analysis with the aim to identify relevant variants that could be implemented in polygenic clinical mucositis prediction models of toxicity.

2. Methods

This systematic review was carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [15]. The protocol of this review and meta-analysis was published on PROSPERO, registration number: CRD42018092843.

2.1 Data sources and search strategy

Both the Medline (Ovid) database and Embase were searched from inception until the 9th of August 2019. The search terms used can be found in **Table S1**. References of all included studies, reviews, meta-analyses, and PharmGKB [16] were screened for additional articles.

2.2 Selection criteria

Studies were eligible when fulfilling the following inclusion criteria: (1) genetic association studies (candidate gene and genome wide association studies), (2) MTX chemotherapy, (3) studies that included mucositis or gastrointestinal toxicity (including mucositis) as an adverse outcome of MTX treatment, (4) studies published in English language, and (5) studies including cancer patients. Studies had to contain a description of an analysis and results of the association between genetic variants (1) and the development of mucositis (3). Reviews, case reports, letters to the editor, conference abstracts, preclinical studies (*in vivo* or *in vitro* studies), and studies after a stem cell transplantation/ bone marrow transplantation were excluded. All the identified records were screened for meeting the inclusion criteria based on the title and abstract. If it was not clear whether to include or exclude a study, based on screening the title and abstract, the article was assessed full-text. The study selection was conducted by one reviewer.

2.3 Data extraction

Data extracted from full-text articles were: last name of first author, year of publication, country, study design (Genome Wide Association Study [GWAS] or candidate gene), chemotherapy (MTX dose, protocol phase, administration of folinic acid rescue), patient population (sample size, age, sex, ethnicity, type of cancer), and mucositis (definition, grading system used, prospectively or retrospectively collected and

graded), genetic variants (name of gene, rs number, position, and genotype frequency). If the rs number was not given in the article, PharmGKB [16], SNPedia [17] or PubMed dbSNP [18] were used to find the rs number. For each studied genetic variant, data on the occurrence of mucositis per genotype and per grade, OR and 95% confidence interval (CI) and p-value was collected. When this information was not available the authors were requested to provide this information. The data were extracted by one researcher and checked by a second researcher.

2.4 Risk of bias and quality assessment

Publication bias was assessed by a Funnel plot and Egger's test if more than 10 studies were included in the meta-analysis for one single SNP. The quality and risk of bias of each article was assessed using a scoring system based on the STREGA recommendations [19] from a previously published study [20] (**Table S2**). The assessment was performed by two researchers independently and discrepancies were discussed to reach consensus.

2.5 Data-analysis

A meta-analysis was conducted for all genetic variations for which in at least two studies a statistically significant association was found. By using the four-genetic model strategy described by Horita and Kaneko, the most applicable genetic model (recessive, allelic, dominant and overdominant) was chosen per genetic variation [21]. For this specific genetic model, the odds ratio was estimated and the 95%-CI using a random-effects model (DerSimonian-Laird method). To correct for multiple testing we used a False Discovery Rate (FDR)-correction. For the primary analysis, the case-control (case-comparison) definition of severe mucositis (grade ≥3) vs less severe or absent mucositis (grade 0-2) was used, if possible. This definition was selected, because grade ≥3 defines a clear and severe mucositis, which can also be easy recognized when graded retrospectively based on notes in patient files and is therefore often used in studies. Only studies with the information available for the specific model and case-control definition were included in the analysis. For each study, SNPs were tested for deviation from Hardy-Weinberg equilibrium (HWE) in a control group. SNPs that were not in HWE were excluded from the meta-analysis. Study heterogeneity was estimated using the Cochran's Q test and reporting of the I-squared statistic.
As a sensitivity analysis to test the robustness of the meta-analysis, the meta-analysis was repeated several times, each time excluding one study (Leave-One-Out analysis).

Subgroup analyses were conducted within pediatric patients, patients using intravenous (IV) MTX (often high-dose MTX) and ALL patients. Furthermore, we tested the influence of using another cutoff value for mucositis grade to define a patient as case or control.
Statistical analysis were performed using R version 3.5.0.

3. Results

3.1 Study eligibility and characteristics

In total, 259 unique records were identified in Medline and Embase and one additional record was found by cross reference check of reviews/meta-analyses, see **Figure 2**. After screening of the titles and abstracts, 106 articles were selected. Of these, 49 articles were excluded based on reading the full-text (**Table S3**). In total 57 studies were included in the qualitative synthesis. Of the 57 studies 26 were included in the meta-analysis. The study characteristics of the 57 included studies are depicted in **Table S4**. All studies were published between 2005 and 2019 and most were set up as candidate gene studies with the exception of the study of Treviño et al. that conducted a genome-wide association study [22]. Only two studies, i.e. Treviño et al. and Stocco et al., included an independent replication cohort [22,23].

3.2 Study population

In total 7269 patients were studied for associations between genetic variations and MTX-induced mucositis. The sample size per study ranged from 15 to 484 patients with a median of 89 patients. Of the studies which provided information on the sex of the patients (n=49), 41 studies included more males than females. In 43 of the 57 studies only pediatric patients were included. The other 14 studies included adults (n=7), included adult and pediatric patients (n=6) or age was not specified (n=1). Ethnicity was specifically reported in 18 studies, which was mainly Caucasian/white in 12 studies and Asian (Chinese) in 6 studies [5,12,22–37]. One study reported the genetic ancestry but did not report self-identified ethnicity [38]. Most studies were carried out in Europe (n=24) and Asia (n=19). Seven studies were conducted in North America and three in South America. Most studies were carried out on patients with ALL (n=42). Other cancer types were osteosarcoma (n=6), NHL (n=3) or multiple cancer types within one study (n=6).

3.3 Chemotherapy protocols

A large range of chemotherapy protocols was used in the included studies. Thirty three of the 57 studies only included patients treated with the same chemotherapy protocol. The protocol number and name

were not reported in 7 studies. Most commonly, one or more Berlin-Frankfurt-Münster protocols were included (n=19). Across the protocols, MTX was administered in various dosages and administration formulas. However, most studies focused on the protocol phase in which IV MTX (n=50) was administered, in which the MTX dosages varied from 0.1 to 12 g/m^2 (n=44 IV MTX of 2-5 g/m2). In all these studies, MTX administration was combined with folinic acid rescue.

3.4 Mucositis

The NCI CTCAE (v1.0 – 5.0) were used in most studies to grade the severity of mucositis (n=38). In addition, the WHO criteria (n=13), CCG toxicity criteria (n=1) and Oral mucositis index (OMI-score; n=1) were used. Three studies did not use a grading system, but gave a description of what they considered as mucositis being present. Two studies did not report the grading system or a case definition. The cutoff value to classify a patient as mucositis case varied between the studies from mucositis of any grade (grade 1-4) to only severe mucositis (grade 3-4). The case definition was clearly described in most studies; however, the control definition was often missing. Not all studies used a case-control setup to test the association. Some studies used the number of variant alleles or a sum score (adding up all grades of the different courses) in a linear regression model. Other studies used the number of cycles with and without a mucositis event, others the number of patients with at least one event with a grade defined as case during total follow-up. Also, the timing of grading varied (26 prospectively [at the moment the event occurred] and 15 retrospectively [later based on notes in patient files]) and was not always defined (n=16).

3.5 Quality assessment

As none of the studies used a control group (not using MTX), the quality of all the included studies was assessed for only the first four items of **Table S2**. We scored the studies on the reporting of important patient characteristics including at least age, sex, and the number of patients per cancer type, per ALL risk group and per ALL immunophenotype, **Table S5**. In many studies, these patient characteristics were not completely reported. In 27 out of the 48 included studies with ALL patients the number of patients per ALL risk group was not reported. Fourteen of the 48 included ALL studies did not report the ALL immunophenotype. Age, sex, and number of patients were reported in most studies. In 6 studies, one or more of these characteristics were missing.

For the characteristics of treatment, we scored the reporting of the protocol name or MTX dose, route of administration of MTX and number of cycles. All studies except one reported this information.

Thirty-three studies reported whether the distribution of genotypes was in Hardy Weinberg Equilibrium. The quality of genotyping was addressed in 21 studies. The methods for checking the quality included having control samples in the genotyping analysis, repeating the genotyping of all samples with a different method, or reporting the percentage of samples which were successfully genotyped or provided call rates. Description of the genotyping methods used was very limited in 12 studies and in two studies completely missing. In a large number of studies, it was not clear which cells and at which time point (diagnosis or remission) DNA was collected. This information was completely missing in 6 studies. In 31 studies, only the type of material was described. In 19 studies the timing of DNA collection and the type of biological specimen was reported.

A sample size calculation was rarely provided (n=2). In the study by Kotnik et al. from 2017 the sample size calculation was based on the MTX toxicity and genotype association. Correction for multiple testing was carried out in a small number of studies (n=9). Multiple testing correction was not required in seven of the other studies, because of the evaluation of a single SNP.

The geographical locations of the studies were either stated in the article or could easily be deducted based on the author's affiliation. Two studies adjusted for self-reported ethnicity in their analyses.

3.6 Genes studied in relationship with mucositis

A large number of SNPs involved in the pharmacodynamics (n=12) and in the pharmacokinetics of MTX (n=11) were studied for their association with mucositis or gastrointestinal toxicity (**Table S6**). In addition, 12 SNPs in non-coding pre-microRNAs and miRNA processing genes were also studied by Gutiérrez-Camino et al. [9,39] and López-López et al. [8]. In total 34 SNPs in 28 (miRNA processing) genes/pre-miRNAs were at least once statistically significantly associated with mucositis ($p<0.05$). Of these, 7 SNPs were replicated in at least one other study (**Table 1**), and were included in our meta-analysis.

3.6.1 MTHFR c.677C>T (rs1801133)

Multiple studies (n=38) have studied the association between MTHFR c.677C>T and mucositis [1,4,5,24,27–29,31,34–38,40–64]. In eight studies a statistically significant association was found [27,34,37,42,46,47,57,62]. A total of 16 studies provided information on the number of cases and controls per genotype using the grade ≥3 versus grade 0-2 definition as mucositis endpoint and were included in the meta-analysis [1,31,34–37,46–48,50,55,56,58–60,62]. The study of Xie et al. was excluded from the meta-analysis, because of deviation from HWE in the control group (grade 0-2) [36]. The data of the other 15 studies were fitted best using a recessive model. Patients with the TT-genotype had a higher chance of

developing mucositis compared to the CT- and CC-genotype (Odds Ratio [OR] 2.53, 95%-CI [1.48-4.32], FDR-corrected p-value 0.011) (**Figure 3**). However, the heterogeneity was relatively large (I^2=45%, moderate heterogeneity) and was mainly caused by the study of Tantawy et al. [62]. When this study was excluded the I^2 dropped to 8% and the OR was 1.86 (1.29-2.67) with an FDR-corrected p-value of 0.011 (**Figure S7**). There was no clear sign of publication bias based on the Funnel plot or with the Egger's test (p=0.116), with the exception of the study of Tantawy et al. The Leave-One-Out analysis showed a stable OR, including Tantawy et al., varying between 1.86 to 2.88 for which none of the 95%-CI's was including the value 1 (**Figure S8**).

Three subgroup analyses were conducted (**Figures S9-11**). The first was of studies in which patients received IV MTX (n=14). The association with mucositis was less pronounced, but still reached statistical significance after correction for multiple testing (OR 2.32, 95%CI [1.37-3.92], FDR-corrected p-value of 0.023). The second included studies with pediatric patients (n=8) which resulted in an OR of 2.90 (1.22-6.89) with an FDR-corrected p-value of 0.130. The third comprised studies with ALL patients only (n=7), in which the OR was 3.31 (1.09-10.04) with an FDR-corrected p-value of 0.242.

Applying different case-control definitions resulted in a non-significant association for the grade ≥1 versus grade 0 definition and a statistically significant association of MTHFR c.677C>T for the grade ≥3 versus grade 0-1 (excluding grade 2) mucositis definition with a OR of 2.39 (1.35-4.25), of which the FDR-corrected p-value was 0.026 (**Figures S12 and S13**).

3.6.2 MTRR c.66A>G (rs1801394)

In total, four studies were identified in which the association between *MTRR c.66A>G* and mucositis was studied [1,27,49,63]. In two studies a statistically significant association was found [27,49]. Only for the case-control definition of grade ≥1 versus grade 0 three of the four studies provided the number of events per genotype [1,27,49].

Applying the four-genetic model strategy to the 3 studies resulted in an overdominant model (AG vs GG+AA). This model showed an OR of 2.08 (1.16-3.73) with an FDR-corrected p-value of 0.042 (**Figure 4**). The heterogeneity was low (I^2 of 11%, p=0.33) and there was no indication of publication bias using 3 studies). The Leave-One-Out analysis showed that only when the study of Faganel Kotnik et al. was excluded the association was still statistically significant (OR 2.59, 95%CI [1.33-5.03]) (**Figure S14**). However, when one of the other two studies was excluded, no statistically significant association was found.

3.6.3 SL19A1 c.80G>A (rs1051266)

Of the 18 studies conducted for *SL19A1* c.80G>A [1,4,12,26,27,29,30,34,35,47,49,50,54–56,59,61,67], three found a statistically significant association [29,55,59]. A total of 10 studies were included for the primary analysis using the grade ≥3 versus grade 0-2 case-control definition [1,30,34,35,47,50,55,56,59,67]. The four-genetic model strategy showed that the allelic model was fitting the data best. The A-allele was associated with a decrease in mucositis when compared to the G-allele (OR 0.52, 95%CI [0.32-0.85], FDR corrected p-value was 0.09, **Figure 5**). However, the heterogeneity was huge (I^2=69%), mainly caused by the study of Salazar et al. When this study was excluded, the heterogeneity almost completely disappeared (I^2=3%) and the association became statistically significant even after FDR correction (OR 0.68, 95%CI [0.52-0.89], FDR-corrected p-value was 0.037, **Figure S15**). The Leave-One-Out analysis showed a stable OR, varying between 0.48 and 0.68 and the 95%CI's did not include the value 1, **Figure S16**. Visual inspection of the funnel plot showed no clear indication of publication bias, except for the fact that the study of Salazar et al. was clearly an outlier in the funnel plot. Three subgroup analyses were conducted. The first covered the studies in which patients received IV MTX (n=9) and resulted in an OR of 0.48 (95%CI [0.29-0.79], FDR-corrected p-value = 0.036, **Figure S17**). The second included the pediatric studies (n=6) and the third comprised patients with ALL only (n=5). Both ORs were close to the overall OR, but not statistically significant after FDR correction for multiple testing (pediatric: OR 0.45, 95%CI [0.23-0.89], FDR-corrected p-value = 0.134, **Figure S18**; ALL: OR 0.56, 95%CI [0.27; 1.18], FDR-corrected p-value = 0.639, **Figure S19**).

No statistically significant association was found when changing the case-control definition into ≥1 versus 0 or ≥3 vs 0-1 (excluding grade 2) (**Figure S20 and S21**).

3.6.4 TYMS 2R>3R (rs34743033)

Of the twelve studies conducted for *TYMS* 2R>3R [27,29,34,35,43,44,47,49,54,56,63,65], seven studies reported an effect size or number of mucositis events in the publication [27,29,43,44,56,63,65]. For six studies additional information was provided after our request [27,34,35,47,56,65]. All studies showed a lower risk of mucositis for the 3R3R (+2R/3R) genotype compared to other genotype(s), except for the study by Oosterom et al. However, in only 2 of the 12 studies this effect was statistically significant [27,56]. For five studies information was available per genotype and for each grade [34,35,47,56,65]. The four-genetic model strategy showed that the overdominant model best fitted the data. However, the result was close to the border between the overdominant and dominant model. Therefore, we have tested both the overdominant and dominant model. Both models did not show any statistically significant association

between *TYMS* 2R>3R and mucositis, and showed a low study heterogeneity (I^2=17% and I^2=0%) (**Figure S22 and S23**).

3.6.5 ABCC2 c.24C>T (rs717620)

Six studies reported on the *ABCC2* c.24C>T SNP in relationship with mucositis [1,12,33,35,56,66]. Two studies found a statistically significant association [12,33]. Three studies provided the data per grade and per genotype [1,35,56]. In total, four studies were included in the analysis using the ≥3 versus 0-2 case-control definition [1,33,35,56]. The dominant model (CT +TT vs CC) was fitting the data best, but the meta-analysis did not show any association. The heterogeneity was very high (I^2=82%). This was probably caused by the deviant findings of Liu et al. [33]. When this study was excluded the heterogeneity disappeared (I^2=0%), but there was still no statistically significant association found between the *ABCC2* c.24C>T and mucositis (**Figure S24 and S25**).

3.6.6 SLCO1B1 c.1865+4846T>C (rs11045879)

Five studies analyzed the association between *SLCO1B1* c.1865+4846T>C and mucositis [12,22,23,56,67]. Two studies found a statistically significant association between *SLCO1B1* c.1865+4846T>C and mucositis [22,23]. Four studies were included in the meta-analysis using the ≥3 versus 0-2 case-control definition [22,23,56,67]. One of these studies both reported the results for the discovery and replication cohort and both cohorts were separately included in the meta-analysis [23]. The data fitted the over-dominant model best using the four-genetic model strategy. However, the result was close to the border between the over-dominant and dominant model. Both the over-dominant and dominant model showed no statistically significant association of the *SLCO1B1* c.1865+4846T>C and mucositis (**Figure S26 and S27**).

3.6.7 miR-1206 A>G (rs2114358)

Gutiérrez-Camino et al. [9] and López-López et al. [8] studied the association of *miR-1206* A>G with mucositis. They both showed a statistically significant association of the GG-genotype and a higher chance of developing mucositis compared to patients with the AA- or AG-genotype. The studies used different case-control definitions ≥3 vs 0-2 (Gutiérrez-Camino et al.) and ≥2 versus 0-1 (López-López et al.). With the provided information we were able to meta-analyze the two studies using the ≥2 versus 0-1 definition. Although the result of the four-genetic model strategy was close to the border with the recessive model, they fitted the over-dominant model better. We have tested both models, but did not find any statistically significant association with mucositis (**Figure S28 and S29**).

3.6.8 Other SNPs identified in review

In addition to the seven genetic variations studied in our meta-analysis, we identified 15 additional SNPs in this literature review that were significantly associated with mucositis. However, these SNPs had been studied in only one study. Most had ORs with a 95%-CI close to 1. The exception was *GGH* c.452C>T (rs11545078) with a very low OR of 0.11 (0.02-0.56), **Table 2**.

In the GWAS of Treviño et al. two SNPs were found to be statistically significant associated with clinically relevant MTX-induced gastrointestinal toxicity [22]: *SLCO1B1* c.1865+4846T>C (rs11045879), included in our meta-analysis, and *SLCO1B1* c.1865+248G>A (rs4149081; OR 15.3, 95%CI [7.9-24.6], p = 0.03). Only the study of López-López also studied the latter SNP, but did not find a significant association [12].

4. Discussion

In the present review, we included 57 articles studying a large number of genetic variations involved in the pharmacodynamics and pharmacokinetics of MTX in relation to mucositis. Twenty-six articles were included in our meta-analysis. Seven genetic variations were found to be associated with mucositis in at least two independent studies. Of these, two SNPs – *MTHFR* c.677C>T and *MTRR* c.66A>G – were found to be statistically significant in our meta-analysis, and one – *SLC19A1* c.80G>A – was statistically significant after reducing heterogeneity by excluding one outlying study.

The data show that carriers of the *MTHFR* c.677C>T TT-genotype and *MTRR* c.66A>G AG-genotype were at increased risk and carriers of the *SLC19A1* c.80G>A A-allele were decreased risk of developing (severe) mucositis compared to patients with (less severe) or no mucositis. Methylenetetrahydrofolate Reductase (*MTHFR*), Methionine Synthase Reductase (*MTRR*) and Solute Carrier Family 19 member 1 (*SLC19A1*) - also known as the reduced folate carrier 1 (*RFC1*) – are important enzymes and a transporter respectively in folate and MTX metabolism. *MTHFR* plays a central role in folate metabolism by catalyzing the conversion of 5,10-methylenetetrahydrofolate (THF) to 5-methylTHF, which is the primary circulating form of folate and is utilized in many subsequent reactions important for purine- and thymidine synthesis and homocysteine remethylation pathway [68]. *MTHFR* c.677C>T causes alanine to be replaced by valine and is associated with a thermolabile *MTHFR* enzyme with reduced activity [68,69]. *MTRR* is involved in remethylating homocysteine to methionine. Methionine is an essential amino acid, necessary for protein synthesis and one carbon metabolism [70]. *MTRR* c.66A>G causes isoleucine to be replaced by methionine and is associated with a decreased enzyme activity [71]. In our study, the overdominant model (AG vs. AA+GG) was significant, whereas we expected to observe an effect of the GG-genotype or G-allele. Given

that studies included in our meta-analysis were relatively small, we recommend this variant to be tested in a large independent validation cohort. *SLC19A1* is one of the primary transporters involved in transporting MTX into the cell. *SLC19A1* c.80G>A results in the substitution of a histidine for an arginine in the protein sequence and has been associated with possibly altered uptake of MTX and higher plasma folate levels [72].

The only GWAS published by Treviño et al. showed that *SLCO1B1* c.1865+4846T>C T-allele (OR 16.4, 95%CI[8.7-26.7]) and c.1865+248G>A G-allele (OR 15.3, 95%CI [7.9-24.6]) were associated with gastrointestinal toxicity [22]. For *SLCO1B1* c.1865+4846T>C we did not find a significant association in our meta-analysis, in which four candidate-gene studies and the GWAS results were taken into account [**Figure S26-27**]. For *SLCO1B1* c.1865+248G>A, another smaller candidate-gene study by López-López et al. also showed the lack of a significant association with mucositis [12]. For both SNPs it would be interesting to study their association with mucositis further in larger patient cohorts. In addition, *GGH* c.452C>T (OR 0.11, 95%CI[0.02-0.56]) showed a large effect in one study and would therefore be valuable to be tested in an independent validation cohort or in a GWAS setting [25].

In our review, we observed that the main focus in the field of pharmacogenomics of MTX-induced mucositis was on pediatric (43/57 studies) ALL (42/57 studies) patients using IV MTX at doses varying between 2 to 5 g/m2 (44/57 studies). When conducting a subgroup analysis in these three categories (IV MTX - pediatric patients - ALL patients) the number of studies included in each decreased. Consequently, statistical significance decreased after FDR-correction. Still, effect sizes remained similar and showed the same directions of effects. These subgroup analyses further emphasized that creating large homogeneous cohorts with similar patient and treatment characteristics are needed to be able to extrapolate results to certain populations. For *MTRR* c.66A>G studies were too few to be able to conduct subgroup analyses.

A large variety of chemotherapy protocols – including often concomitant chemotherapy – and endpoints was used. Differences between treatment protocols could lead to differences in the risk of mucositis. Furthermore, the definition of a mucositis case was different between the studies, which might have influenced the results. In our study, we tried to take this factor into account by only analyzing studies with a clear and comparable case-control definition and by mostly focusing on grade 3 or higher. A grade 3 mucositis requires medical intervention, such as a hospitalization, extra pain management or a nasal tube to aid nutrition [73]. Given that these interventions - especially when studied retrospectively - are often mentioned in the patient record file, it becomes less likely that cases were missed. For *MTHFR* c.677C>T and *SLC19A1 c.*80A>G, it was possible to analyze other case-control definitions than the grade 3 or higher

versus grade 0-2 definition, because of available data per grade. When using the grade 1 or higher versus grade 0, the effect became less pronounced for both SNPs. When using the grade 3 or higher versus grade 0-1 (excluding grade 2) definition, the effect became more pronounced for *SLC19A1* c.80A>G. This comparison was unfortunately impossible for *MTRR* c.66A>G, where only grade 1 or higher versus the grade 0 definition were available to be analyzed. It remains an important factor to take into account in future studies that mucositis should preferably be assessed in a prospective, valid and reliable manner. For future pharmacogenomic studies in which the mucositis is prospectively graded, it would be interesting to use a case definition of grade ≥2 instead of grade ≥3, because it is clinically relevant to find genetic risk factors that predict an increased risk of developing grade ≥2 mucositis. High-risk patients can then be closely monitored to allow timely interventions to prevent severe mucositis from occurring. For controls only grade 0 should be used to compare real controls (patients without any grade of mucositis). For our meta-analysis we didn't use this definition for cases (grade ≥2 mucositis) and controls (grade 0 mucositis), because this would have substantially reduced the number of included studies.

In a large proportion of the included studies, important baseline information on the cancer type, immunophenotype or risk classification was missing. In over one-fifth of the studies, the description of the laboratory methods used was very limited or completely missing. In more than half of the studies information on the quality of genotyping was missing. Sample size calculations were almost never provided and corrections for multiple testing were only carried out in a small proportion of the studies. Finally, only two studies adjusted for self-reported ethnicity. We therefore concluded that the number of high-quality studies remains limited and that abovementioned factors are important to be taken into account and to be described in future studies.

With regard to the results of our meta-analysis, it is important to note that effect sizes range between ORs of 0.52 and 2.63, never exceeding a 2.6-fold increased or decreased risk MTX-induced mucositis. Most effect sizes of risk alleles in meta-analyses are relatively small. This is often observed in pharmacogenomics of complex traits such as the multifactorial process of developing MTX-induced mucositis [74,75], but also could be due to improper study design issues as noted. Studies were often underpowered, studied cohorts were heterogeneous with regard to clinical and treatment characteristics and all but one [22] cohort studied genes in a candidate gene approach. It is known, that these types of studies are suboptimal for studying multiple genetic variants and rare genetic variants leading to extreme phenotypes [74,76,77]. In future studies, large sample sizes with clearly defined genetic materials, as well as prospectively collected treatment and toxicity data of good quality allowing for subgroup analyses,

should be studied to further elucidate the role of the five polymorphisms identified in this study – *MTHFR* c.677C>T, *MTRR* c.66A>G, *SLC19A1* c.80G>A, *SLCO1B1* c.1865+4846T>C and *SLCO1B1* c.1865+248G>A – and to identify possible new polymorphisms. In these studies, complex gene-gene and gene-environment interactions should be taken into account as the strategy of studying single variant associations has not proven to be very successful [75]. To date, oral and gastrointestinal mucositis/toxicity were often studied in one combined measure of toxicity, due to power issues. In future studies it might be valuable to study these two phenotypes separately since genetics might influence oral mucositis and gastrointestinal mucositis/toxicity differently.

In conclusion, we reviewed all reported genetic variation studies regarding the development of MTX-induced mucositis in the adult and pediatric cancer population. *MTHFR* c.677C>T is a valuable candidate to study in future genetic prediction models. The role of *MTRR* c.66A>G, S*LC19A1* c.80G>A, *SLCO1B1* c.1865+4846T>C and c.1865+248G>A remains unclear and should be further studied in large, homogenous cohorts with clear case-control definitions.

CRediT author statement

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Conflict of interest

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Dr. Bruce Carleton is Director of the Pharmaceutical Outcomes Programme at the BC Children's Hospital and Professor of Pediatrics, Medical Genetics, Pharmaceutical Sciences, Population & Public Health at the University of British Columbia. He is the Chief of the Division of Translational Therapeutics, in the Department of Pediatrics and a Senior Clinician Scientist at the BC Children's Hospital Research Institute in Vancouver, Canada.

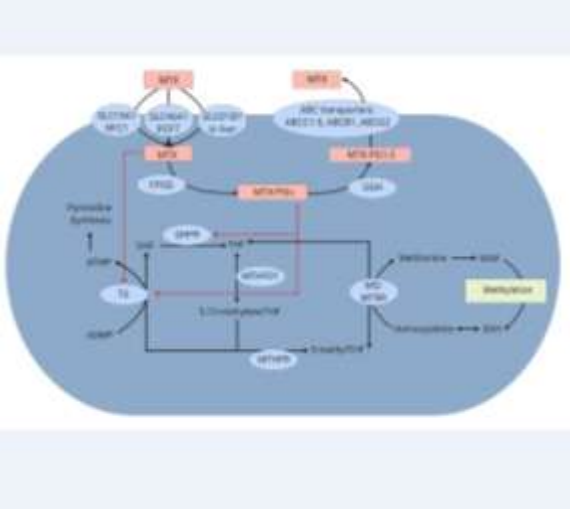

Figure 1. Important transporters and enzymes in MTX- and folate metabolism

Mechanism of action of MTX. MTX is transported into the cell by Reduced Folate Carrier 1 (SLC19A1/RFC1) / Proton-Coupled Folate Transporter (SLC49A1/PCFT). In the liver, MTX is transported by SLCO1B1. MTX is then polyglutamated by Folylpolyglutamate Synthetase (FPGS) and inhibits Dihydrofolate Reductase (DHFR) / Thymidylate Synthase (TS). MTX is de-polyglutamated by gamma-glutamyl hydrolase (GGH) and then transported out of the cell by ABCC1-5, ABCB1 and ABCG2. Methylene tetrahydrofolate reductase (MTHFR), Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase And Formyltetrahydrofolate Synthetase 1 (MTHFD1) and Methionine Synthase / Methionine Synthase Reductase (MS/MTRR) are important enzymes in the folate / one-carbon metabolism cycle.

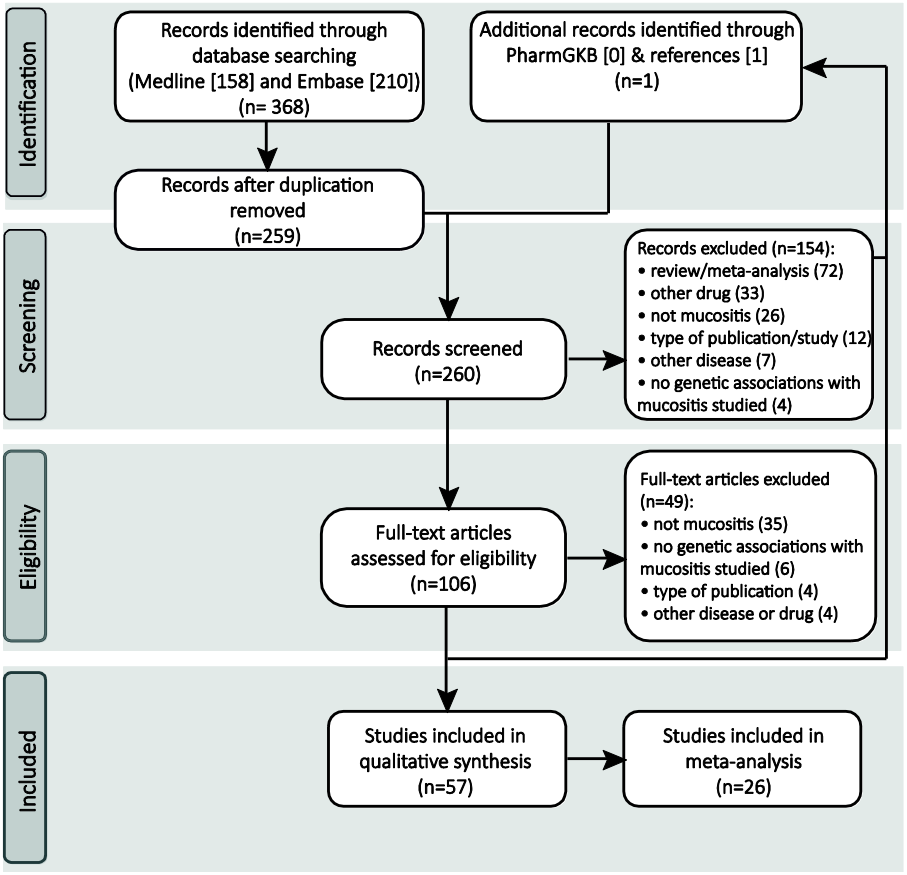

Figure 2. Flowchart

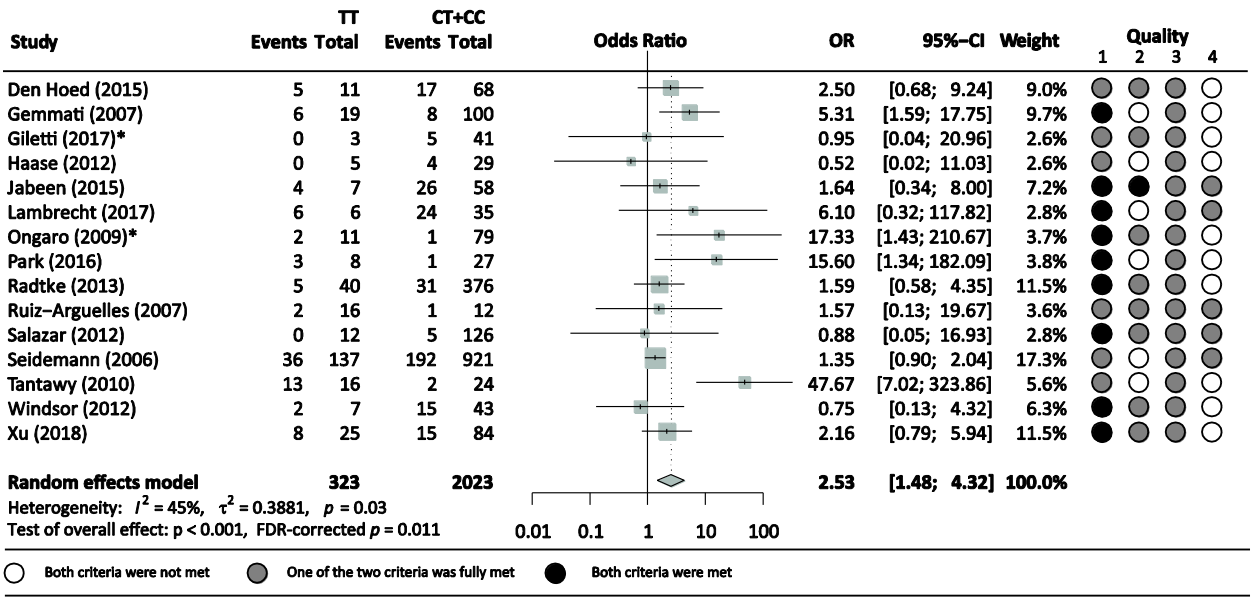

Figure 3. Meta-analysis results for *MTHFR c.677C>T* (rs1801133) grade ≥3 versus grade 0-2 mucositis or gastrointestinal toxicity

Abbreviations: OR *odds ratio*, CI *confidence interval, * outcome was gastrointestinal toxicity, not mucositis*. Quality: item 1 = quality of clinical information; item 2 = quality of genotyping; item 3 = quality in reporting of study population origin; item 4 = quality in terms of sample size calculation and statistical correction for multiple testing (for more detailed information, see table S3 and S5).

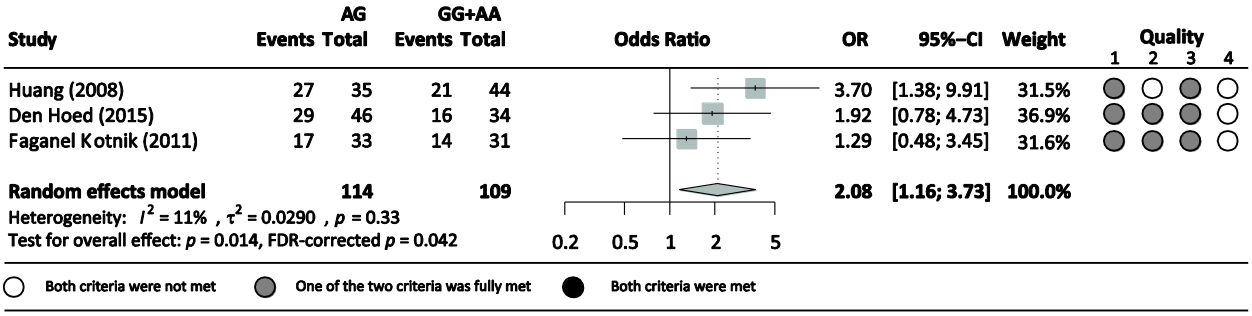

Figure 4. Meta-analysis results for *MTRR c.66A>G* (rs1801394) grade ≥1 versus grade 0 mucositis or gastrointestinal toxicity

Abbreviations: OR *odds ratio*, CI *confidence interval*. Quality: item 1 = quality of clinical information; item 2 = quality of genotyping; item 3 = quality in reporting of study population origin; item 4 = quality in terms of sample size calculation and statistical correction for multiple testing (for more detailed information, see table S3 and S5).

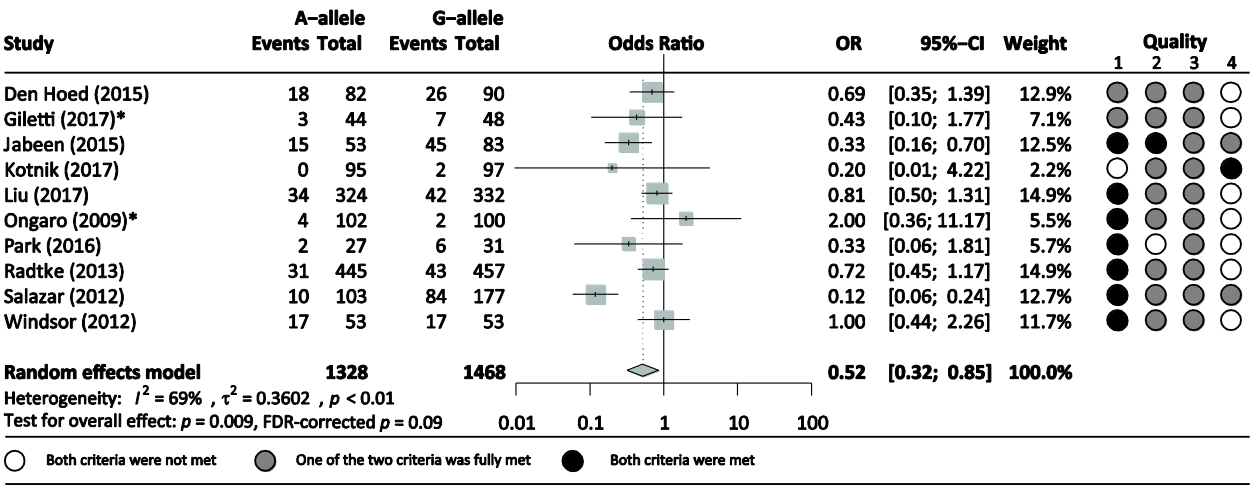

Figure 5. Meta-analysis results for *SL19A1* c.80G>A *(rs1051266)* grade ≥3 versus grade 0-2 mucositis or gastrointestinal toxicity

Abbreviations: OR *odds ratio*, CI *confidence interval, * outcome was gastrointestinal toxicity, not mucositis*. Quality: item 1 = quality of clinical information; item 2 = quality of genotyping; item 3 = quality in reporting of study population origin; item 4 = quality in terms of sample size calculation and statistical correction for multiple testing (for more detailed information, see table S3 and S5).

Table 1. SNPs with at least one statistically significant replication

SNP	Number of studies that found a statistically significant association	Total number of studies [ref.]
MTHFR c.677C>T (rs1801133)	8	38 [1,4,5,24,27–29,31,34–38,40–64]
MTRR c.66A>G (rs1801394)	2	4 [1,27,49,63]
TYMS 2R>3R (rs34743033)	2	12 [27,29,34,35,43,44,47,49,54,56,63,65]
ABCC2 c.24C>T (rs717620)	2	6 [1,12,33,35,56,66]
SLC19A1 c.80G>A (rs1051266)	3	18 [1,4,12,26,27,29,30,34,35,47,49,50,54–56,59,61,67]
SLCO1B1 c.1865+4846T>C (rs11045879)	2	5 [12,22,23,56,67]
miR-1206 G>A (rs2114358)	2	2 [8,9]

Table 2. SNPs assessed in one study and with a statistically significant association

Gene name/miRNA and rs number	Genotype comparison	OR (95%CI)	p-value (corrected p-value)	Study
DHFR (rs1650723)	NR	NR	0.005	[50]
CNOT1 (rs11866002)	TT vs CC + CT	6.68 (1.74-25.70)	0.0056 (0.3956)	[8]
hsa-mir 4268 (rs4674470)	AA + AG vs GG	0.31 (0.12-0.80)	0.0093 (0.011)	[39]
hsa-mir 3683 (rs6977967)	AA vs AG + GG	0 (0.0)	0.0098 (0.003)	[39]
GGH (rs11545078)	CT+TT vs CC	0.11 (0.02-0.56)	0.010	[25]
ABCG2 (rs2231135)	NR	NR	0.01	[50]
ABCC1 (rs2230671)	NR	NR	0.0105	[12]
XPO5 (rs7755135)	AG + AA vs GG	2.68 (1.14-6.32)	0.0251	[8]
EIF2C1 (rs595961)	NR	NR	0.0294	[8]
miR-146a (rs2910164)	CG + CC vs GG	2.54 (1.08-5.97)	0.0309	[8]
miR-1307 (rs7911488)	AG + GG vs AA	2.80 (1.04-7.54)	0.0311	[8]
TNRC68 (rs139919)	NR	NR	0.0327	[8]
SND1 (rs322825)	CT + TT vs CC	0.41 (0.17-0.96)	0.0394	[8]
SLC22A6 (rs4149172)	NR	NR	0.0397	[12]
ABCC3 (rs9895420)	AA + AT vs TT	0.22 (0.05-0.97)	0.044	[38]

Abbreviations: OR *odds ratio*, CI *confidence interval*, NR *not reported*.