MTHFR variants 677TT and 677CT/1298AC are associated with decreased tolerance to methotrexate in pediatric acute lymphoblastic leukemia

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Abstract

Background Methotrexate (MTX) remains a critical component in the treatment of pediatric acute lymphoblastic leukemia (ALL), exerting its antileukemic effect through interference of the folate metabolic pathway. MTHFR is an enzyme that serves as the rate limiting step within this pathway and there has been speculation that certain MTHFR single nucleotide polymorphisms (SNPs) alter physiologic responses to MTX and affects drug toxicity. **Methods** We performed a retrospective analysis of pediatric patients treated at our institution to assess correlation between different MTHFR genotypes and MTX induced toxicities. Specifically, we examined maximum tolerated Capizzi and oral MTX doses, MTX clearance times during high dose MTX (HDMTX), and frequency of MTX-associated toxicities. **Results** Within our study population, 46 out of 242 patients were tested for MTHFR SNPs with 33 resulting positive for a known MTHFR polymorphism. Patients with MTHFR genotypes including those who were homozygous 677TT and compound heterozygous 677CT/1298AC demonstrated significantly decreased tolerance to oral MTX as demonstrated by decreased maximum tolerated MTX dosing relative to control and the 677TT genotype also demonstrated reduced tolerance to IV MTX (Capizzi MTX). Clinically significant MTHFR genotypes were likely to be detected in the presence of myelosuppression, but no other known MTX adverse effects demonstrated predictive ability. Lastly, no genotype was associated with increased risk of developing MTX leukoencephalopathy or thrombosis with any SNP. **Conclusions** MTHFR genotypes including homozygous 677TT and compound heterozygous 677CT/1298AC are associated with decreased tolerance to both Capizzi and oral MTX, manifested by increased myelosuppression.

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Abbreviations:

6-MMP	6-methylmercaptopurine
6-MP	6-Mercaptopurine
6-TG	6-thioguanine
ALL	Acute Lymphoblastic Leukemia
CAR	Chimeric Antigen Receptor
C-MTX	Capizzi Methotrexate
CNS	Central Nervous System
COG	Children's Oncology Group
DNA	Deoxyribonucleic Acid
EHR	Electronic Health Record
HDMTX	High-Dose Methotrexate
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
SNP	Single Nucleotide Polymorphism

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Conclusions

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Introduction

Pediatric acute lymphoblastic leukemia and lymphoma (ALL) is the most common childhood malignancy, accounting for approximately 25% of all pediatric cancer diagnosis. Chemotherapy inhibiting folate metabolism, particularly methotrexate (MTX), has remained a core component of ALL therapy since the first published remissions as a monotherapy in 1948. Currently within Children's Oncology Group (COG)-based protocols, MTX is administered in by multiple modes of delivery including intravenous, intrathecal, and oral administration during all phases of ALL treatment. One of the major contributions of MTX is to combat leukemia in sanctuary sites, namely the CNS and testicles, thereby preventing local relapse.

The antileukemic activity of MTX is attributed to its ability to disrupt folate metabolism. MTX enters the cell through the reduced folate carrier and then polyglutamated which helps retain the drug intracellularly. It is also believed that the polyglutamated form is more bioactive, inhibiting MTX's main targets, Dihydrofolate Reductase and Thymidylate Synthase, which are important for DNA synthesis. Through the disruption of folate metabolism, there is also reduction in the conversion of homocysteine to methionine resulting in decreased protein synthesis and methylation. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that also exists in this pathway and serves as the irreversible, rate limiting step of the conversion of 5,10-methylentetrahydrofolate (5,10-CH_2-THF) to 5-methyltetrahydrofolate (5-CH-THF). Two important single nucleotide polymorphisms (SNPs), C677T and A1298C, are frequently identified in the general population and thought to be clinically significant based upon relative enzymatic function. The C677T SNP is thought to reduce MTHFR activity to 60-70% and 30% in heterozygous and homozygous individuals respectively. This SNP has also been associated with elevated homocysteine, which may lead to a prothrombotic state. The A1298C SNP is more active with 60 and 80% in its homozygous and heterozygous form, respectively. However, for those with one copy of the A1298C SNP, activity is estimated to be slightly decreased at 50-60% when coexisting with a single C677T SNP, otherwise known as compound heterozygosity.

Patients treated with MTX can experience a variety of multisystem toxicities including, but not limited to, myelosuppression, transaminitis, subacute leukoencephalopathy, mucositis, and renal toxicity. The influence of MTHFR SNPs on toxicity has been examined previously and results have remained controversial. It should be noted that most studies to date have either focused on high dose MTX (HDMTX) with typical doses of 5 g/m^2/dose given intravenously, examined ethnically homogenous populations, or have not differentiated between differing modalities of MTX treatment. In a retrospective analysis of CCG-1891 after analyzing for common polymorphic genes involved in drug metabolism, the MTHFR C677T genotype was determined to have no significant effect on cumulative oral MTX received during maintenance, although a 25-30% reduction of cumulative dose was observed across the population. This method of analysis, however, can be misleading as it fails to account for several confounders, including medication holds, average dose received, and compliance.

The aim of our study was to directly examine the influence of MTHFR SNPs on methotrexate tolerance at various phases of treatment, with particular attention given to oral methotrexate which is given during the maintenance phase of treatment, the last and longest phase of treatment on COG-based ALL treatment protocols. Dosing of MTX in maintenance along with oral 6-mercaptopurine (6-MP), is regulated by both degree of myelosuppression (goal absolute neutrophil count 500-1500/ul and Platelet count $>$ 50,000 cell/ul) and to a lesser extent liver toxicity. There is extreme variability between patients likely from a multitude of factors including individual pharmacogenomics. As part of the folate metabolic pathway, MTHFR has the potential to influence MTX efficacy, as well as toxicity, and those with certain MTHFR genotypes may be at increased risk for toxicity.

Our overarching goal was to utilize our local ALL patient population to identify clinically significant genotypes that would identify individuals who need reduced MTX dose adjustment upfront and therefore provide guidance to providers taking care of these patients. Furthermore, we also examined the influence of MTHFR

genotypes on other phases of treatment as well as risk for developing additional toxicities such as subacute leukoencephalopathy and thrombosis.

Methods

Patient Selection and Evaluation of Clinical Data

This study was approved by the Johns Hopkins Institutional Review Board (IRB00282395). Data were extracted from EPIC, the electronic medical record (EHR) used by Johns Hopkins Hospital. We retrospectively reviewed an existing database of pediatric ALL patients treated at Johns Hopkins Hospital from January 2012 to March 2021. From this database, we first searched the EHR to identify individuals who had MTHFR genetic testing sent and further categorized based upon result. Patients were grouped as not tested, wildtype, heterozygous 677CT, homozygous 677TT, compound heterozygous 677CT/1298AC, heterozygous 1298AC, and homozygous 1298CC. We then examined each individual EHR for basic demographic information including age at diagnosis, ALL phenotype, treatment protocol utilized, and outcome (remission, relapsed/refractory disease, death). Patients whose disease relapsed or progressed before receiving standard of care MTX were excluded from further analysis. For each remaining individual, we examined the record to best determine causal reason for sending MTHFR genetic testing which was further generalized to broader categories for comparison. We also identified patients who received Capizzi MTX (C-MTX) and/or HDMTX dosing to record maximum dose achieved and/or clearance time with those treatment regimens respectively. We also examined maximum tolerated dose for oral agent 6-MP and MTX given during maintenance therapy. Here, we defined maximum tolerated dose for oral agents as stable dosing for > 12 weeks after at least 6 months of therapy. In addition to the above, we also collected information regarding the occurrence of thrombosis or MTX leukoencephalopathy anytime during their treatment course.

Statistical Analysis

Patients were grouped based upon MTHFR genotype as stated above. With exception of analysis related to the causal reason for sending MTHFR genetic testing, patients with wildtype MTHFR results were combined with those not tested, presuming a majority have wildtype MTHFR, and served as a control group for analysis. The control group was then compared to each individual MTHFR genotype. When analyzing the causal reason for sending of MTHFR genetic testing, those with wildtype MTHFR were solely utilized as control and compared to each MTHFR genotype. Statistical comparisons were performed using GraphPad Prism Version 9.3.1 (San Diego, CA, USA). For comparison of numerical data between control and each individual MTHFR genotype, Welch's t-test was applied. For comparison of categorical data, we utilized Fisher's exact test. Significance was defined as $p<0.05$.

Results

Patient characteristics and MTHFR genotype status

A total of 242 patients were identified as being treated at our institution on treatment protocols for ALL between January 2012 and March 2021. Table 1 summarizes patient characteristics. We excluded 38 patients with relapsed or refractory disease who did not receive standard upfront therapy. Genetic testing for MTHFR SNPs was performed in 46 patients, with 33 resulting positive for a known variant. Of these, 8 patients were reported to be heterozygous for the C677T SNP (677CT), 9 were homozygous for the C677T SNP (677TT), 9 were heterozygous for both the C677T and A1298C SNPs (also known as compound heterozygous; 677CT/1298AC), 6 were heterozygous for the A1298C SNP only (1298AC), and 1 did not report a specific SNP. Interestingly, no patients homozygous for the A1298C SNP (1298CC) were identified. An additional 158 patients were not evaluated for MTHFR SNPs and in combination with subjects with the wildtype MTHFR genotype were evaluated as a control group for comparison.

MTHFR genotypes and toxicity to C-MTX and HDMTX

During consolidation, MTX is utilized in interim maintenance cycles, where patients receive either escalating intravenous doses (Capizzi-MTX) or high intravenous doses of MTX (HDMTX). Given the potential

intolerance to MTX found with both the homozygous 677TT and compound heterozygous 677CT/1298AC genotypes, we assessed tolerance to IV MTX given through these dosing strategies. Overall, 100 patient records were available to assess maximum C-MTX dosing. Compared to control group, the homozygous 677TT group showed significantly decreased maximum dose of Capizzi with an average maximum Capizzi dose being (174 ± 89 vs. 285 ± 90 mg/m^2, p<0.05) (Fig. 1A). No other group, including the compound heterozygous group met statistical significance. For HDMTX, 99 patients were documented as receiving. Compared to control group, no genotype showed significant increase or decrease in average time to clearance of HDMTX (Fig. 1B)

Tolerance to oral MTX based on MTHFR genotype

During maintenance therapy for ALL, patients continue a regimen of oral chemotherapy, including 6-MP and MTX, which can be frequently adjusted based upon hematologic parameters. Here, we had 190 patients who met criteria for evaluation. The MTX dosing for patients with the homozygous 677TT genotype was significantly decreased when compared to patients with either wildtype or not tested (11.9 ± 9.5 vs. 19.9 ± 7.5 mg/m^2, $p < 0.05$) (Fig. 2A). In addition, patients classified as compound heterozygous 677CT/1298AC, being heterozygous for both SNPs of interest, were also significantly decreased compared to patients identified as wildtype or not tested (11.4 ± 6.1 vs. 19.9 ± 7.5 mg/m^2, $p < 0.01$). In comparison, patients with either heterozygous SNP, although with an overall decreased average MTX dose, did not reach statistical significance (heterozygous 677CT = 14.4 ± 7.8 mg/m^2, $p = 0.11$ and heterozygous 1298AC = 17.5 ± 6.9 mg/m^2, $p = 0.50$). Considering that lower dosing could overall reflect greater patient sensitivity to medication in general, we also examined the effect of specific MTHFR genotypes on 6-MP dosing. When compared to patients with control, no group required significantly lower doses of 6-MP (Fig. 2B). Additionally, we assessed 6-MP metabolites, 6-TG and 6-MMP, while on 100% recommended stating dose for both oral MTX and oral 6-MP for each patient where data was available. Overall, there was no significant correlation between either 6-TG or 6-MMP levels and maximum tolerated oral MTX ($R^2 < 0.1$) or 6-MP ($R^2 < 0.1$) (Fig. 2C and 2D). However, there was an overall negative trend between 6-MP metabolite level and oral 6-MP dosing, which would be expected whereas there is no negative trend seen when correlated with oral MTX. These findings support the notion that patients carrying either the homozygous 677TT genotype or the compound heterozygous 677CT/1298AC genotype have decreased tolerance to oral MTX.

Toxicities associated with MTHFR genotypes

MTX can cause a variety of adverse effects including, but limited to myelosuppression, elevated liver function tests (LFTs), mucositis, renal toxicity, and neurotoxicity. The mechanism by which MTHFR genotypes would influence MTX-induced toxicity is unclear. Therefore, we examined the EHR to best determine the causal reason for sending MTHFR genetic testing for each patient to better elucidate how MTHFR genotypes can influence intolerance to MTX. As detailed in Fig. 3, a significant proportion of patients with both the homozygous 677TT (8/9) and compound heterozygous 677CT/1298AC (6/9) genotypes were found to have myelosuppression as a primary reason for sending. This is in comparison to patients harboring the heterozygous 677CT (3/8), heterozygous 1298AC (3/6), and wildtype (5/13) genotypes which all had a lower frequency of myelosuppression as a cause for sending. Combining those with 677TT and 677CT/1298AC genotypes and comparing to those with wildtype, 677CT, and 1298AC genotypes, myelosuppression was significantly associated with identification of clinically meaning SNP (OR= 5.4, 95% CI 1.3-17.5, p< 0.02). In our data set, there were several causes attributed to the sending of MTHFR genetic testing, but were varied and only attributed to 1-2 patients at most with exception of elevated LFTs in the MTHFR wildtype group which was attributed to 3 patients.

We also examined two potential adverse effects experienced by ALL patients, thrombosis and MTX leukoencephalopathy (Table 2). Thrombosis risk is increased in patients with MTHFR SNPs and generally attributable to high homocysteine levels, however, this may only be modestly elevated for most individuals regardless of MTHFR genotype. In our population, we did not find any significant increase in the occurrence of thrombosis in any SNP group when compared to the control group. MTX leukoencephalopathy is also an important adverse effect commonly attributed to the intrathecal and high dose intravenous administrations

of MTX. Likewise, when compared to the control group, there was no significant increase in the occurrence of MTX leukoencephalopathy.

Discussion

It has long been known that individual treatment responses and tolerance in pediatric ALL are influenced by genetics. Certain SNPs in the TPMT gene were previously identified as a predictor of 6-MP and 6-TG toxicity in ALL treatment and assessment of TPMT genotype is now standard of care in the upfront setting. The role of MTHFR SNPs in ALL pathogenesis and treatment has been extensively studied yet remains controversial. Many studies are small, contain a multitude of patients on different treatment protocols, contain genetically and ethnically homogenous populations, or have been limited by their retrospective nature. Here, our study examines a large number of patients sourced from a genetically heterogenous population treated with similar COG-based protocols and suggests two MTHFR genotypes, homozygous 677TT and compound heterozygous 677CT/1298AC, are associated with increased MTX toxicity and require decreased oral MTX dosing during ALL maintenance therapy. Based upon this data, one could surmise that appropriate dosing of oral MTX for patients with either homozygous 677TT or compound heterozygous 677CT/1298AC would benefit from initial dose reductions as final average dosing was around 50% of the recommended starting dose for both groups. With both homozygous 677TT and compound heterozygous 677CT/1298AC genotypes having known reduced MTHFR enzymatic activity (30% and 50-60% respectively) relative to heterozygous 677CT and heterozygous 1298AC genotypes (60-70% and >80% respectively), our findings correlate well with known enzymatic function. Surprisingly, our results did not completely correlate with toxicity to IV methotrexate formulations, although could be a limitation of our study given the limited sample size of patients receiving either C-MTX or HDMTX. As far as predicting MTHFR genotype, myelosuppression was the only clinical factor significantly associated with detection of a clinically significant variant. No other toxicities demonstrated statistical significance and were not as common. We also assessed the association of specific MTHFR genotypes and other MTX toxicities, namely methotrexate leukoencephalopathy and thrombosis. Here we found that no MTHFR genotype was shown to increase risk of either toxicity. It should be noted that thrombosis risk in relation to MTHFR polymorphisms is attributed to high homocysteine levels and previously literature suggests that these levels may be mildly elevated at most. Transaminitis and mucositis are also major toxicities associated with methotrexate as well as other chemotherapeutic agents received by ALL patients. We found a large proportion of patients regardless of MTHFR status developed mucositis at some point during treatment although were not able to elaborate on the severity or causal relationship with MTX versus other therapies given limitations of information in the EHR.

There is also evidence that MTHFR status can influence outcomes, namely the likelihood of relapse or eventual death from ALL disease. Within our cohort of patients tested for MTHFR SNP, only one had refractory disease (1 patient with heterozygous 677CT SNP) and ultimately achieved remission after CAR T cell therapy and bone marrow transplant. Compared to most recent statistics showing greater 90% survival among ALL patients, this certainly does not demonstrate worse outcome for patients with clinically meaningful MTHFR genotypes, although would not speculate with regards to a clinical benefit given the small sample size, large variation of time since diagnosis and treatment, as well as significant population of patients within cohort that never received oral or IV methotrexate or had MTHFR genetic testing evaluated given early relapsed/refractory disease.

Being retrospective in nature, there are significant limitations to our study, mainly that information extracted is reliant on accurate medical documentation which is sometimes incomplete. We also combined patients whose MTHFR genotyping was not tested with those who tested wildtype, which was an assumption that may not have been true. In addition, it is likely certain biases played a role in the decisions to adjust oral MTX dosing. By knowing MTHFR status, providers may have preferentially decreased oral MTX dosing in response to toxicity whether it was a clinically significant genotype or not. Given the frequency of MTHFR SNPs in the population combined with targeted testing and the fact this was a single institutional cohort, our sample sizes were limited, especially among different genotype groups. It is very likely additional patients with MTHFR variants, both clinically significant and insignificant, exist within the control population and

were never tested. Although this would be expected to further reinforce the significance of our results, it does limit our ability to extrapolate information such as targeted oral MTX dosing and it also may limit some of our other comparisons that did not achieve statistical significance.

Nonetheless, our study was able to demonstrate significantly decreased tolerance to oral MTX among patients with homozygous 677TT and compound heterozygous 677CT/1298AC genotypes as demonstrated by level of myelosuppression. These results do warrant further investigations. Ideally, this would be done through a prospective study. However, a more practical approach could involve a multi-institutional effort where genetic data can be harvested through existing biobanks and MTX dose and toxicity could retrospectively be reviewed for each sample. As was demonstrated with TPMT related to 6-MP toxicity, MTHFR may also influence MTX toxicity and therefore, identification of MTHFR genotypes upfront may lead to reduced ALL treatment complications in the future.

Conflicts of Interest: The authors declare no conflict of interest.

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References

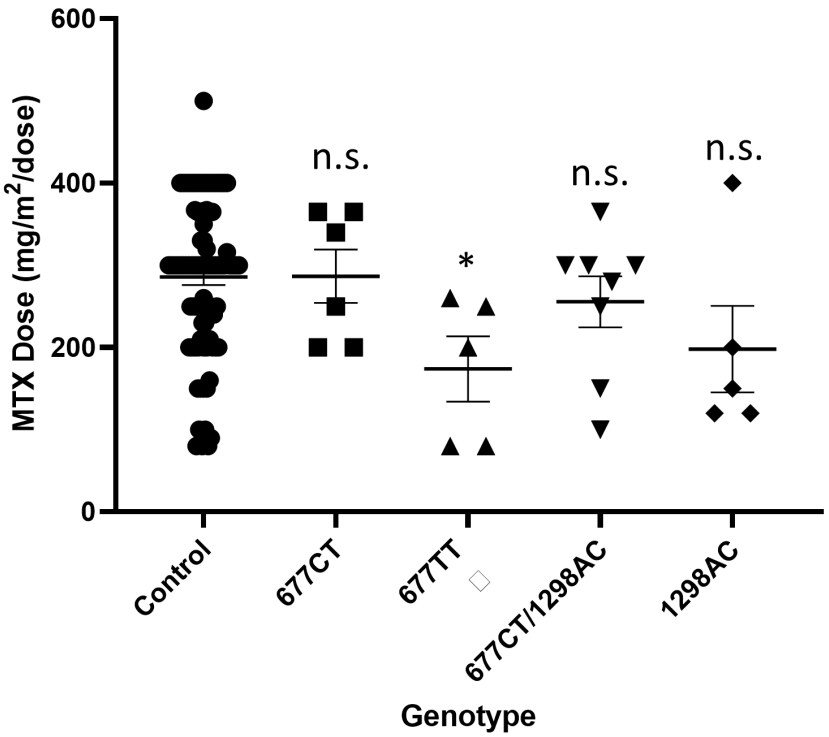
FIGURE LEGENDS

FIGURE 1: MTHFR genotype effect on maximum tolerated C-MTX and HDMTX clearance times. Maximum tolerated C-MTX (A) and HDMTX clearance times for each patient were plotted according to specific MTHFR genotype. *, $p < 0.05$; n.s., not significant. MTX, methotrexate.

FIGURE 2: Oral MTX and 6-MP during maintenance therapy. Maximum tolerated doses of oral MTX (A) and oral 6-MP (B) were obtained for each patient and plotted according to specific MTHFR genotype. (C & D) 6-MP metabolite data for 6-TG and 6-MMP were obtained from patient records while they were on 100% recommended protocol dosing and compare to maximum tolerated oral 6-MP dose (C) or maximum tolerated oral dose (D). *, $p < 0.05$; **, $p < 0.01$; n.s., not significant: MTX, methotrexate; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; 6-MMP, 6-methylmercaptopurine; RBC, red blood cell.

FIGURE 3: Toxicities triggering the assessment of MTHFR genotype status. The causal reason for sending MTHFR genetic testing for each patient was obtained from the medical record and broadly categorized into groups listed in figure. Note: for patients who had no documentation for sending of MTHFR genetic testing, classification group unknown was used.

A



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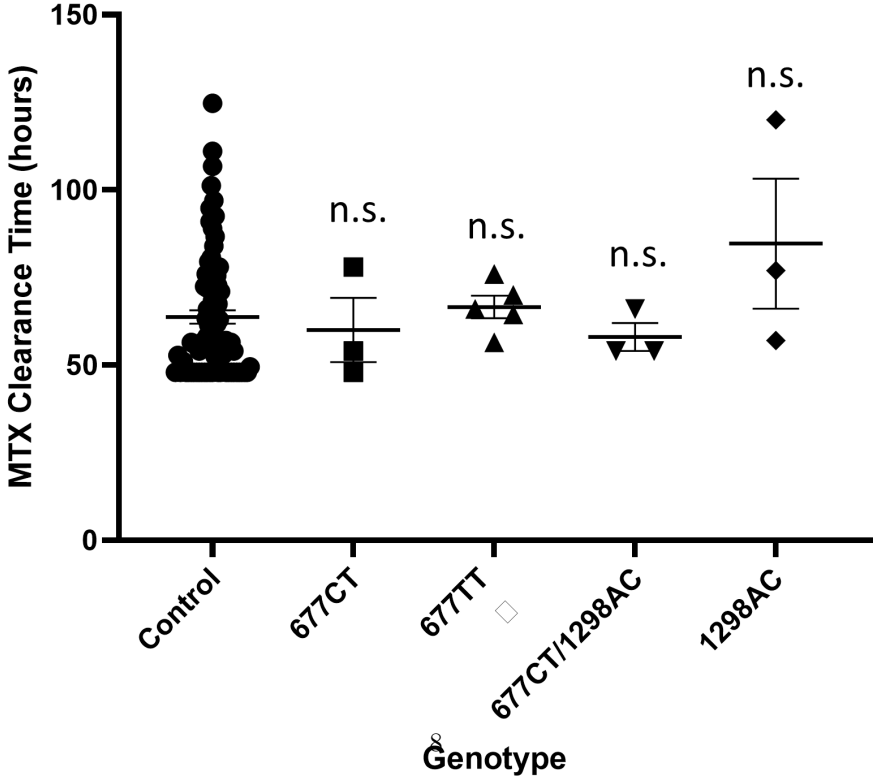

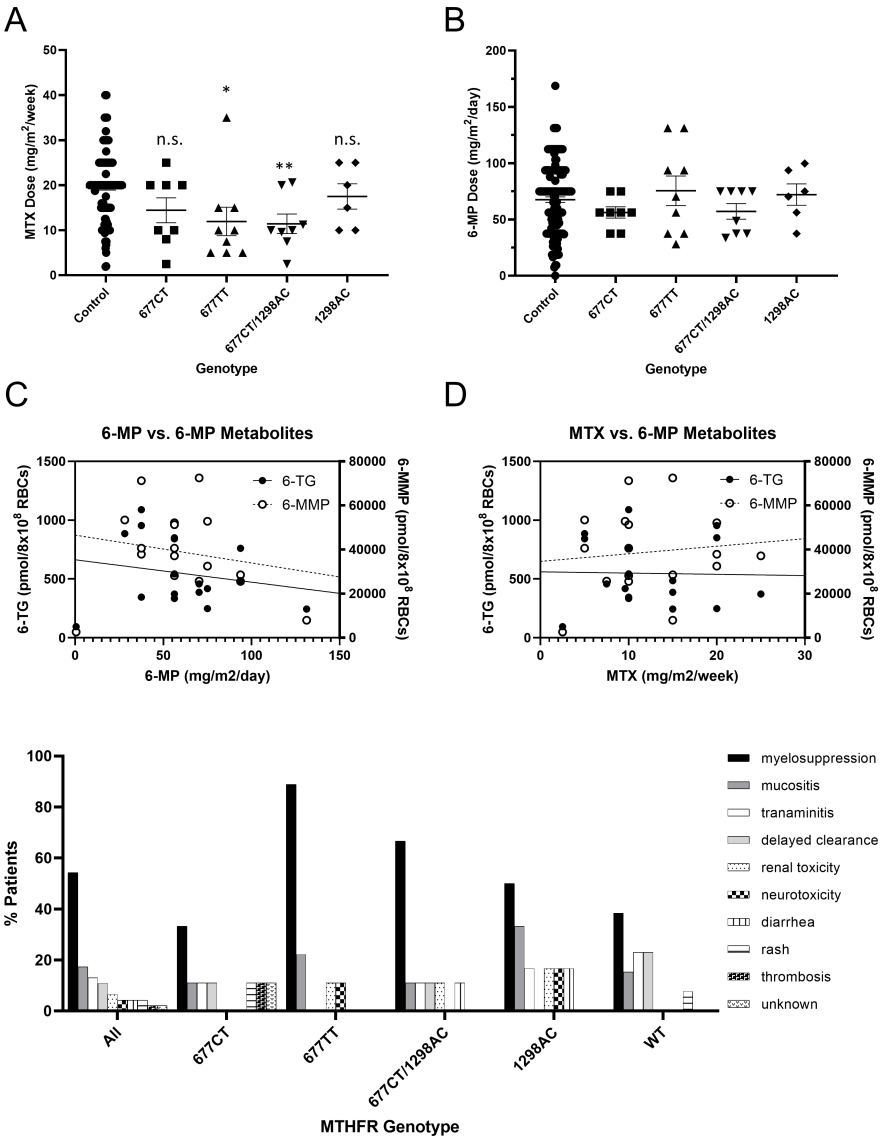

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