Genome-Wide Meta-Analysis Identifies a S1PR1 Genomic Region Associated with Microtubule Targeting Agent-Induced Sensory Peripheral Neuropathy

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Conflicts of interest: The authors declare no conflicts of interest.
Support: Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Numbers R01 CA192156 (D.L.K), U10 CA180821, U10 CA180882, and U24 CA196171 (Alliance for Clinical Trials in Oncology), UG1 CA233320 and UG1 CA233327 (NCTN). Research was also supported by a grant from the Breast Cancer Research Foundation and support from Give Breast Cancer the Boot through the Helen Diller Family Cooperative Cancer Center (D.L.K.). K.C.C. was supported in part by NIH grant T32 GM007175. K.O. was supported in part by NCI grant P01CA142538. Also supported in part by Abraxis, Bristol-Myers, and Celgene for CALGB 40502 (https://acknowledgments.alliancefound.org). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Key words: chemotherapy, sensory peripheral neuropathy, chemotherapy-induced peripheral neuropathy, genome-wide association, pharmacogenetics, S1PR1
Abstract

Microtubule targeting agents (MTAs) are anticancer therapies commonly prescribed for breast cancer and other solid tumors. Sensory peripheral neuropathy (PN) is the major dose-limiting toxicity for MTAs and can limit clinical efficacy. The current pharmacogenomic study aimed to identify genetic variations that explain patient susceptibility and drive mechanisms underlying development of MTA-induced PN. A meta-analysis of genome-wide association studies from two clinical cohorts treated with MTAs (CALGB 40502 and CALGB 40101) was conducted. A Cox regression model with cumulative dose to first instance of grade 2 or higher PN was used in the association analysis of European subjects (n = 469) in CALGB 40502 to study the cause-specific risk of PN; these data were meta-analyzed with additional subjects of European ancestry (n = 855) from CALGB 40101 that previously studied the risk of PN. Novel single nucleotide polymorphisms in a predicted regulatory genomic region downstream of sphingosine-1-phosphate receptor 1 (S1PR1; e.g., rs74497159, $\beta_{\text{CALGB 40101}}$ per allele log hazard ratio (95% CI) = 0.591 (0.254 - 0.928), $\beta_{\text{CALGB 40502}}$ per allele log hazard ratio (95% CI) = 0.693 (0.334 - 1.053); $P_{\text{META}} = 3.62 \times 10^{-7}$) were the most highly ranked associations based on P-values with risk of developing grade 2 and higher PN. In silico functional analysis identified multiple regulatory elements and potential enhancer activity for S1PR1 within this genomic region. Inhibition of S1PR1 function in iPSC-derived human sensory neurons shows partial protection against paclitaxel-induced neurite damage. Further validation may lead to a novel strategy for prevention and/or treatment of MTA-induced neuropathy.
Introduction

Microtubule-stabilizing agents (MTAs) such as taxanes and epothilones, are widely prescribed for treatment of various solid tumors. These mitotic inhibitors disrupt microtubule dynamics via stabilization of microtubules to primarily target and block rapidly dividing cells, and are effective in treating primary and metastatic breast cancer. However, MTA therapy often causes significant dose-limiting toxicities, including sensory peripheral neuropathy. This nerve damage presents as distal axonal degeneration, and clinically manifests as numbness, tingling or painful sensations in a “glove and stocking” distribution. Up to 50% of patients experience some degree of sensory peripheral neuropathy with as many as 30% reaching severe peripheral neuropathy (grade 3 or 4). These symptoms manifest as early as the first dosing cycle, and in severe cases, the symptoms persist for years after the last course of therapy. Reported risk factors for drug-induced neuropathy include prior treatment with neurotoxic agents, frequency of chemotherapy dosing, high cumulative chemotherapy exposure, preexisting neuropathy and age. However, these risk factors do not fully account for the observed incidence of chemotherapy-induced peripheral neuropathy (CIPN). There are currently no neuroprotective strategies that provide adequate clinical efficacy in preventing this toxicity and only duloxetine has been recommended for treatment of existing CIPN.

Human genetic association studies have been used as tools to identify critical genes involved in the pathophysiology of CIPN. In genetic association studies, consideration of the highest-ranking single nucleotide polymorphisms (SNPs) with respect to P-value suggested a potential role for genes related to the regulation of neuron morphogenesis and neurodegeneration (EPHA4/5/6, FGD4, FZD3, ARHGEF10, VAC14) in the pathogenesis of taxane-induced peripheral neuropathy.
neuropathy\textsuperscript{5}. While most of these genetic association findings did not reach genome-wide significance, candidate gene analyses in independent populations support the associations with ephrin receptor genes, \textit{FGD4}, and \textit{ARHGEF10}\textsuperscript{6}. The primary goal in the current study was to conduct a meta-analysis with two cohorts of breast cancer patients (CALGB 40502 and CALGB 40101) to extend these genomic findings and further elucidate this complex phenotype.

Cancer and Leukemia Group B (CALGB) 40502 was a phase III randomized three-arm study comparing nanoparticle albumin-bound (nab) paclitaxel or ixabepilone once per week to weekly paclitaxel as first-line therapy for patients with advanced breast cancer\textsuperscript{7}; bevacizumab was administered in all arms of the study. CALGB 40101 was a phase III randomized trial with 2x2 factorial design to test the noninferiority of single agent paclitaxel with doxorubicin and cyclophosphamide (AC) and the superiority of six cycles of treatment over four cycles\textsuperscript{8}. The most common grade 3 to 4 nonhematological toxicity in both studies was sensory peripheral neuropathy\textsuperscript{7,8}. Findings from the pharmacogenomic association meta-analysis were functionally evaluated \textit{in vitro} to probe the mechanistic basis of chemotherapy-induced sensory neuron damage.

\textbf{Materials and Methods}

\textit{Participants}

All study participants were enrolled in either CALGB 40502 or CALGB 40101. Trial design and eligibility criteria for enrollment for each clinical trial have been previously described\textsuperscript{7,8}. CALGB is now a part of the Alliance for Clinical Trials in Oncology. All subjects provided written informed consent for both the treatment and companion pharmacogenetic protocols that
met state, federal, and institutional guidelines. Only subjects in CALGB 40101 receiving paclitaxel every two weeks were included in pharmacogenetic studies. Pharmacogenetic analysis was approved by Institutional Review Boards at the National Cancer Institute and the University of California San Francisco. Toxicity data was collected by the Alliance Statistics and Data Center, sample genotyping was performed at Riken Center for Genomic Medicine and statistical analysis was done at the University of California San Francisco.

**Genotype Data**

Study characteristics of CALGB 40502 and CALGB 40101 are shown in Table S1. From the 799 patients randomized in CALGB 40502, 633 consented patients with DNA samples were genotyped using the Illumina HumanOmniExpressExome-8 BeadChip, interrogating 964,055 SNPs with coverage of common variants and additional exonic content. Genotyping data were filtered using a standard quality control (QC) pipeline (Figures S1-S3). Detailed sample and variant quality control are described in Supplementary Methods. Samples were filtered for low call rate or low genotyping performance, relatedness, and sex check as a measure for genotyping quality. Using principal components analysis and self-reported ethnicity, a total of 485 subjects with estimated European ancestry were selected for primary analysis to prevent population stratification bias. Genetic imputation was performed with 902,927 autosomal SNPs using the Michigan Imputation Server\(^9\). After post-imputation filtering, a total of 23,210,471 imputed SNPs remained and a total of 469 samples from CALGB 40502 with complete phenotypic data were used in the primary analysis. All samples from CALGB 40101 used in the current meta-analysis were described in a prior GWAS\(^10\) and are briefly described in Supplementary Methods. A total of 14,676,818 post-QC SNPs with 855 samples from CALGB 40101 with complete phenotype information were used for the meta-analysis.
Phenotype Data

Adverse events, including chemotherapy-induced peripheral neuropathy, were graded according to the NCI Common Terminology Criteria for Adverse Events (NCI-CTCAE v3 in CALGB 40502; NCI-CTCAE v2 in CALGB 40101), defining the range of severity of neuropathy cases as grade 0-5. Since the incidence of the toxicity is dependent on cumulative drug exposure, sensory peripheral neuropathy was assessed with a dose-to-event phenotype. An MTA-induced sensory peripheral neuropathy event was defined as the cumulative MTA dose (mg/m²) to first instance of grade 2 or higher sensory peripheral neuropathy. In CALGB 40502, patients were treated until unacceptable toxicity, other complicating disease, alternative therapy, patient withdrawal, treatment completion, progression or death. Patients for whom no neuropathy event was reported were informatively censored at time of occurrence with one of the other competing risks. Patients in CALGB 40101 for whom no neuropathy event was reported were uninformatively censored at completion of treatment (i.e., four or six treatment cycles).

Statistical Analysis for Genome-wide Analysis

Genome-wide association analyses were individually completed for each CALGB cohort. In CALGB 40502, each SNP was tested for an association with cause-specific hazard of neuropathy event within the framework of a Cox model using the Wald statistic, stratifying for treatment arm and adjusting for age. In CALGB 40101, the association between genotypic variation for each SNP and hazard of neuropathy event was tested within the framework of a Cox model using the score statistic, assuming uninformative censoring. In each case, genotypic variation was inferred using imputed allele dosage for untyped SNPs and associations were tested assuming an additive
genotype-phenotype effect. This analysis was conducted under the R statistical environment\textsuperscript{11} 3.3 with the GenABEL\textsuperscript{12}, survival\textsuperscript{13}, and cmprsk\textsuperscript{14} packages. Per SNP summary statistics were further used to conduct an inverse-variance weighted meta-analysis using the METAL software\textsuperscript{15}. The reported P-values and confidence interval estimates have not been adjusted to account for multiple testing or imputation error. This discovery analysis used ranking of unadjusted P-values for prioritization for additional analyses.

\textit{In vitro neurotoxicity studies}

Human induced pluripotent stem cells (iPSCs) were differentiated into mature sensory neurons (day 35+; iPSC-SNs) according to a published protocol\textsuperscript{16}. iPSC-SNs were treated with paclitaxel (Sigma-Aldrich, cat. no. T7402; St. Louis, MO) with or without FTY720 (Cayman Chemical; cat. no. 11975) or W146 (Cayman Chemical, cat. no. 10009109; Ann Arbor, MI), and compared to those treated with 0.2% DMSO (Sigma-Aldrich, cat. no. D2650; St. Louis, MO) as a vehicle control. After 48 hours of drug treatment, sensory neurons were fixed in 4\% paraformaldehyde for 15 min at room temperature. After washing with phosphate buffered saline (PBS), cells were permeabilized with 0.25\% Triton-X (Sigma-Aldrich; Saint Louis, MO) in PBS for 10 min at room temperature. Cells were then blocked with the addition of 10\% goat serum (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA) in 1\% bovine serum albumin and 0.5\% Tween-20 blocking solution for 1 hour. Fixed cells were incubated overnight at 4\(^\circ\)C with anti-TUJ1 antibody (Covance, cat. no. MRB-435P; Princeton, NJ). Goat anti-rabbit secondary antibody (Life Technologies, cat. no. A-11008; Carlsbad, CA) was added in blocking buffer for 1 hour. After PBS washes, 4\',6-diamidino-2-phenylindole (DAPI) (ThermoFisher, cat. no. D1306;
Waltham, MA) was added to stain for nuclei. Imaging was performed at 20X magnification using the IN Cell Analyzer 2000 (GE Healthcare Life Sciences; Pittsburgh, PA).

**Imaging Data Analysis**

Workflow of the imaging analysis is shown in Figure S4. Nine raw images were generated from each well, representing a field-of-view of 15.95 mm$^2$ (2048 x 2048 pixels; 47% of well area). These images were batch processed through an imaging processing software, MIPAR™, with a custom-built algorithm to analyze measurements for chemotherapy-induced neuronal damage. This algorithm generates optimized grayscale images by reducing overall noise and minimizing the amount of non-specific staining to identify and quantify the neurite networks within each field-of-view image. A subsequent segmentation algorithm was performed to estimate nuclei within each field-of-view image. After processing, each image or field-of-view yielded measurements of total neurite area and neuron count. Neurite area was defined by the total area of pixels captured within the identified TUJ1-stained network for each image. Cell counts were designated with DAPI-stained nuclei, rejecting DAPI-stained particles less than 50 pixels to exclude nonspecific DAPI staining. To get a global measurement for each well, total neurite area and total cell count were generated by summing measurements across the nine field-of-view images. Processed images included further in the analysis were required to pass quality control on a per-well basis to assess the quality of the neurons and images (Figure S4). For quality control purposes, all nine field-of-view images were stitched together using an in-house script to generate per-well images, using the Grid/Collection Stitching plugin in Fiji. Wells were only included if neurites covered $\geq 50\%$ of the entire well, no more than 3 field-of-view images (out
of 9) contained out-of-focus images, and a majority of the signal intensities captured were not from artifacts.

**Statistical Analysis for Image Analysis**

For each experiment, all drug treatments were completed with 6-8 replicates on the same plate and raw neurite area measurements and cell counts from imaging data were averaged to obtain a mean total neurite area and cell count per condition. Mean total neurite areas and mean total cell counts were expressed as a ratio of drug-treated to vehicle-treated samples. Differences between relative ratios for the treatment groups were tested for significance by one-way ANOVA test using the function `aov` and subsequent post hoc multiple comparisons using unpaired, two-sided Student’s *t* test with the function `t.test` in *R*\(^1\) version 3.5.3. The effects of S1PR\(_1\) modulators on paclitaxel neurotoxicity were assessed by comparison to paclitaxel-treated cells. Experiments were repeated three times with independent neuron differentiations and the results represent the mean phenotype measurements from each differentiation. The reported P-values and confidence interval estimates have not been adjusted for multiple testing.

**Results**

Of the 799 individuals randomized to the three-arm treatment in CALGB 40502, only 615 subjects were genotyped and had complete phenotype information. Patient characteristics of the CALGB 40502 subjects are listed in Table 1; the distribution of age, race, ethnicity, prior taxane status, and tumor subtype in the pharmacogenetics cohort was similar to the entire clinical trial cohort. Patient characteristics for the CALGB 40101 subjects were previously summarized\(^10\), and differences in sample size, disease stage, drug therapy, and genotyping arrays between the two
cohorts are described in Table S1. The main non-hematological toxicity in CALGB 40502 and CALGB 40101 was sensory peripheral neuropathy with reported event rates of grade 2 or higher of 49% and 24%, respectively (Table S1). Within CALGB 40502, a similar cumulative incidence of peripheral neuropathy was observed regardless of treatment arm (Figure 1; Figure S5), where risk of developing peripheral neuropathy was a function of cumulative chemotherapy dose. The main competing risk for developing peripheral neuropathy in the nab-paclitaxel arm was disease progression or death, while other competing risks, such as other adverse events, other complicating disease, alternative therapy, patient refusal, and treatment completion were more likely to lead to censoring for the peripheral neuropathy phenotype in the paclitaxel and ixabepilone arms.

The meta-analysis of the SNP associations from CALGB 40502 and CALGB 40101 was filtered for SNPs with a minor allele frequency of ≥ 5% in at least one of the two cohorts. None of the resulting SNPs reached genome-wide significance, although 18 linkage disequilibrium-pruned SNPs (r² ≥ 0.7) had P < 10⁻⁵ (Table 2; Figures S6-S7). The 18 SNPs with the lowest P-values were filtered for further support of association from SNPs in high linkage disequilibrium with the identified SNP and expression in human dorsal root ganglion (DRG)¹⁷. SNPs in genomic regions annotated to *C9orf106*, *SLITRK1*, *KLHL1*, *LOC100129716*, and *SEPT5* had limited linkage support from visual inspection of LocusZoom¹⁸ plots and 11 SNPs were annotated to genes that are not detected in human DRG¹⁷ (*ZFPM2* (three independent SNPs), *C9orf106*, *KLHL1*, *SUGCT* (two independent SNPs), *ZBBX*, *LOC100129716*, *ADGRB3* and *CNGB1*). Based on the expression and linkage support filtering, additional analyses were only considered for the genomic regions around five SNPs (rs74497159, rs10771973, rs11076190, rs9623812,
rs2060717; Figure 2, Figures S8-11). Four of the remaining five SNPs were associated with increased risk of MTA-induced peripheral neuropathy, while a single SNP was protective. Cumulative incidence plots for MTA-induced peripheral neuropathy stratified by each of the five SNPs of interest are shown in Figure 3 and Figures S12-S15. Interestingly, the association of rs11076190 with peripheral neuropathy is driven by the paclitaxel-treated patients.

Bioinformatic analysis of the genomic regions surrounding the five SNPs chosen for further analysis was carried out to understand the potential functional effects of genetic variation on gene expression and function; the results from these in silico analyses are summarized in Tables 3 and S2. Examination of ENCODE data tracks (UCSC Genome Browser; https://genome.ucsc.edu) identified histone acetylation and methylation marks, DNase peaks, multiple transcription factor binding sites, and predicted functional activity from genome segmentation algorithms within the genomic regions surrounding rs74497159, rs10771973, rs11076190, rs9623812 and rs2060717 (Figures S16-20). Further evidence that these SNPs are located in transcriptionally active regions includes classification as super-enhancers that interact with multiple genes. In some cases, the predicted enhancer region is expected to directly interact with the annotated gene to control its expression (Table 3). For example, a SNP annotated to S1PR1 (rs74497159) is located downstream of the 3’ end of this gene in a super-enhancer region that interacts with S1PR1. Similarly, the intronic SNP of FGD4 (rs10771973) lies adjacent to the last exon within a predicted transcriptional transition or elongation region and interacts directly with FGD4.
Intronic SNP rs10771973 is significantly associated with splicing quantitative trait loci in tibial nerve tissue ($P = 1.2\times10^{-11}$, GTEx$^{19}$), suggesting that this variant may regulate alternative splicing of pre-mRNA levels and affect the overall $FGD4$ gene expression. rs11076190 is located in a genomic cluster with several chemokines (Figure S9) and is annotated within a FOXA1 transcription factor binding site linked to $CX3CL1$ (Open Regulatory Annotation, ORegAnno, track; Figure S18). SNPs within linkage disequilibrium with rs10771973 ($FGD4$), rs11076190 ($CX3CL1$) and rs2060717 ($CALU$) are each associated with expression quantitative trait loci and/or splicing quantitative trait loci, indicating potential relevance for regulation of gene expression.

While the bioinformatic analysis highlights the potential functional activity for five SNPs of interest, the annotated genes from three of the five SNPs (rs74497159/$SIPR1$, rs10771973/$FGD4$, and rs11076190/$CX3CL1$) are linked to CIPN (Table 3). For functional validation, we focused on the gene annotated to the genomic region with the highest ranking based on P-value for association to MTA-induced peripheral neuropathy from our genome-wide meta-analysis, $SIPR1$. Since rs74497159 is annotated to the $SIPR1$ gene and this SNP and others in linkage disequilibrium are in a super-enhancer region that controls the expression of $SIPR1$, the effect of modulation of $S1PR_1$ functional activity was tested in human iPSC-SNs.

The human iPSC-SNs were generated following a published protocol and yield neurons expressing expected sensory neuron markers$^{16}$. Based on paclitaxel dose-response studies, paclitaxel treatment (1 µM) for 48 hours in the iPSC-SNs had no significant effect on caspase-3/7 activity and decreased cellular ATP levels by <30%, indicating limited cytotoxicity under the
conditions used (data not shown). Drug-induced neurotoxicity is phenotypically characterized by a distinctive loss of neurites and a reduction in neurite network complexity without a decrease in total cell count (Figure 4; Figure S21-S22), which was quantified by total neurite area stained for βIII-tubulin and number of DAPI-stained nuclei. There was no significant effect of any treatment on cell numbers, consistent with limited cytotoxicity from paclitaxel and other chemicals. Treatment with 1 μM paclitaxel alone resulted in more than 50% decrease in neurite staining (49-64% reduction, \( P < 0.01 \); Figure 4) compared to vehicle-treated sensory neurons, demonstrating paclitaxel-induced damage to the overall neurite networks. Treatment with 1 μM S1PR functional antagonist FTY720 (0.3-29% reduction) and 1 μM S1PR1 antagonist W146 (0.6-14% reduction) had little to no effect on neurite area (Figure 4 and Figure S21). Combined treatment of the iPSC-SNs with paclitaxel and FTY720 resulted in partial protection against paclitaxel-induced neuronal damage (33-55% increase in neurite area relative to paclitaxel treatment, \( P < 0.05 \) ) (Figure 4 and Figure S21). The combination of paclitaxel and W146 had minimal effect on the paclitaxel-induced loss of neurite area (4-21% increase in neurite area relative to paclitaxel treatment; Figure 4 and Figure S21).

**Discussion**

We identified multiple SNPs that implicate genes of potential relevance to MTA-induced sensory peripheral neuropathy, even though no SNP associations achieved genome-wide significance. Because the study focuses on sensory neuronal mechanisms involved in MTA-induced PN, five independent SNP associations with linkage disequilibrium support for association and whose nearest gene is expressed in human DRG\(^{17} \) were prioritized for *in silico* functional analysis to determine if the SNP lies in a potential regulatory genomic region. Three of the five SNPs
(rs74497159, rs10771973, rs11076190) had the strongest in silico evidence for predicted functional activity and previous reports linking their annotated genes (S1PR120,21, FGD410,22 and CX3CL123–25) to chemotherapy-induced neurotoxicity. Importantly, S1PR1 function was linked to neurotoxicity of paclitaxel in sensory neurons in vitro.

Among the three genomic regions identified from the primary meta-analysis, the highest-ranking association based on P-values revealed the genomic region in chromosome 1 annotated to S1PR1, a gene that encodes for sphingosine-1-phosphate receptor 1 (S1PR1). S1PR1 is a member of a G-coupled receptor family that is known for its roles in cell proliferation, migration and differentiation26. S1PR1 has been shown to be directly involved in mediating inflammatory responses through activation with its signaling ligand sphingosine-1 phosphate (S1P). S1P signaling has been targeted for autoimmune diseases27 such as multiple sclerosis, psoriasis, and chronic inflammatory neuropathy. Most notably in peripheral neurons, the S1P-to-S1PR1 axis has been associated with increased neuronal excitability28, reduction in neuronal growth through Rho GTPase signaling29, and increased hyperalgesia30 and other pain-like behaviors31,32. The association of S1PR1 G-coupled receptor signaling with Rho GTPase-mediated signaling in peripheral neurons is noteworthy since other genes involved in RhoA signaling have been previously implicated in genome-wide and sequencing studies of MTA-induced PN6,10. In this study, the leading SNP annotated to S1PR1 associated with a higher risk of peripheral neuropathy regardless of which MTA was administered. Additionally, this genomic region is encompassed in a functionally predicted enhancer region that may be acting directly on S1PR1 expression. Alongside these results and recent evidence highlighting S1PR1 as a drug target for prevention of chemotherapy-induced neuropathic pain in vivo20,21, functional studies were
focused on investigating if modulating S1PR\textsubscript{1} function in sensory neurons would protect against MTA-induced damage.

Functional studies were performed using a human iPSC-derived sensory neuron model that displays paclitaxel-induced neurodegeneration. The addition of S1PR\textsubscript{1} functional antagonist fingolimod (FTY720) to paclitaxel in these sensory neurons attenuates paclitaxel-induced neurotoxicity with similar effect sizes as previous functional validation studies in cellular models of chemotherapy-induced neuronal damage\textsuperscript{33,34}. Additionally, functional antagonists of S1PR\textsubscript{1} have consistently alleviated CIPN and other pain-like symptoms \textit{in vitro} and \textit{in vivo}\textsuperscript{20,21,35,36}, and have led to ongoing phase I clinical trials investigating the use of fingolimod to prevent and treat chemotherapy-induced neuropathy (NCT03943498, NCT03941743). While studies have also shown treatment with the S1PR\textsubscript{1} antagonist W146 mitigates paclitaxel-induced neuropathic pain\textsuperscript{20} and S1P-induced hypersensitivity and thermal sensitivity \textit{in vivo}\textsuperscript{20,30}, minimal effect is observed with W146 treatment in the current \textit{in vitro} studies. Since studies have primarily focused on targeting S1PR\textsubscript{1} in the spinal cord and have implicated its role as astrocyte-specific\textsuperscript{20,21}, it is possible that a decrease in S1PR\textsubscript{1} expression from internalization and degradation\textsuperscript{27} is essential to mitigate the effects of paclitaxel in peripheral neurons. This need for degradation may explain why only blocking S1PR\textsubscript{1} function with W146\textsuperscript{37} does not have the same pronounced protective effect. Interestingly, activation of S1PR\textsubscript{3} may also be involved in sensory neurite retraction, nociceptor excitability, and pain-like symptoms\textsuperscript{28,35,38}. FTY720 has also been shown to bind to S1PR\textsubscript{3} and other receptors\textsuperscript{27}, although its functional activity is largely attributed to S1PR\textsubscript{1} binding. While expression of both S1PR\textsubscript{1} and S1PR\textsubscript{3} are known in primary DRG\textsuperscript{39} and iPSC-derived sensory neurons used in these studies (Figure S23), the exact role of these
receptors in sensory peripheral neuropathy is not yet clear. These functional studies are the first step in understanding the role of S1PR1 signaling in peripheral sensory nociceptors under chemotherapy exposure and warrant further investigation to fully elucidate the role of sphingosine signaling in MTA-induced neuropathy.

This genome-wide association study has also highlighted SNPs in a genomic region near CX3CL1 and other chemokine genes. The top SNP (rs11076190) in this region lies just downstream of CX3CL1, encoding for a small chemokine ligand fractalkine (CX3CL1) that exclusively binds to CX3CR1 on lymphocytes. Paclitaxel treatment in preclinical models has been shown to increase levels of monocyte infiltration and inflammatory macrophage activation within peripheral nerves through CX3CL1-CX3CR1 crosstalk, releasing pro-inflammatory cytokines (TNF-α, IL-1β) and initiating peripheral neuropathic pain\(^40\). However, recent work by Yu et al. has demonstrated that nerve injury signals the local expansion of CX3CR1+ macrophages within the DRG itself and these DRG resident macrophages are responsible for neuropathic pain \textit{in vivo}\(^41\). Additionally, previous work suggests that increased recruitment of transcription factors (i.e., NF-κB) to the CX3CL1 promoter region is heightened in \textit{in vivo} models of CIPN\(^24\). Since rs11076190 is in a predicted transcription factor binding site and potential promoter, it may modulate the contribution of CX3CL1 to this toxicity. While little is known about the interaction between CX3CL1 and S1PR1, their robust roles in signaling downstream cytokine release (e.g., TNF-α and IL-1β) that results in peripheral neuropathic pain suggests that regulation of neuroimmune interactions are important to the clinical symptoms in the periphery, and further validation may allow for interesting strategies to monitor CIPN.
The intronic SNP rs10771973 of *FGD4* was in the top SNPs from the meta-analysis and was the only variant identified in the previous GWAS in CALGB 40101 that remained in this meta-analysis\(^\text{10}\). Of note, *FGD4* is a critical gene for peripheral nerve development and a known causal gene of Charcot-Marie-Tooth subtype 4H, which is characterized by distal muscle weakness, severe foot deformities, sensory weakness or loss, and gait instability\(^\text{42}\). With this prior knowledge, it is conceivable that those harboring common genetic variation in *FGD4* may be more susceptible to peripheral neurotoxicity upon drug exposure. Importantly, in another genetic association study in an independent population, rs10771973 was linked with increased risk of paclitaxel dose reductions\(^\text{22}\). Investigation of the effects of paclitaxel on *FGD4* function in peripheral sensory neurons warrants further investigation.

While only three of the five genomic regions associated with MTA-induced peripheral neuropathy have been previously recognized to play a role in CIPN, other genomic regions may also be important for unveiling biological mechanisms underlying this toxicity. rs2060717 lies in a highly predicted promoter site within an intronic region of *CALU*, a gene that encodes for calcium-binding protein calumenin, which localizes to the endoplasmic reticulum with potential roles in early neuronal development\(^\text{43}\). The rs9623812 SNP lies in a potential enhancer site within an intronic region of *SCUBE1*, encoding for a cell surface glycoprotein that is secreted during brain injury\(^\text{44}\). Further studies are needed to understand the roles of these genes in sensory neurons and may lead to novel mechanisms of MTA-induced PN.

While this pharmacogenetic study using human genomic and cellular data has identified potential genes that have a translatable relevance to the CIPN phenotype, there are several limitations.
Although a total of 1,324 samples from the discovery cohorts was used in the meta-analysis, the pharmacogenetic study presented is still insufficiently powered and increasing sample size may provide a more robust analysis. The main limitation is genetic validation of our top-ranking SNPs. None of the top-ranking SNPs \( (P < 10^{-5}) \) from our meta-analysis were replicated in the taxane-treated ECOG-5103 European cohort or in the UK BioBank (data not shown). In the case of ECOG-5103, paclitaxel treatment is different from both CALGB 40502 and CALGB 40101 and this sample was also limited by cohort size. While the UK BioBank is proving to be a useful resource for replication of genetic associations with common phenotypes, the largest number of drug-induced polyneuropathy cases identified was 122 subjects for two ICD9 codes classified as “polyneuropathy due to drugs” and “polyneuropathy due to toxic agents”. The other main limitation is the use of NCI-CTCAE grading for phenotyping peripheral neuropathy events, which has been shown to underestimate the progression of neuropathy symptoms and embody inconsistency in scale interpretation\(^{45}\). It is likely that phenotyping a combination of patient-reported and physician-reported outcomes will yield more comprehensive information. Lastly, our functional studies were limited to studying effects of S1PR\(_1\) in sensory neurons and did not investigate potential cross-talk between neurons and other cell types in the periphery. As CX\(_3\)CL\(_1\) and S1PR\(_1\) both play established roles in peripheral lymphocytes, it is possible that their effects on paclitaxel-induced damage to peripheral nerves are initiated by external cues from other cell types. This phenomenon is true with \( FGD4 \), where the interplay of the frabin (\( FGD4 \))-Cdc42 Rho GTPase axis in Schwann cells causes peripheral nerve demyelination in CMT4H\(^{46}\). Additionally, other genes identified by this genome-wide study (e.g., \( CALU, SCUBE1 \)) not functionally explored also warrant further studies to investigate their roles in peripheral neurons, which may reveal interesting mechanisms underlying MTA-induced neuropathy.
In conclusion, this genome-wide association meta-analysis has identified potential genetic markers of MTA-induced peripheral neuropathy. This pharmacogenetic study highlights the importance of S1PR$_1$ receptor signaling from a genome-wide discovery analysis using clinical samples and functional validation using human iPSC-derived sensory neurons. Of note, S1PR$_1$ signaling functionally intersects between both Rho-GTPase signaling and neuroinflammation, which have been well-documented to play roles in MTA-induced peripheral neuropathy. Further genomic and functional validation of sphingosine-1-phosphate signaling may lead to a novel and exciting strategy for prevention and/or treatment of chemotherapy-induced neuropathy.
Study Highlights

What is the current knowledge on the topic?
Microtubule targeting-agent induced sensory peripheral neuropathy is a dose-limiting toxicity that can impact clinical benefit and significantly hinder quality of life. While some key genes involved in development of MTA-induced PN have been discovered from previous human genetic association studies, there is still limited understanding of the mechanisms underlying this common toxicity.

What question did this study address?
This genome-wide meta-analysis was motivated to extend known genomic findings and discover novel targets that further elucidate the biology involved in MTA-induced PN.

What does this study add to our knowledge?
Our pharmacogenetic approach using genome-wide data from clinical samples and functional studies in human sensory neurons has identified sphingosine-1-phosphate receptor signaling as a potential molecular driver involved in susceptibility of developing MTA-induced PN.

How might this change clinical pharmacology or translational science?
The implication of sphingosine-1-phosphate receptor signaling may lead to more investigations into targeting this pathway for prevention or treatment of chemotherapy-induced peripheral neuropathy.
ClinicalTrials.gov Identifier: NCT00785291 (CALGB 40502) and NCT00041119 (CALGB 40101)

Acknowledgements

The authors would like to thank all the patients and clinicians who took part in the study.

Author Contributions

K.C.C., L.N.S, M.J.R, H.L.M, K.O., and D.L.K wrote the manuscript. K.C.C, K.O., and D.L.K. designed the research. K.C.C., C.X., C.H., T.M., C.J., F.M., P.N.F, H.S.R, L.N.S, and M.K. performed the research. K.C.C., C.H., C.J., K.O., and D.L.K analyzed data.
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**Figure 1.** Cumulative incidence plot of chemotherapy-induced peripheral neuropathy (A: paclitaxel; B: nab-paclitaxel; C: ixabepilone) and informative competing events as a function of cumulative dose (mg/m^2) to event for all subjects in the pharmacogenetics discovery cohort of CALGB 40502. Top left insert displays the entire range of cumulative doses, where the boxed area denotes where ~75% of the data lies (Figure S5) and is the dose range represented in the larger plot.

**Figure 2.** Associations with microtubule targeting agent-induced peripheral neuropathy in the genomic region around rs74497159 located downstream of S1PR1, a gene which encodes for sphingosine-1-phosphate receptor 1. Associations with cumulative dose to first instance of grade 2+ peripheral neuropathy for analyzed SNPs are shown on a –log_{10}(P-value) scale. Dot color indicates the strength of linkage disequilibrium (r^2) between the indicated SNP and each SNP in this genomic region. Plot was produced using LocusZoom (http://locuszoom.sph.umich.edu/).

**Figure 3.** Cumulative incidence plot for chemotherapy-induced peripheral neuropathy stratified by rs74497159 genotype in CALGB 40101 (A, paclitaxel) and CALGB 40502 (B, paclitaxel; C, nab-paclitaxel; D, ixabepilone). Top left insert displays the entire range of cumulative doses, where the boxed area denotes where ~75% of the data lies (Figure S5) and is the dose range represented in the larger plot. The number of individuals with each genotype is noted in parentheses. The allele risk for peripheral neuropathy events without other competing events are shown in the plots for CALGB 40502 (B-D).

**Figure 4.** Inhibition of S1PR signaling attenuates paclitaxel-induced neuronal damage. (A) Representative per-well images of differentiated sensory neurons (D35+) derived from induced
pluripotent stem cells used to investigate S1PR signaling in paclitaxel-induced neuronal damage. Differentiated neurons were treated with 1 μM paclitaxel for 48 hours in the absence and presence of a S1PR1 inhibitor (W146; 1 μM) or a S1PR1 functional antagonist (FTY720; 1 μM). The cells are stained for βIII tubulin and staining was quantified as total neurite area. All images shown are from a single experiment. Scale bar indicates 1 mm. (B) Quantification of mean total neurite area from βIII tubulin staining after drug treatments in three independent differentiations. Each data point represents the mean measurement of 6-8 replicates from a single independent differentiation and expressed relative to vehicle controls. The coefficient of variation in vehicle-treated neurites ranges from 11-24%. Raw values used to calculate the means are shown in Figure S21. Relative mean neurite areas were tested for differences across treatments by one-way ANOVA ($P = 6E-05$) with post-hoc comparisons using unpaired, two-sided Student’s $t$ test (*$P < 0.05$).
|                  | Pharmacogenetic Cohort* | Clinical Trial Cohort |
|------------------|-------------------------|----------------------|
| **Age (yrs)**    |                         |                      |
| 20-49            | 164 (27)                | 218 (27)             |
| 50-69            | 390 (63)                | 500 (63)             |
| 70+              | 61 (10)                 | 81 (10)              |
| **Race**         |                         |                      |
| White            | 501 (81)                | 640 (80)             |
| Black            | 81 (13)                 | 113 (14)             |
| Other            | 23 (4)                  | 32 (4)               |
| Unknown          | 10 (2)                  | 14 (2)               |
| **Ethnicity**    |                         |                      |
| Hispanic or Latino | 31 (5)                | 47 (6)               |
| Non-Hispanic     | 548 (89)                | 712 (89)             |
| Unknown          | 36 (6)                  | 40 (5)               |
| **Taxane as adjuvant therapy** | | |
| Yes              | 270 (44)                | 352 (44)             |
| No               | 345 (56)                | 447 (56)             |
| **Tumor subtype** |                       |                      |
| ER or PgR unknown/missing | 0 (0)                | 16 (2)               |
| ER or PgR positive | 443 (72)              | 573 (72)             |
| ER and PgR negative | 172 (28)            | 210 (26)             |

ER, estrogen receptor; PgR, progesterone receptor
*Genotyped samples with complete phenotype information
**Self-reported race and ethnicity
Table 2. Top ranking SNPs for meta-analysis using cumulative dose to first instance of Grade 2 or higher peripheral neuropathy event.

| SNP*        | Chr | Alleles† | Gene                                      | CALGB 40101 | CALGB 40502 | META |
|-------------|-----|----------|-------------------------------------------|-------------|-------------|------|
| rs74497159  | 1   | T>G      | 168 kb 3' of SPRR1                       | 0.065       | 0.055       | 3.62E-07  ++ |
| rs3110366   | 8   | T>A      | 142 kb 5' of ZFPM2                       | 0.204       | 0.198       | 1.07E-06   -- |
| rs77526807  | 9   | G>T      | 39 kb 3' of C9orf106                     | 0.053       | 0.059       | 1.66E-06   ++ |
| rs17076837  | 13  | C>G      | 382 kb 3' of SLITRK1                     | 0.135       | 0.124       | 1.85E-06   ++ |
| rs61963755  | 13  | T>A      | intronic region of KLHL1                 | 0.050       | 0.055       | 1.88E-06   ++ |
| rs2342780   | 8   | T>A      | 68 kb 5' of ZFPM2                       | 0.069       | 0.064       | 2.06E-06   -- |
| rs10771973  | 12  | G>A      | intronic region of FGD4                  | 0.311       | 0.301       | 2.15E-06   ++ |
| rs2342791   | 8   | T>C      | 43 kb 5' of ZFPM2                       | 0.164       | 0.162       | 2.53E-06   -- |
| rs11076190  | 16  | T>C      | 8 kb 3' of CX3CL1                       | 0.060       | 0.063       | 2.55E-06   ++ |
| rs78777495  | 7   | A>T      | 67 kb 3' of SUGCT                        | 0.107       | 0.097       | 2.99E-06   ++ |
| rs9623812   | 22  | A>T      | intronic region of SCUBE1                | 0.330       | 0.335       | 3.23E-06   -- |
| rs2060717   | 7   | G>A      | intronic region of CALU                 | 0.069       | 0.066       | 3.48E-06   ++ |
| rs6788186   | 3   | T>C      | 696 kb of 3' ZBBX                       | 0.267       | 0.257       | 5.08E-06   ++ |
| rs78017515  | 7   | A>G      | 87 kb 3' of SUGCT                       | 0.069       | 0.059       | 5.72E-06   ++ |
| rs13168251  | 5   | T>G      | 718 kb 3' of LOC100129716               | 0.111       | 0.105       | 6.54E-06   ++ |
| rs777619    | 6   | T>C      | 487 kb 5' of ADGRB3                     | 0.193       | 0.189       | 8.13E-06   ++ |
| rs2188156   | 22  | G>A      | 65 kb 5' of SEPT5                       | 0.051       | 0.052       | 8.23E-06   ++ |
| rs57940640  | 16  | G>A      | intronic region of CNGB1                | 0.064       | 0.068       | 8.74E-06   ++ |

SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency
*SNPs listed are filtered for P < 10^{-5} and LD-pruned (r^2 ≥ 0.7) to the top-ranking SNP within each genomic region.
†Alleles are denoted Major>Minor allele
### Table 3. Summary of *in silico* functional analysis on LD ($r^2 \geq 0.6$) block of rs74497159, rs10771973, rs11076190, rs9623812, and rs2060717.

| SNP          | rs74497159 | rs10771973 | rs11076190 | rs9623812 | rs2060717 |
|--------------|------------|------------|------------|-----------|-----------|
| Gene         | SIPR1      | FGD4       | CX3CL1     | SCUBE1    | CALU      |
| **Presence of regulatory marks** | Yes* | Yes* | Yes* | Yes* | Yes* |
| eQTL         | Not found  | DNM1L, FGD4*, BICD1 | COQ9*, CIAPIN1, DOK4, FAM192A* | Not found | FAM71F1, METTL2B, CCDC136 |
| sQTL         | Not found  | FGD4*, DNM1L | COQ9 | Not found | CCDC136, CALU |
| Super-enhancer regions detected | Yes† | Yes† | Yes | Yes | Yes |
| Previous reports of gene linked to chemotherapy-induced peripheral neuropathy (CIPN) | [22,23] | [10, 24] | [25-27] | Not found | Not found |

*a Gene annotated using RefSeq genes

*b SNPs $r^2$ with $\geq 0.6$

# Presence of promoter and enhancer histone marks, DNAse peaks, and proteins bound are from the Roadmap Epigenomics Consortium 2015 data consolidated and queried from Haploreg v4.1. Further details can be found in Table S2.

†eQTL and sQTL information was queried from GTEx v8.

‡Super-enhancer regions are from genetic and epigenetic annotation information analyzed and queried from SEdb v1.03. Further details can be found in Table S2.

*Includes brain tissue

†Includes tibial nerve

§Super-enhancer regions are predicted to interact with annotated gene

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*Gene annotated using RefSeq genes

*SNPs $r^2$ with $\geq 0.6$

†Presence of promoter and enhancer histone marks, DNAse peaks, and proteins bound are from the Roadmap Epigenomics Consortium 2015 data consolidated and queried from Haploreg v4.1. Further details can be found in Table S2.

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*Includes brain tissue

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§Super-enhancer regions are predicted to interact with annotated gene
