PHARMACOGENETIC PREDICTORS OF TAXANE-INDUCED PERIPHERAL NEUROPATHY

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ABSTRACT

DANIEL HERTZ: Pharmacogenetic Predictors of Taxane-Induced Peripheral Neuropathy
(Under the direction of Dr. Howard L. McLeod)

Peripheral neuropathy is an adverse event of taxane treatment that is related both to the patient's cumulative drug exposure and their inherent sensitivity to neurotoxicity. Discovery and validation of genetic loci that determine neuropathy risk is an important first step towards individualization of taxane treatment with the ultimate goal of maximizing treatment efficacy and minimizing the risk of severe adverse events.

Paclitaxel exposure is regulated by enzymes and transporters that have common variants known to influence protein expression or activity. Paclitaxel is primarily metabolized by the CYP2C8 enzyme, and prior research from our group and others suggests that patients who carry a common low-activity variant, *CYP2C8*3*, may be at increased risk of neuropathy. Using a cohort of paclitaxel-treated breast cancer patients, I was able to confirm the association between *CYP2C8*3* and increased risk of paclitaxel-induced peripheral neuropathy.

I then attempted to use a genotyping platform that interrogates thousands of variants in hundreds of genes relevant to drug metabolism, elimination, and transport to identify polymorphisms that influence risk of neurotoxicity after accounting for the *CYP2C8*3* variant. Surprisingly, I discovered a polymorphism

in a gene not thought to be relevant to paclitaxel pharmacokinetics, *ABCG1*, which was associated with neuropathy risk.

Less is known about the clinical or genetic factors that modulate docetaxel-induced neuropathy risk. I performed genome-wide association in a large cohort of docetaxel-treated patients to discover genetic loci that modulate risk of neuropathy. I discovered several candidates, one of which was an intergenic polymorphism that surpassed genome-wide significance after adjustment for relevant clinical covariates.

I then attempted, unsuccessfully, to replicate these discoveries in independent cohorts of taxane-treated patients. This inability to replicate indicates that either the associations of these variants are limited to the cohort in which they were discovered or that they were merely spurious discoveries. Replication should be attempted in independent patient cohorts that are more similar to those in which these discoveries were made to validate the influence of these variants on neuropathy risk, enabling translation into routine clinical practice.

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Translational research is by its nature a collaborative endeavor and there are a number of other individuals whom I must recognize. I have had the opportunity to work with many collaborators at Lineberger Comprehensive Cancer Center but I must thank Dr. Lisa Carey in particular. Lisa has been an incredibly helpful collaborator, granting me access to her patient database and samples in which the first two studies were carried out. She has also contributed substantially to the conception and execution of these studies and the abstracts and manuscripts that have been and continue to be produced from this work. I am also thankful for the funds she provided from her Breast Cancer Research Foundation grants and for giving me the opportunity to contribute to the process of writing these grant proposals and progress reports. Lisa has been helpful in many other ways including providing letters of recommendation, giving me guidance during my faculty search, and introducing me to her colleagues who are some of the most highly esteemed researchers in breast cancer. Additionally, I would like to thank other members of the cancer center (Shelley Earp, Billy Irvin, Carey Anders) and the LCCC 9830 team (Amy Drobish, Patricia Basta, Jim Bensen, and Chuck Perou) who have contributed to these and other projects.

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PREFACE

All of the work within this dissertation was done in collaboration with other scientists, clinicians, and statistical geneticists. Each chapter represents a separate anticipated publication that is either submitted or in preparation. Prior to writing this thesis Chapter 2 was submitted for publication in Annals of Oncology. Chapters 3 and 4 are both in preparation for submission to scientific journals. The appendix includes manuscripts that were published prior to the writing of this dissertation. All copyrighted material included in this dissertation is used with permission from the relevant copyright holders.

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LIST OF ABBREVIATIONS

95% CI	95% Confidence Interval
AAG	Alpha 1-acid glycoprotein
ABCB1	ATP-binding cassette sub-family B member 1
ABCG1	ATP-binding cassette sub-family G member 1
AC	Doxorubicin (Adriamycin) and cyclophosphamide
ADME	Absorption, distribution, metabolism, and excretion
AF	Allele Frequency
ArPIKfyve	Associated Regulator of PIKfyve
ASW	African Ancestry in Southwest USA
AUC	Area under the curve
BMI	Body mass index
BSA	Body surface area
CALGB	Cancer and Leukemia Group B
cCR	Clinical complete response
cPD	Clinical progressive disease
cPR	Clinical partial response
cRR	Clinical response rate
cSD	Clinical stable disease
CDC42	Cell division control protein 42
CEU	Utah residents with Northern and Western European Ancestry
CHB	Han Chinese in Beijing, China
CIPN	Chemotherapy-induced peripheral neuropathy

CMT	Charcot-Marie-Tooth disease
CTCAE	Common Terminology Criteria for Adverse Events
CYPXYZ	Cytochrome P450 XYZ (3A4, 3A5, 2C8 etc.)
D.f.	Degrees of freedom
DNA	Deoxyribonucleic acid
DOK6	Docking protein 6
EBF3	Early B-cell factor 3
ER	Estrogen Receptor
ERMBT	Erythromycin Breath Test
FANCD2	Fanconi anemia group D2
FDA	Food and Drug Administration
FGD3	FYVE, RhoGEF and PH domain-containing protein 3
FGD4	FYVE, RhoGEF and PH domain-containing protein 4
Foxj1	Forkhead/winged-helix J1
GCRC	General Clinical Research Center
Grade 2+	Grade 2 or higher
Grade 3+	Grade 3 or higher
GWAS	Genome-wide association study
HER2	Human Epidermal Growth Factor Receptor 2
HLA	Human leukocyte antigen
HR	Hazard ratio
HWE	Hardy Weinberg Equilibrium
IRB	Institutional Review Board

JPT	Japanese in Tokyo, Japan
K399R	Lysine-to-Arginine substitution at amino acid 399
LCCC	Lineberger Comprehensive Cancer Center
Ln	Log_e
MAF	Minor Allele Frequency
MTD	Maximum tolerated dose
NAV1	Neuron navigator 1
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
OPCML	Opioid-binding protein/cell adhesion molecule-like
OR	Odds ratio
P-gp	P-glycoprotein
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PGx	Pharmacogenetics or pharmacogenomics
PI(3,5)P2	Phosphatidylinositol 3,5-biphosphate
PI5P	Phospatidylinositol 5-phosphate
PK	Pharmacokinetic(s)
RECIST	Response Evaluation Criteria in Solid Tumors
$Rosi_3$	Rosiglitazone concentration at 3 hours
SD	Standard Deviation
SLCO1B3	Solute carrier organic anion transporter family member 1B3
SNP	Single nucleotide polymorphism

T	Paclitaxel (Taxol)
TIPN	Taxane-induced peripheral neuropathy
TUBB2A	β-Tubulin IIa
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1
UNC	University of North Carolina
VKORC1	Vitamin K epoxide reductase complex subunit 1
YRI	Yoruban in Ibadan, Nigeria

CHAPTER I

INTRODUCTION

TAXANE DISCOVERY AND FORMULATION

The taxanes are a class of chemotherapeutic agents that are highly efficacious for the treatment of a wide array of cancer types. The original taxane, paclitaxel (Taxol), was isolated from the Pacific Yew tree Taxus brevifolia in the early 1970s after a crude extract of the bark demonstrated cytotoxic activity in a screening protocol(1). Due to solubility issues, paclitaxel was developed with a formulation vehicle, Cremophor EL, which enabled intravenous administration(2). Even before approval there were foreseeable issues with Cremophor, leading to investigations into alternative vehicles such as triacetin(3) and a continued effort toward developing alternative paclitaxel formulations such as encapsulation in liposomes(4) or bound to albumin (Abraxane) which has received Food and Drug Administration (FDA) approval(5).

Production of a kilogram of pure paclitaxel required the destruction of thousands of Taxus brevifolia trees, spawning understandable fears about the continued supply of drug(6). This provoked efforts toward culturing bark in the lab, producing synthetic paclitaxel, or producing paclitaxel from similar compounds found in the endlessly renewable needles of the Yew tree(7). These investigations led to the development of the semi-synthetic docetaxel (Taxotere)

which is based off of a starting material isolated from the needles of the Taxus baccata(8) and demonstrated superior *in vitro* cytotoxicity in pre-clinical structure-activity studies of paclitaxel analogs(9, 10). Docetaxel had solubility issues of its own, which were overcome by formulation with polysorbate (Tween) 80(11). Almost a half decade later a fully synthetic pathway for paclitaxel production was discovered(12, 13), assuaging fears of a taxane supply shortage. Taxoids with superior pharmacological or pharmaceutical properties continue to be developed, such as the recently FDA approved cabazitaxel (Jevtana)(14), or the currently in-development tesetaxel (DJ-927)(15). Due to the lack of clinical experience and knowledge of these agents only paclitaxel and docetaxel in their original formulations (herein referred to as taxanes) will be considered for the remainder of this dissertation.

ADME AND PHARMACOKINETICS

Because it was the first in its class, reports of paclitaxel mechanism, pharmacokinetics, and optimal clinical utilization generally outnumber those of docetaxel. The absorption, distribution, metabolism and excretion (ADME) properties of the two taxanes are more similar than different, beginning with the intravenous-only administration of both compounds necessitated by the lack of solubility(2). After introduction into the systemic circulation, in which the taxanes are highly protein bound to both albumin and alpha 1-acid glycoprotein (AAG) (16, 17), their strong hydrophobicity leads to extensive drug diffusion throughout the body(18). Both drugs, in addition to passive diffusion, are substrates for a number of transporters including active uptake by OATP1B3(19, 20) into

hepatocytes. Here, however, is a major difference between the ADME of the taxanes. Docetaxel, like many other drugs, is metabolized mainly by CYP3A4/5(21) while paclitaxel is metabolized primarily by CYP2C8(22) with a minor contribution from CYP3A4(23, 24) at a site distinct from that metabolized on docetaxel(25). Systemic concentrations of the hydroxylated metabolites of both taxanes are negligible as they are eliminated immediately after formation via MRP2 transport into the biliary system(26, 27); and the metabolites are not thought to contribute to either the efficacy or toxicity of the parent compound(28-30). Pre-clinical rat studies revealed that very little paclitaxel is eliminated via the kidneys(31), which is consistent with estimates of <5% renal elimination in early clinical studies of both paclitaxel(32) and docetaxel(33). Another route of taxane elimination is direct P-glycoprotein (P-gp) efflux(34, 35), which is important in a variety of ways. P-gp is expressed in the intestine, and a large portion of taxane elimination occurs through this direct intestinal efflux(36). Perhaps just as important for taxane pharmacology is that P-gp is over-expressed in many tumors and functions as a cancer resistance mechanism by extruding cytotoxic agents from the cell cytoplasm(34, 37).

The similarity in taxane ADME leads to general pharmacokinetic (PK) similarity. Both taxanes follow three-compartment pharmacokinetics, though docetaxel PK is linear(38, 39) while paclitaxel demonstrates saturable kinetic behavior(40) caused by micellular encapsulation in Cremophor EL(41). More recent models that account for Cremophor concentration or measure only free paclitaxel demonstrate linear kinetic behavior of the free parent compound(42).

Population pharmacokinetic models have improved our ability to detect and understand factors that influence pharmacokinetic behavior. For both paclitaxel and docetaxel, patient age, BSA, liver function (bilirubin level), and AAG influence drug exposure but it is unclear whether the influence of other factors such as sex(43) or hormone levels(44) are limited to only one taxane. Regardless, after accounting for these variables 15-25% of inter-individual variability in estimates of free paclitaxel or docetaxel clearance remain unexplained(40, 45).

MECHANISM AND INDICATIONS

The taxanes work by binding to microtubules(46); protein pipes that form the mitotic spindle during chromosomal separation, among other tasks necessary for cellular growth and replication(47, 48). Microtubules, comprised of two subunits (α and β), exist in a state of 'dynamic stability' in which assembly and disassembly remain in balance. The taxanes specifically bind to the same binding site(49) on the β-subunit(50, 51) and interfere with microtubule disassembly(52). Disruption of dynamic stability leads to accumulation of microtubules and inability to form the spindle centromeres(53) which is necessary for mitosis. This causes cells to remain in the G2/M replication phase(54) inducing cellular apoptosis(55).

Both paclitaxel and docetaxel have been tested pre-clinically and in early clinical studies in a variety of tumor types. Despite the broadly positive results of these early clinical studies, Food and Drug Administration (FDA) approval for both paclitaxel (breast, ovarian, non-small cell lung cancer, AIDS-related

Kaposi's Sarcoma)(56) and docetaxel (breast, non-small cell lung, hormone refractory prostate, gastric adenocarcinoma, squamous cell carcinoma of the head and neck)(57) is limited to specific indications in just a few tumor types.

TOXICITY PROFILE AND DOSE-LIMITING TOXICITY

Similar to other chemotherapeutic agents, the taxanes are highly toxic and are administered at the highest dose that is associated with an acceptable risk of severe toxicity (maximum tolerated dose [MTD]). During the original dose-escalation studies, the primary dose limiting toxicities of paclitaxel were infusion-related 'hypersensitivity'(58) and leukopenia(59). In subsequent studies pretreatment with granulocyte colony-stimulating factor (Filgrastim) was used to overcome the hematologic toxicity(60). The infusion reaction, which may not be true hypersensitivity, can also be circumvented through the use of prophylactic antihistamines(61) and successful re-challenge with pretreatment and slower infusion has been reported without incident(62). Prevention of these original dose-limiting toxicities enabled further escalation, to a dose at which peripheral neuropathy is the most common, severe toxicity associated with paclitaxel treatment(32).

Similarly, patients taking docetaxel are pretreated to limit certain adverse events, such as fluid retention(63) and hypersensitivity(64). MTD studies with docetaxel reported concurrent severe granulocytopenia and mucositis(65), and identified neutropenia, a specific myelotoxicity characterized by a decrease in neutrophils, as docetaxel's predominant dose limiting toxicity(33). Severe neutropenia is sometimes associated with a fever of unknown origin, a

dangerous syndrome called febrile neutropenia that often requires rehospitalization and can be fatal(66). Though it's not often treatment limiting, docetaxel is also associated with sensory peripheral neurotoxicity, but for reasons not entirely clear the incidence of severe, grade 3 or higher (grade 3+) peripheral neuropathy tends to be lower with docetaxel (1%-7%) than paclitaxel (2.5%-9%) in head-to-head studies(67-70).

TAXANE-INDUCED PERIPHERAL NEUROPATHY

Mechanism

Microtubules are necessary for formation of peripheral neurons, specifically the development of neuronal axons(71). It is unsurprising, then, that neuropathy is a characteristic toxicity of many microtubule targeting agents, including those outside of the taxane class such as the vinca alakaloids and the epothilones(72, 73). However, despite a vast research effort within *in vitro* and animal systems, it is not clear what the true mechanism of taxane-induced peripheral neuropathy (TIPN) is. Early in pre-clinical development it was discovered that *in vitro* paclitaxel treatment of spinal neurites from dorsal route ganglia led to microtubule accumulation(74) and inhibition of neurite branching and growth(75). There is also evidence from these *in vitro* studies that Schwann cells may be a primary site of action for taxanes(76) leading to decreased myelin production and myelination of neuronal axons(77). These effects on neurons and Schwann cells have been confirmed in studies of paclitaxel treated rats in which both neuronal axons and Schwann cells accumulated microtubules(78). Interestingly, all of these effects resolved over time, normal Schwann cell

function and axonal myelination were restored within 6 months of treatment discontinuation(79, 80). Our knowledge of the neurotoxic effects in humans is very limited but a biopsy of a sural nerve from a paclitaxel treated patient confirmed that axonal demyelination and atrophy occur *in vivo*(81).

Description

TIPN manifests in a “glove-and-stocking” presentation with symptoms beginning symmetrically in the fingers and toes and spreading inward to the hands and feet(73, 82). Most patients originally describe a tingling sensation(83) which will progress to numbness and loss of function of the affected extremities with continued treatment(84). Discontinuation of therapy typically impedes the progression of neuropathy and in most cases the patient’s symptoms will dissipate over time(85, 86), recapitulating the findings from the previously described animal studies. Descriptively the neuropathy seen with paclitaxel and docetaxel are very similar and it is unclear why there is a difference in incidence between the agents. The paclitaxel vehicle Cremophor EL is known to be somewhat neurotoxic(11), however, paclitaxel formulations that do not include Cremophor still induce neuropathy(87), indicating that paclitaxel itself is the principal neurotoxin(88). Perhaps differences in distribution, specifically to the dorsal route ganglia, or affinity for or uptake within neurons or Schwann cells explains the difference between taxanes, though head-to-head comparison in a rat model indicated similar neuropathy incidence and severity between the two taxanes(89) so perhaps the mechanism is specific to humans.

Risk Factors

Risk factors for taxane-induced neuropathy, particularly for paclitaxel, have been reported from analyses in large patient cohorts. The primary risk factor is increased drug exposure, with cumulative dose administered being the strongest predictor for neuropathy development(85, 90-93). Beyond cumulative dose, the exposure to a single dose as measured by either the paclitaxel area under the curve (AUC)(94) or the amount of time paclitaxel concentration remains above a threshold level(95, 96) influence risk of neuropathy, suggesting that cumulative exposure to the free compound is the most important risk factor. Some comparative clinical studies of paclitaxel infusion times and frequencies have suggested differences in neurotoxicity for 24 hour vs. 1 hour infusions and tri-weekly vs. weekly schedules(97, 98). However, the results of these studies are not entirely consistent(99), and are difficult to interpret due to non-uniformity in the cumulative dose received between the comparator arms. Interestingly, in a meta-analysis of the studies comparing the weekly vs. 3-weekly schedule the dose intensity (mg/m^2/week) was a stronger predictor of neuropathy risk than the schedule(100), suggesting that differences in exposure from treatment schedules and/or non-linear pharmacokinetics likely explain the differences in neuropathy seen among the different treatment schedules, duration times, and doses. By extension, the previously discussed factors that influence drug exposure such as age, BSA, drug binding, and sex are likely to influence neuropathy risk. The data on the association with patient age is somewhat inconclusive(101-103); while most of the other factors have not been directly studied.

Aside from differences in drug administration or exposure, some patients are inherently sensitive to taxane-induced neuropathy. One group of patients who are at increased risk are those with prior neuropathy(92, 104) either from previous chemotherapy(105) or secondary to comorbidities such as diabetes or alcohol abuse(106, 107). There is also evidence that the risk of paclitaxel-induced neuropathy is higher in African-Americans than Caucasians(108, 109). Whether this reflects an inherent sensitivity or there are currently unappreciated differences in drug exposure between races is currently unknown.

GERMLINE GENETIC VARIABILITY AND PHARMACOGENETICS (PGX)

Based on the available evidence, neuropathy risk is determined in part by exposure to the offending agent and in part by inherent patient sensitivity. Both of these factors are themselves influenced by a multitude of inputs which form a complex network of processes that ultimately results in the unpredictable and unexplainable variability in the development of taxane-induced peripheral neuropathy.

One factor that may be responsible for some of the observed variability is variation in germline genetics(110). Small changes in the DNA code can lead to dramatic changes in gene transcription(111), post-transcriptional processing(112), or protein activity(113) among other possible influences on biology(114). These seemingly minor changes in DNA sequence can have clinically relevant downstream effects on drug pharmacokinetics or pharmacodynamics(115).

Examples of putative genetic alterations that influence pharmacotherapy outcomes are catalogued and evaluated by various organizations(116-118). Inclusion of this information into the drug package insert by the FDA is viewed as regulatory validation that the genetic marker could be useful in clinical practice(119). A number of pharmacogenetic markers have surpassed this stringent level of validation, including the use of genetic markers in common drug metabolizing enzymes or transporters such as: *CYP2C19*(120), CYP2D6(121), *CYP2C9*(122), *UGT1A1*(123) and *SLCO1B3*(124). Similarly, markers in genes relevant to drug mechanism or etiology of adverse events have also been validated including: *VKORC1*(125), IL28B(126), and the HLA system(127-129).

Pharmacogenetics of Taxane-Induced Peripheral Neuropathy

Discovery and validation of the genetic loci that determine the risk of TIPN are the first steps toward the clinical use of patient genetics in treatment individualization. Early work utilized a candidate-gene approach and focused primarily on single nucleotide polymorphisms (SNPs) involved in taxane metabolism and elimination in small patient cohorts. Because of the lack of rigid statistical methodology, many of the findings of these studies are likely to be false positives(130), necessitating replication in independent cohorts of patients. The first SNP reported to modulate risk of paclitaxel-induced neuropathy is the *CYP2C8*3* (rs10509681 K399R, rs11572080 R139K) variant(131). This finding was replicated once by Leskela et al.(132) and I was able to replicate it in a sub-analysis of a small patient cohort (**Appendix 1**)(133). As with most PGx associations, other studies have not replicated these results(134-137),

necessitating replication in additional cohorts of patients with *a priori* defined analysis plans and rigorous statistical methodology. Leskela et al. also reported that the *CYP2C8* haplotype-C SNP (rs1113129) and *CYP3A5*3C* are protective for risk of paclitaxel-induced neuropathy, however these findings could not be replicated by Gréen et al.(138). Similarly inconsistent findings have been reported for three linked SNPs in the *ABCB1* gene and their influence on paclitaxel(139) and docetaxel(140) induced neuropathy.

More recently groups have reported significant associations with neuropathy for SNPs in genes that are relevant to taxane pharmacodynamics (PD) such as β-Tubulin IIa (*TUBB2A*)(141) and *FANCD2*(142), though attempted replication of these findings has not been reported. Because our understanding of taxane pharmacology and neuropathy etiology is so limited, groups have attempted to use a genome-wide approach to discover SNPs that are associated with paclitaxel-induced neuropathy risk. The first genome-wide association study (GWAS) was published earlier this year by Baldwin et al. Using a large cohort of breast cancer patients they identified a single SNP in the *FGD4* gene that they were able to replicate in two smaller independent patient cohorts(143). Preliminary results of one other paclitaxel-induced neuropathy GWAS have been reported(108), however, attempted replication of these unpublished SNPs was unsuccessful(144).

Validation of these associations would enable translation into clinical practice where they could be used to predict which patients will experience neurotoxicity. These patients could be treated with modified taxane regimens or

alternative therapies to maximize efficacy while minimizing toxicity. The first step in this process, the discovery of these genetic loci, is a formidable task given the millions of known polymorphisms, thousands of relevant genes, and our limited understanding of taxane pharmacokinetics and pharmacology.

PERSPECTIVE

The taxanes are an effective class of chemotherapeutic agents that are associated with development of peripheral neurotoxicity. This adverse event provokes a great deal of patient suffering and often necessitates discontinuation of therapy. Part of the variability in neuropathy risk is explained by differences in drug exposure or clinical factors, both of which are likely to be influenced, at least in part, by variation in germline genetics. The overall hypothesis of this dissertation is that germline variants that modulate a patient's risk of experiencing taxane-induced peripheral neuropathy can be discovered and validated using a variety of pharmacogenetic analysis techniques in independent cohorts of taxane-treated patients. Despite previous attempts to discover and validate genetic loci that modulate risk of taxane-induced neuropathy there are critical gaps in our knowledge in this area. Clinical validation of variants could improve our understanding of the etiology of taxane-induced peripheral neuropathy and more importantly could directly improve patient care by enabling identification of patients at high risk of taxane-induced neuropathy who should be treated with modified taxane doses or non-taxane containing regimens.

SPECIFIC AIMS

I. **Confirm in an independent cohort that patients who carry *CYP2C8*3* are at increased risk of paclitaxel-induced peripheral neuropathy.**

Hypothesis. Patients from the Lineberger Comprehensive Cancer Center (LCCC) 9830 database who carry *CYP2C8*3* are at increased risk of experiencing grade 2+ peripheral neurotoxicity by a cumulative dose of paclitaxel.

II. **Discover and validate variants in genes relevant to drug metabolism, elimination, and transport that increase a patient's risk of experiencing grade 2+ neuropathy during paclitaxel treatment.**

Hypothesis. Interrogation of variants on the Affymetrix DMET™ Plus Chip in Caucasian patients from the LCCC 9830 database will identify variants in genes relevant to paclitaxel pharmacokinetics that modulate a patient's risk of experiencing grade 2+ peripheral neurotoxicity during treatment.

III. **Discover variants anywhere in the genome that are associated with modulated risk of experiencing grade 3+ neuropathy by a cumulative dose of docetaxel through competing-risks analysis and genome-wide association.**

Hypothesis. Competing-risks analysis of all variants on the Illumina 610 Quad Chip in genetically-defined European patients from the Cancer and Leukemia Group B (CALGB) 90401 study will identify variants that are associated with modulated likelihood of experiencing grade 3+ peripheral neurotoxicity during treatment with docetaxel.

CHAPTER II

CONFIRM IN AN INDEPENDENT COHORT THAT PATIENTS WHO CARRY CYP2C8*3 ARE AT INCREASED RISK OF PACLITAXEL-INDUCED PERIPHERAL NEUROPATHY

INTRODUCTION

Paclitaxel is one of the most highly effective therapies in the treatment of breast cancer, improving disease free survival when added sequentially to anthracycline based combination therapy in the adjuvant setting(145, 146). Many patients, however, are unable to tolerate the full course of paclitaxel therapy due to the appearance and progression of sensory neurotoxicity. In large trials, the rate of grade 2 and higher (grade 2+) sensory neuropathy is 15-20%(146-148). Grade 2 neuropathy manifests as a tingling or burning sensation, which can progress to paresthesia that interferes with activities of daily living (grade 3 toxicity)(149). Paclitaxel-induced neuropathy typically resolves over time if treatment is discontinued, but may be irreversible beyond a certain level of severity(85). For this reason paclitaxel therapy is often discontinued once a patient experiences grade 2+ neurotoxicity.

There are known risk factors for development of paclitaxel-induced neuropathy; patients who have prior neuropathy, either from diabetes(150) or neurotoxic chemotherapeutic treatment(106), are at increased risk. There also may be an increased risk for patients who are older(101) or African-

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Hertz DL, Roy S, Motsinger-Reif AA, Drobish A, Clark LS, McLeod HL, Carey LA, Dees EC. *CYP2C8*3* increases risk of neuropathy in breast cancer patients treated with paclitaxel. Annals of Oncology 2013; (In press)

American(108), though these associations may reflect some other causal factor. The progressive nature of paclitaxel-induced neuropathy suggests toxicity development may be attributed to cumulative drug exposure. Indeed, increased cumulative dose(90) and an increase in the time that the drug concentration remains above a threshold for a given dose(95) are both associated with increased neuropathy risk.

Paclitaxel is primarily metabolized by CYP2C8(22), with a contribution from CYP3A4(23), and exposure to paclitaxel in cancer patients is correlated with CYP2C8 activity(151) (**Appendix 2**). Thus, any factor which modulates the activity of CYP2C8 is likely to influence the patient's exposure to paclitaxel. Fortunately, CYP2C8 has few inhibitors and inducers with which paclitaxel would have drug interactions. Despite the relative lack of interactions, there is still appreciable inter-patient variability (19-26%) in clearance of unbound paclitaxel left unexplained after accounting for baseline factors such as body size and bilirubin(45).

Some common single nucleotide polymorphisms (SNPs) in the *CYP2C8* gene yield changes in amino acid sequence. *CYP2C8*3*, the most common variant found in European individuals, refers to two non-synonymous exonic SNPs (rs11572080 Arginine139Lysine (R139K), and rs10509681 Lysine399 Arginine (K399R)) which are very often co-inherited. However, in some individuals only the K399R mutation is found(152). Based on *in vitro* data the K399R amino acid substitution is responsible for the change in enzyme activity; only this variant demonstrates decreased paclitaxel metabolism when each are

tested in isolation(153, 154). It was recently reported that patients carrying the K399R variant exhibit decreased clearance of the free parent compound, and increased overall exposure(155).

Gréen et al. were the first to suggest a potential increase in neuropathy risk for patients who carried the *CYP2C8*3* variant(131). We recently reported results from a small pharmacogenetic study demonstrating that breast cancer patients treated with neoadjuvant paclitaxel who carried the *3 allele were more likely to achieve clinical complete response from paclitaxel treatment (55% versus 23%; OR=3.92, 95% CI: 1.46-10.48, corrected p=0.046), but tended to have higher incidence of grade 3+ neuropathy (22% vs. 8%; OR=3.13, 95% CI: 0.89-11.01, uncorrected p=0.075)(133) **(Appendix 1**). Leskela et al. also detected a significant increase in risk of experiencing neuropathy in patients who were homozygous for the *3 allele(132). Therefore we hypothesized that these findings could be replicated in a larger, independent cohort of European-American breast cancer patients treated with paclitaxel, then replicated again in a cohort of African-American patients.

METHODS

Patients and Treatments

*CYP2C8*3* K399R (referred to as *CYP2C8*3* from now on) was genotyped in a cohort of patients treated between 2005 and 2011 and derived from the University of North Carolina Lineberger Comprehensive Cancer Center (UNC LCCC) Breast Cancer Database, which includes prospective collection of demographic data, including self-reported race, treatment details, and toxicities.

Eligible women received neoadjuvant and/or adjuvant paclitaxel-containing regimens and enrolled in an IRB approved clinical trial that collected genomic DNA from all newly diagnosed patients. In most cases patients received paclitaxel on a familiar neoadjuvant or adjuvant treatment protocol, with a predefined dose, schedule, and duration. Some patients received treatment concurrent with paclitaxel, most commonly with a biological agent for HER2 over-expressing tumors. Toxicities were evaluated during paclitaxel treatment, recorded prospectively, and coded by National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) based on the physician's description(149). Use of supplemental neuropathy prevention (glutamine, vitamin B complex, or vitamin B6) or treatment (gabapentin or amitriptyline) was at the discretion of the treating clinician and prospectively recorded. All patients signed informed consent to participate and agreed to allow DNA to be collected for additional pharmacogenetic studies. The study protocol was approved by the UNC Institutional Review Board.

SNP Genotyping

A 30 mL blood sample was collected from each subject at the time of study enrollment. DNA used for genotyping was extracted by the UNC Biospecimen Processing Facility and plated at 60 ng/uL. Genotyping was carried out blinded to clinical data using the Affymetrix DMET™ Plus Chip (Affymetrix, Inc., Santa Clara, CA, USA) at Gentris Corp. (Gentris Corp. Morrisville, NC) following the manufacturer's protocol with known genomic DNA controls provided by Affymetrix to monitor inter- and intra-assay performance. Any sample or assay

with call rate <98% was excluded from analysis. *CYP2C8*3* K399R (rs10509681) (AM_10125) was the only SNP analyzed for this replication study; all non-*3 loci are assumed to be wild-type (*1) enabling classification of every subject as CYP2C8*3 variant (*3/*3), heterozygote (*1/*3), or wild-type (*1/*1).

Statistical Analysis

The primary analysis was carried out in a cohort of self-reported European-American patients who were not included in the previous analysis(133). African-American patients were analyzed separately in a cross-race replication. These two groups were then combined with patients of other races and previously reported patients to create a large mixed-race cohort. *CYP2C8*3* was assessed for concordance with Hardy-Weinberg Equilibrium (HWE) using Fisher's exact test. The primary toxicity endpoint was the cumulative paclitaxel dose at which grade 2+ neuropathy was first reported and any patient not experiencing grade 2+ toxicity was censored at their cumulative dose received. The primary analysis plan was to use the log-rank test to determine whether there is a difference in risk of grade 2+ neuropathy across European-American patients classified by *CYP2C8*3* K399R genotype. A standard $\alpha=0.05$ was utilized due to the single SNP-phenotype association tested in the primary analysis.

Secondary analyses with grade 2+ or 3+ neurotoxicity incidence, without consideration of dose, were carried out using a Fisher's Exact test across the 3 genotype groups (*1/*1, *1/*3, *3/*3). Following log-rank analysis additional covariates (age [continuous variable], prior diagnosis of diabetes [yes vs. no],

taxane schedule [80-90mg/m^2 weekly vs. 175mg/m^2 every 2 or 3 weeks], use of prophylactic or therapeutic neuropathy treatment [yes vs. no]) were included in a multiple Cox proportional hazards model to adjust for their potential influence on neuropathy risk. Backward selection using Akaike Information Criteria (AIC) as a selection criterion was used to select the final model. AIC balances model goodness of fit and complexity by penalizing the inclusion of extra covariates; it has been shown to be an effective model selection tool(156). Replication of positive findings in the European-American cohort was attempted via log-rank analysis in the self-reported African-American patients. Finally, the entire patient cohort was analyzed in a multiple Cox proportional hazards model which included self-reported race (European vs. Non-European) in addition to the previously described covariates. All statistical analyses were carried out in R Statistical Software, version 2.13.0 (R Development Core Team, Vienna, Austria).

RESULTS

Patient Population

411 paclitaxel treated patients were eligible for analysis and successfully genotyped for *CYP2C8*3* by DMET™ Plus. Demographic data including patient and treatment characteristics for the European-American (N=209), African-American (N=107), and a combined, mixed-race cohort (N=411) can be found in **Table 1**.

CYP2C8*3

The distribution of the *CYP2C8*3* variant conformed to Hardy-Weinberg Equilibrium separately in the European-American (p=0.79) and African-American (p=0.77) cohorts(157). Allele frequency (AF) in the European- (AF=0.14) and African- (AF=0.03) American patients were consistent with that previously reported for each reference population in the International Hapmap Project : CEU (Utah residents with Northern and Western European ancestry) AF=0.14 and ASW (African Ancestry in Southwest USA) AF=0.04, respectively(158, 159). The number of *1/*1, *1/*3, and *3/*3 women in each cohort is displayed in **Table 1** along with the frequencies of grade 2+ (17%-21%) and grade 3+ (8-14%) neuropathy, which were similar to those reported in prior paclitaxel studies(146-148).

Neuropathy by Genotype

Analysis in European-American cohort

In the primary analysis the log-rank test demonstrated a difference in risk of grade 2+ neuropathy across genotype groups, as displayed in the Kaplan-Meier curves in **Figure 1**. As expected, risk of neuropathy was highest in patients who were homozygous for the *3 variant and lowest in patients homozygous for the wild-type allele (log-rank p=0.006). The hazard ratio for the *3 homozygotes vs. other individuals (*3/*3 vs. *1/*3 & *1/*1, recessive genetic model) was statistically significant (Hazard Ratio(HR)=7.16, 95% Confidence Interval (95% CI): 1.70-30.17, p=0.002). Assuming an additive effect for each *3 variant an

individual carries (0, 1, or 2) also gave significant results (HR=1.93, 95% CI: 1.05-3.55, p=0.032) but the hazard ratio for comparing carriers of the *3 variant vs. the wild-type homozygotes (*3/*3 & *1/*3 vs. *1/*1: dominant genetic model) was not statistically significant (HR=1.74, 95% CI: 0.88-3.45, p=0.110). In the secondary analysis, which did not account for cumulative dose, the incidence of grade 2+ neurotoxicity differed across the three genetic groups (Variant: 67%, Heterozygote: 22%, Wild-type: 14%, p=0.042) while the results from the grade 3+ analysis were consistent in trend but did not reach significance (p=0.313, data not shown).

Clinically relevant covariates: age, prior diabetes diagnosis, use of neuropathy prophylaxis or treatment, and paclitaxel schedule, were included in a multiple Cox proportional hazards model. The only covariate that was kept in the backwards selection procedure was diabetes history (**Table 2**). Similar to the unadjusted analysis, in the final model the association between *CYP2C8*3* genotype and risk of grade 2+ neuropathy was significant assuming a recessive (HR = 6.88, 95% CI: 1.62-29.14, p=0.009) or additive (HR=1.95, 95% CI: 1.06-3.58, p=0.031), but not a dominant (HR = 1.77, 95% CI: 0.89-3.52, p = 0.102), genetic effect.

Replication in African-American Cohort

107 self-reported African-American individuals were evaluable in the cross-race replication. As expected the variant allele was substantially less common in this cohort (AF=0.03) and there were no *3 homozygous individuals. Comparing patients carrying one *CYP2C8*3* allele with wild-type homozygous

patients again showed greater risk of grade 2+ peripheral neuropathy in the log-rank analysis (HR=3.30, 95% CI:1.04-10.45, p=0.043) and a trend in the same direction using the Fisher's exact test (50% vs. 20%, p=0.112) (**Figure 2**).

Cox Proportional Hazards Model in Mixed-Race Cohort

411 paclitaxel treated women were available for inclusion in a Cox proportional hazards model that included self-reported race (European vs. non-European). In the final model there was a higher risk of grade 2+ neuropathy in non-European women (HR=1.76, 95%CI: 1.05-2.93, p=0.031) (**Figure 3**) and a non-significant, negligible increase in risk as patient age increases (HR=1.02, 95% CI: 1.00-1.04, p=0.102) (**Table 2**). After adjustment for these covariates the association between increased risk of grade 2+ neuropathy and *CYP2C8*3* was significant (**Figure 4**) in either a dominant (HR = 2.04, 95% CI: 1.21-3.43, p=0.007) or additive genetic model (HR = 1.98, 95% CI: 1.25-3.13, p=0.004) but not in a recessive model (HR = 3.41, 95% CI: 0.82-14.16, p=0.092). In follow-up exploratory model building an interaction between race and diabetes history was discovered, in which a diagnosis of diabetes increased risk of neuropathy in European patients but decreased risk in non-European patients (p=0.029, **Figure 5**).

DISCUSSION

*CYP2C8*3* had been previously suggested as a risk factor for increased neuropathy occurrence in breast cancer patients treated neoadjuvantly with paclitaxel(133) (**Appendix 1**). In that exploratory toxicity analysis within an efficacy study, the data and cohort were not optimized for the neuropathy

endpoint. Only grade 3+ toxicities were considered and the cumulative dose received at the time of toxicity was not accounted for. Also, because there were other variants besides CYP2C8*3 being interrogated, and the cohort was relatively small, the analysis was not stratified by race *a priori*. Despite these limitations a trend was discovered for greater grade 3+ neuropathy incidence in patients carrying the *CYP2C8*3* variant (Odds ratio (OR)=3.13, 95% CI: 0.89-11.01, uncorrected p=0.075). Based on this exploratory finding, and two previously published concurring studies, we attempted to replicate the association in two independent cohorts from the same database, and then combined these replication sets with other available patients to more comprehensively investigate this association.

The current study provides replication of an association between the *CYP2C8*3* K399R variant and increased risk of grade 2+ paclitaxel-induced neuropathy in European-American patients. The association was then replicated separately in the African-American patients. Finally, a model was built that included all patients and relevant clinical covariates, and after adjusting for race, age, and diabetes, the risk of grade 2+ neuropathy was greater in women carrying *CYP2C8*3*.

Leskela et al. previously reported that *CYP2C8*3* increased risk of paclitaxel-induced neuropathy(132). Their study was carried out in a cohort of Spanish paclitaxel-treated cancer patients with dose-to-grade 2+ neuropathy used as the primary endpoint and taxane schedule and patient age adjusted for. Our analysis plan, endpoints, and covariates were similar to theirs and our

results confirm their findings. Although they assumed an additive model, their data suggested that a recessive effect may best characterize the influence of the *3 variant. The results from our European-American cohort also suggested a recessive model but the results in the larger mixed-race cohort are significant with either the dominant or additive model. Indeed, the approximate doubling of risk associated with the addition of each *3 allele, found both by Leskela et al. and us, suggests an additive genetic effect may be most appropriate.

While the association between CYP2C8*3 and paclitaxel-induced neuropathy has now been replicated multiple times, there are previous reports which did not demonstrate this association(134-137). Differences in patient inclusion, study design, and end point likely explain these discrepant findings, similar to that seen with other inconsistently demonstrated pharmacogenetic associations(160). Specifically, one study combined patients on either paclitaxel (24%) or docetaxel (76%) and analyzed these groups together(135). While docetaxel has both structural and mechanistic similarity to paclitaxel, it has a lower incidence of neurotoxicity and is not metabolized by CYP2C8. The other three studies utilized patient cohorts that were treated concomitantly with carboplatin. Carboplatin is less neurotoxic than other platinum compounds but is known to induce sensory neuropathy(161). Neuropathy risk from docetaxel or carboplatin would not be modulated by CYP2C8, thus confounding these previous analyses. In the present study all patients were treated with paclitaxel and only 22% received concurrent treatment, the vast majority of which was non-neuropathic biological treatment (bevacizumab, trastuzumab, lapatinib); only five

patients (1.2%) were concurrently treated with a drug associated with neuropathy (carboplatin). Moreover, patients who received treatment prior to paclitaxel (78%) received almost exclusively doxorubicin/cyclophosphamide (98%), which is not neurotoxic, and only one patient was previously treated with a known neurotoxin (docetaxel) (**Table 1**).

Hapmap reference populations indicate that the *3 variant is not found in patients of African descent (Yoruban in Ibadan, Nigeria [YRI] AF=0.00), however, the allele frequency in our African-American patients (AF=0.03) was very similar to that reported for individuals of African ancestry living in the United States (ASW AF=0.04), corroborating past reports that African-American patients harbor varying amounts of European and African genetic loci(162) (**Figure 6**). Based on our findings, not only is the *3 variant found in individuals from a wide range of self-reported races, the increase in neuropathy risk it confers is consistent across racial groups.

Despite the lower frequency of the high risk variant in non-European individuals, these patients were at an increased risk of neuropathy overall (HR=1.76, 95% CI: 1.05-2.93, p=0.031). This finding confirms a recent publication from Schneider et al.(109) and suggests that while *CYP2C8*3* is one factor that influences a patient's risk of paclitaxel-induced neuropathy, perhaps along with age and diabetes history, there are other currently unappreciated factors at work. An inter-race difference in paclitaxel exposure is possible, but to our knowledge this has never been reported and does not exist with docetaxel(163), which again is not metabolized by CYP2C8. It is noteworthy that

the risk of HIV-associated distal neuropathy(164) and diabetes-related neuropathy(165) are greater in African-Americans than European-Americans; thus there seems to be a general predisposition to neuropathy in African-Americans regardless of etiology. We hypothesize that there are inter-race differences in frequencies of genetic loci responsible for this phenotype that are not currently known. We also identified an unexpected interaction between race and diabetes in which diabetes history increased neuropathy risk in Europeans but decreased risk in non-Europeans, which caused diabetes history to be eliminated from the final Cox model in the mixed race cohort. However, this finding may be merely a statistical artifact secondary to testing many interactions in exploratory model building in a relatively small number of diabetic individuals (n=48, **Figure 5**).

The major limitation of this study is the retrospective use of a clinical registry instead of a prospective clinical study. This manifests in a number of ways, most notably the differences in paclitaxel treatment and schedule, the use of neuropathy prophylaxis or treatment, and the non-uniformity in toxicity collection. We have attempted to adjust for these factors when possible. In meta-analyses comparing the risk of neuropathy for the weekly vs. 3-weekly schedules, the dose intensity was found to be a more important factor than schedule itself(100). The patients in this analysis were treated with one of three standard paclitaxel regimens: 3-hour infusion of 175 mg/m^2 every three weeks (58.3 mg/m^2/wk) or every two weeks (87.5 mg/m^2/wk) or a 1-hour weekly infusion (80-90 mg/m^2/wk). Despite attempts to include this data in Cox models, we could

not detect a significant influence on neuropathy risk for paclitaxel dose, schedule, or infusion time, all of which are highly collinear in this dataset.

Use of supplementary agents to prevent or treat neuropathy was somewhat common in this patient cohort (35%, **Table 1**). The majority of these patients (78%) received the non-FDA approved amino-acid supplement glutamine, which has shown inconsistent efficacy in clinical studies(166, 167). Perhaps due to differences in effectiveness of the various agents administered, or the timing of neuropathy development and supplement use, we did not see an association with the risk of neuropathy.

The neuropathy data was collected and recorded by the treating oncologist or nurse at each treatment visit. This relies on patient reporting, as no validated measure of sensory neuropathy is used consistently in outpatient paclitaxel treatment(168). Though this may limit the accuracy of data collection, relative to a validated test performed at pre-specified times during therapy, the treating clinician's decision to switch drugs, delay treatment, or decrease the dose also relies on patient reports, thus clinically relevant toxicities will be captured by this method. The use of grade 2+ neuropathy, the severity at which alternative treatment strategies are considered, improved our study power by approximately doubling our event rate as compared to using grade 3+ toxicity, and avoided the possible confounding of patients discontinuing treatment when grade 2 neuropathy is encountered. Moreover, because grade 2 neurotoxicity often progresses to grade 3, it is clinically useful to identify patients at risk of

experiencing grade 2+ neuropathy before therapy is initiated, and analyses with this endpoint are free of the confounding present in assessing grade 3+ toxicity.

We have previously reported that patients carrying the *3 variant experience superior clinical response from paclitaxel treatment, consistent with the demonstrated correlation between paclitaxel exposure and treatment efficacy(39, 169). This apparent shift of the therapeutic window suggests that the optimal dose of paclitaxel may need to be stratified in the general population based on *CYP2C8*3* status, similar to a recent Phase I study which identified maximum tolerated irinotecan doses based on the patient's *UGT1A1*28* genotype(123). Further work with population pharmacokinetic models that account for key patient factors in addition to *CYP2C8* genotype(155, 170) may enable appropriate dose selection for a patient, bypassing stratified therapy and realizing truly individualized therapy.

In conclusion, we have replicated a previous finding that *CYP2C8*3* carriers are more likely to experience sensory peripheral neuropathy when treated with paclitaxel in two racially homogenous populations. This association remained significant after adjustment for clinical covariates that are thought to modify risk of neuropathy; age, treatment schedule, and diabetes. In our mixed-race cohort the risk of neuropathy doubled for a *3 homozygote compared to a heterozygote and a heterozygote compared to a wild-type homozygous patient, suggesting an additive or gene-dose effect. Although the *3 variant is less commonly found in non-European individuals, the increased risk of paclitaxel-induced neuropathy is consistent across racial groups. Future work should focus

on translating these findings to optimize paclitaxel dosing so that patients achieve the greatest possible therapeutic benefit with an acceptable risk of severe toxicity.

TABLES

Table 1 Characteristics of LCCC 9830 CYP2C8*3 Patient Cohort

		European-American	African-American	Mixed-Race (n=411)
Age (Years)	Median	51	46	50
	Range	24-84	25-68	22-84
Self-reported Race	European	209	0	287
	African-American	0	107	107
	Other	0	0	17
Neuropathy	Grade 2+	35 (17%)	23 (21%)	76 (18%)
	Grade 3+	16 (8%)	15 (14%)	42 (10%)
CYP2C8*3 (K399R) Genotype^	Wild-type (*1/*1)	155 (74%)	101 (94%)	330 (80%)
	Heterozygous (*1/*3)	51 (24%)	6 (6%)	76 (18%)
	Variant (*3/*3)	3 (1%)	0	5 (1%)
Treatment Prior to Paclitaxel	AC (Doxorubicin/Cyclophos)	154 (74%)	87 (81%)	316 (77%)
	AC + Bevacizumab	2	0	2
	A (Doxorubicin)	2	0	2
	AC + Docetaxel	1	0	1
Treatment Concurrent to Paclitaxel	Trastuzumab	33 (16%)	21 (20%)	71 (17%)
	Bevacizumab	4 (2%)	4 (4%)	9 (2%)
	Carboplatin	1	1	2
	Carboplatin + Bevacizumab	1	2	3
	Trastuzumab + Lapatinib	4	0	4
	Trastuzumab + Cyclophos.	0	0	1
Paclitaxel Schedule & Dose	80-90 mg/m^2 Weekly	64 (31%)	34 (32%)	131 (32%)
	175 mg/m^2 Every 2 weeks	130 (62%)	64 (60%)	235 (57%)
	175 mg/m^2 Every 3 weeks	15 (7%)	9 (8%)	45 (11%)
Total Paclitaxel Received	Median (mg/m^2)	700	700	700
	Range (mg/m^2)	80-1280	160-1280	80-1280
Diabetes	Prior diagnosis	16 (8%)	24 (22%)	48 (12%)
	No prior diagnosis	193 (92%)	83 (78%)	363 (88%)
Neuropathy Prophylaxis or Treatment	Gabapentin	6 (3%)	7 (7%)	15 (4%)
	Amitriptyline	5 (2%)	1	9 (2%)
	Glutamine	62 (30%)	22 (21%)	112 (27%)
	Vitamin B Complex	1	0	1
	Vitamin B6	4 (2%)	2	7 (2%)
	Total	78 (37%)	32 (30%)	144 (35%)
Treatment Modality^	Neoadjuvant	58 (28%)	47 (44%)	188 (46%)
	Adjuvant	153 (73%)**	61 (57%)**	226 (55%)**

^: Differences due to known allele frequency differences between races.
**: Three patients were treated with paclitaxel neoadjuvantly and adjuvantly

Table 2 Cox Proportional Hazards Model for Grade 2+ Neuropathy

	European Cohort (n=209)			Mixed-race Cohort (n=411)		
	Hazard Ratio	95% Confidence Interval	P-value	Hazard Ratio	95% Confidence Interval	P-value
CYP2C8^ *1/*3 vs. *1/*1	1.55	0.75-3.21	0.234	1.95	1.14-3.32	0.015*
CYP2C8^ *3/*3 vs. *1/*3	5.01	1.09-23.02	0.038*	2.14	0.50-9.27	0.307
CYP2C8^ *3/*3 vs. *1/*1	7.78	1.80-33.62	0.004*	4.17	0.99-17.60	0.052
Diabetes Diagnosis	2.18	0.84-5.63	0.110	Not included in final model		
Age	Not included in final model			1.02	1.00-1.04	0.102
Self-Reported Race**	No racial heterogeneity			1.76	1.05-2.93	0.031*

^CYP2C8 comparisons are made between individual genotype groups, not based on assumptions of genetic effect (recessive, additive, or dominant).
*Statistically Significant Difference
**Non-European-American (African-American + Other) vs. European-American

FIGURE LEGENDS

Figure 1 Incidence curve for grade 2+ neuropathy across genotype groups in the European cohort (n=209). The highest risk was seen in the variant homozygotes and the lowest risk in the wild-type homozygotes.

Figure 2 Incidence curve for grade 2+ neuropathy across genotype groups in the African-American replication cohort (n=107). Higher risk was seen in the carriers of the CYP2C8*3 variant homozygotes as compared to the wild-type homozygotes (p=0.043). No homozygous variant individuals were found in this cohort.

Figure 3 Incidence curve for grade 2+ neuropathy across racial groups in the entire mixed-race cohort (n=411). Higher risk was seen in the non-European-American (n=124) as compared to the European-American (n=287) women after adjusting for CYP2C8 and age (p=0.031).

Figure 4 Incidence curve for grade 2+ neuropathy across genotype groups in the mixed-race cohort (n=411). This data indicates that each *3 variant approximately doubles a patient's risk of grade 2+ neuropathy, supporting an additive genetic effect.

Figure 5 Incidence curve for grade 2+ neuropathy for the entire patient cohort (n=411) stratified by race and diagnosis of diabetes. Risk of neuropathy

increased in European patients with diabetes but decreased for non-European patients.

Figure 6 Principal components analysis of DMET™ genotype data for entire cohort (N=411, circles) and reference Hapmap populations (triangles): CEU (Utah residents with Northern and Western European ancestry), CHB (Han Chinese in Beijing, China), JPT (Japanese in Tokyo, Japan), and YRI (Yoruban in Ibadan, Nigeria). The self-reported white patients (orange circles) predominantly cluster with the European Hapmap samples (orange triangles). The self-reported black patients (red circle) cluster near the Hapmap African samples (red triangles) however; they are shifted toward the Europeans demonstrating that genetically they represent a continuum between the two groups. Some self-reported white individuals don't cluster with the European samples, but instead cluster with the black patients or fall between the European and Asian samples while some self-reported black patients cluster with the European samples. 9830 Patients who reported their ethnicity as 'other' (blue circles) are a mixture of Asians, who cluster with the Asian Hapmap samples (green and purple triangles), Hispanics, American Indians, and other ethnicities.

FIGURES

Figure 1 Neuropathy by CYP2C8 Genotype in LCCC 9830 Europeans

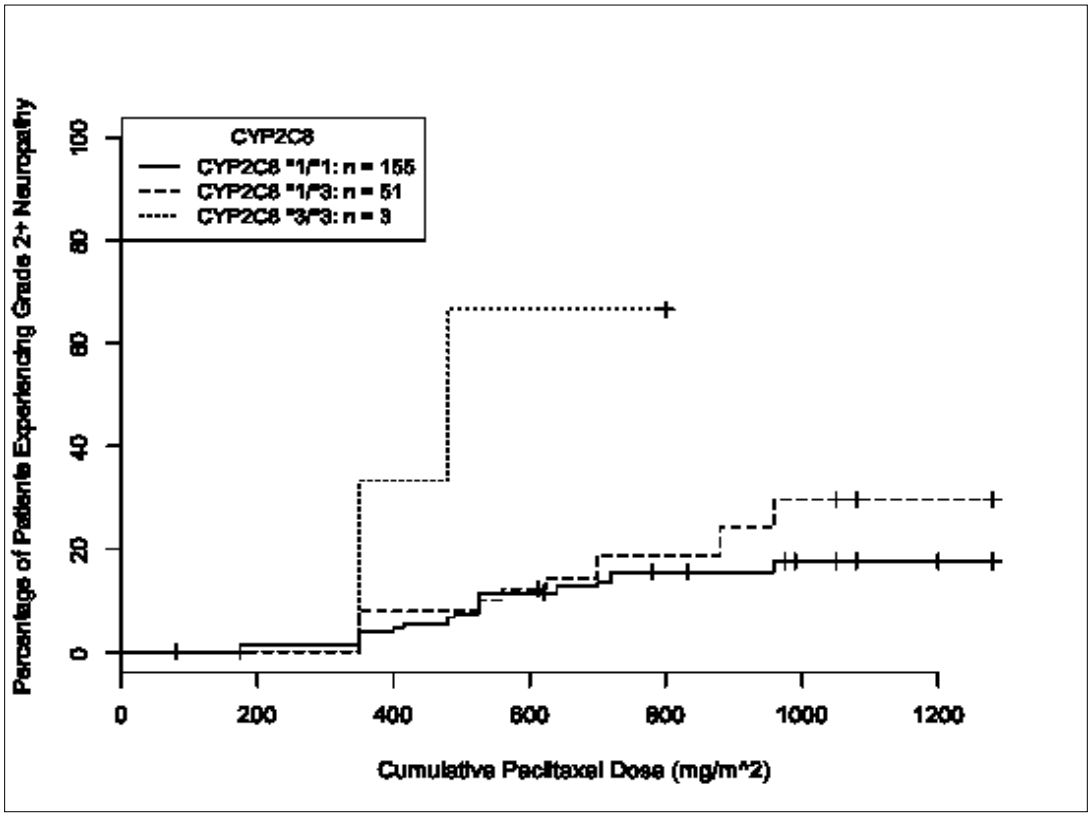


Figure 2 Neuropathy by CYP2C8 Genotype in LCCC 9830 African-Americans

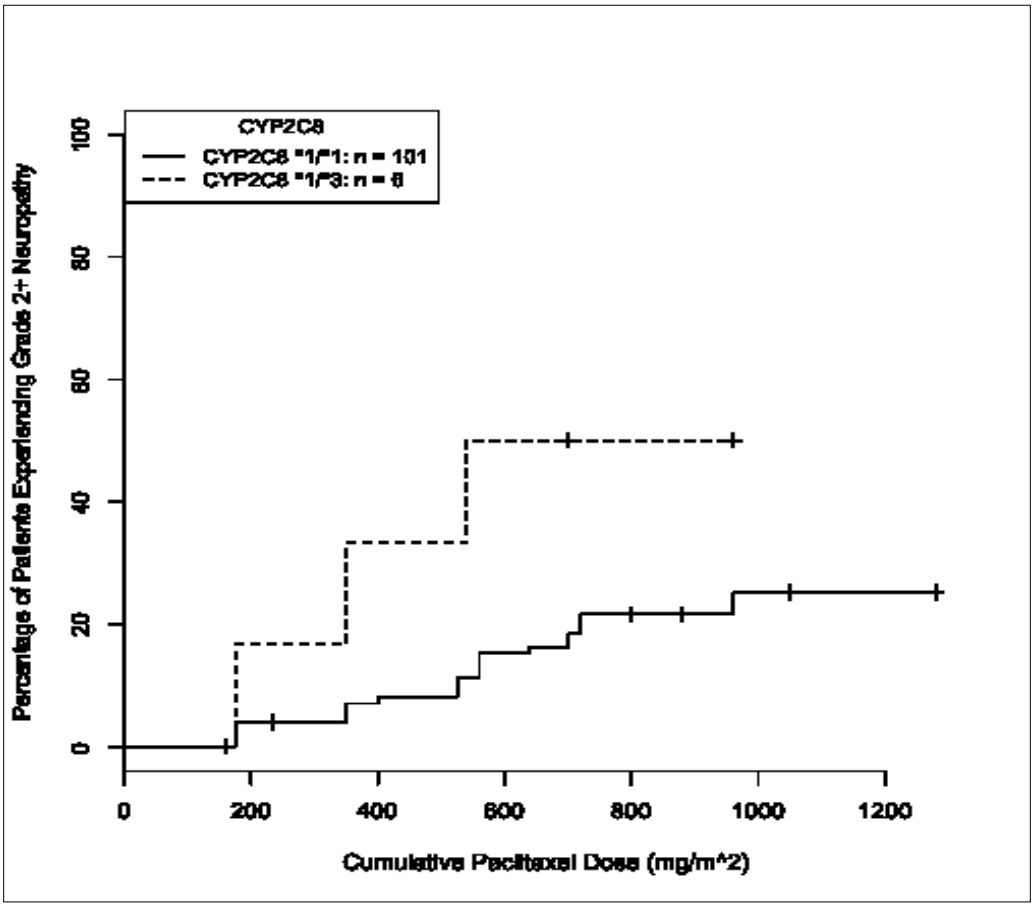


Figure 3 Grade 2+ Neuropathy Incidence by Race in LCCC 9830

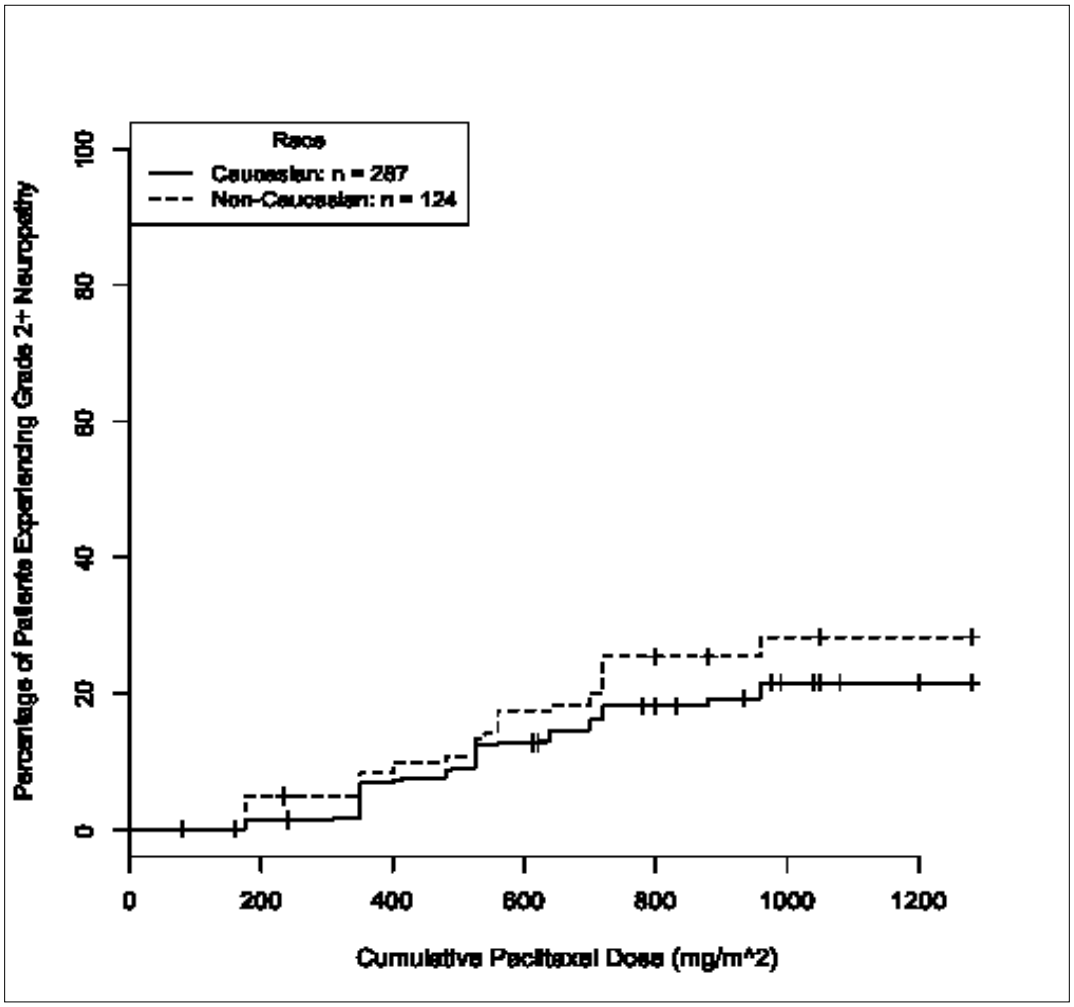


Figure 4 Neuropathy by CYP2C8 Genotype in LCCC 9830 Cohort

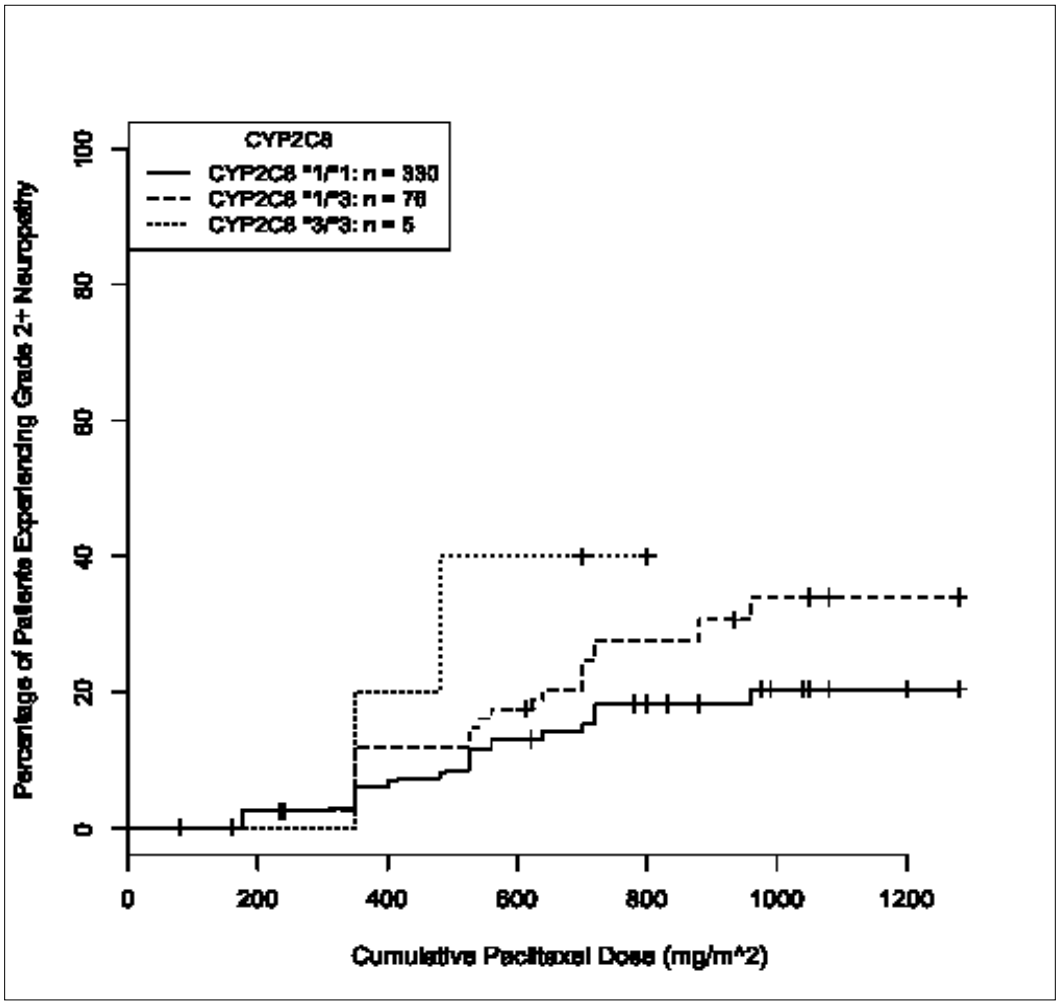


Figure 5 Neuropathy Incidence Stratified by Race and Diabetes

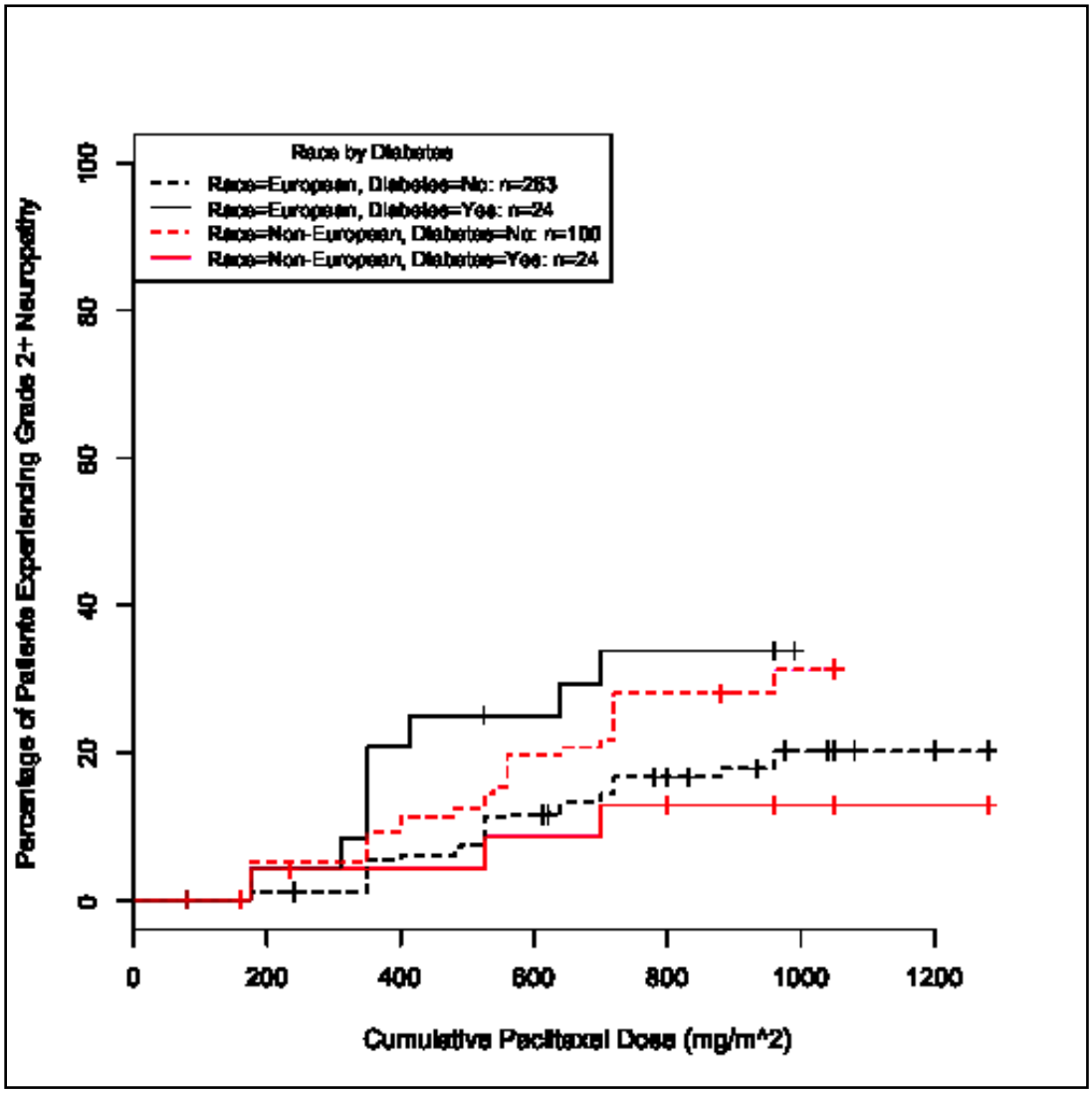
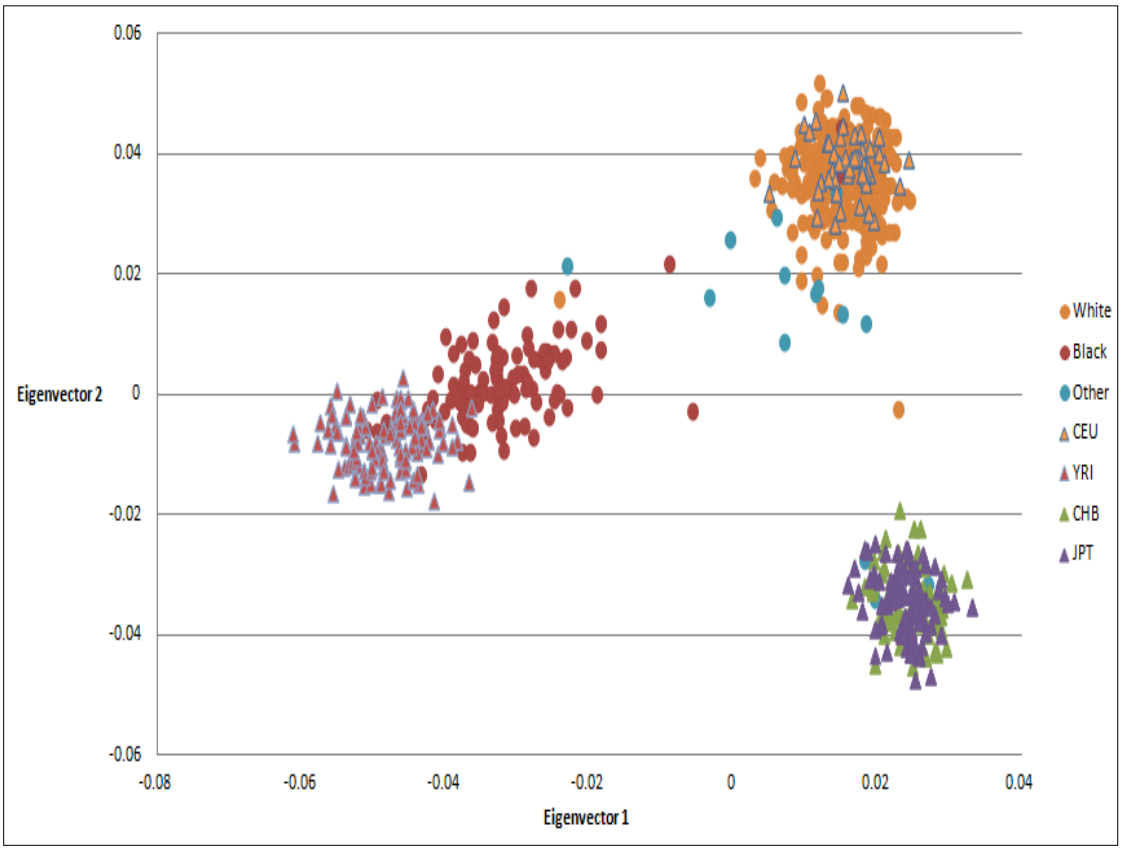


Figure 6 Principal Components Analysis of LCCC 9830 Patients with Hapmap Controls



CHAPTER III

DISCOVER AND VALIDATE VARIANTS IN GENES RELEVANT TO DRUG METABOLISM, ELIMINATION, AND TRANSPORT THAT INCREASE A PATIENT'S RISK OF EXPERIENCING GRADE 2+ NEUROPATHY DURING PACLITAXEL TREATMENT

INTRODUCTION

In breast cancer treatment, the sequential addition of paclitaxel to standard anthracycline therapy has improved rates of pathological complete response in neoadjuvant treatment(171) and overall survival in the adjuvant setting(145, 146). Along with its impressive efficacy, paclitaxel treatment is associated with a variety of severe adverse events, including the development of peripheral sensory neuropathy which typically presents as tingling in the fingers and toes that will often resolve if treatment is discontinued(85). However, mild neuropathy will progress with continued treatment to potentially irreversible loss of tactile function and balance(84).

Neuropathy development is partly determined by the cumulative paclitaxel dose administered over the course of therapy(85, 90-93), and may also depend in part on the exposure to a given dose(95). Patients who carry the *CYP2C8*3* variant experience greater exposure to paclitaxel than wild-type homozygous individuals(155) and are at increased risk of paclitaxel-induced neuropathy(132, 133)(Chapter 2). In addition to *CYP2C8*, there are a number of enzymes(23) and

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Daniel L. Hertz, Siddharth Roy, Alison A. Motsinger-Reif, Amy Drobish, L. Scott Clark, Howard L. McLeod, Lisa A. Carey, E. Claire Dees. Affymetrix DMET™ Plus Chip Identifies SNP in ABCG1 that Modulates Risk of Paclitaxel-Induced Neuropathy in Caucasians

transporters(20, 26, 36) known to be involved in paclitaxel pharmacokinetics. Variability in the activity of these enzymes and transporters may also contribute to variability in paclitaxel exposure and consequently modify the risk of paclitaxel-induced neuropathy.

Many prior studies have investigated whether there is an association between variants in these candidate genes and risk of neuropathy(132, 134, 137, 139, 141, 142, 172-175), however, none have accounted for the underlying influence of *CYP2C8*3* or used an approach that can comprehensively and directly interrogate thousands of variants that are most likely to influence paclitaxel exposure. The Affymetrix (Santa Clara, CA) DMET™ Plus Chip is a commercially available genotyping panel that interrogates nearly 2,000 genetic variants within genes that are responsible for Drug Metabolism, Elimination, and Transport (DMET™)(176). We hypothesized that using the DMET™ Plus Chip to further genotype a cohort of patients who have previously been analyzed specifically for the *CYP2C8*3* variant, we could identify additional genetic loci that influence a patient's risk of paclitaxel-induced neuropathy. By conditioning our analysis on *CYP2C8*3,* we can eliminate the known effect of this variant and more directly investigate the genetic sources of the remaining variability, potentially validating prior results from candidate gene studies or discovering genes not previously recognized to influence the risk of paclitaxel-induced neuropathy.

METHODS

Patients and Treatments

The subjects, toxicity, and covariate data for this analysis were the same as those used in Chapter 2. The primary analysis for this aim was restricted to the self-reported Caucasian patients; all non-Caucasian patients were included in a secondary cohort for potential replication. The study protocol was approved by the UNC Institutional Review Board.

Genotyping

A 30 mL blood sample was collected from each subject at the time of study enrollment. DNA used for genotyping was extracted by the UNC Biospecimen Processing Facility and plated at 60 ng/uL. Genotyping was carried out blinded to clinical data using the Affymetrix DMET™ Plus Chip (Affymetrix Inc., Santa Clara, CA, USA) at Gentris Corp. (Gentris Corp. Morrisville, NC) following the manufacturer's protocol with known genomic DNA controls provided by Affymetrix Inc. to monitor inter- and intra-assay performance. Any patient sample or assay with successful call rate <95% or <90%, respectively, was excluded from analysis. Variants were also excluded if the minor allele frequency was <5% in the entire population or if the p-value for the Fisher's Exact estimate of Hardy-Weinberg proportions was <0.05 in either the Caucasian or non-Caucasian cohort.

Statistical Analysis

The primary toxicity endpoint was the incidence of grade 2+ neuropathy during paclitaxel treatment (Yes vs. No). Exact testing, conditioned on previously

analyzed results for the *CYP2C8*3* (K399R, rs10509681) variant, was used to compare the risk of neuropathy incidence across genotype groups for each marker that passed quality control. Due to the exploratory nature of this study, uncorrected p-values were compared with a significance threshold $\alpha=0.001$ which was selected for consistency with prior pharmacogenetic discovery studies utilizing the DMET™ Plus Chip(177).

Variants that surpassed the exploratory significance threshold were tested in a log-rank analysis utilizing the dose-at-onset of grade 2+ neuropathy in order to account for the known effect of cumulative paclitaxel treatment. Any patient not experiencing grade 2+ neuropathy was censored at the cumulative dose they were administered over the course of therapy. Variants with significant findings in the dose-to-event analysis were then tested in a cross-race replication in the non-Caucasian subjects (n=124). Finally, covariates that are thought to be relevant to neuropathy risk including: age (continuous), race (Caucasian vs. non-Caucasian), paclitaxel dose and schedule (80-90mg/m^2 weekly vs. 175mg/m^2 every 2-3 weeks), diabetes (yes vs. no), and supplemental neuropathy treatment (as defined in the methods above, yes vs. no) were tested in a multiple Cox proportional hazards model with backward elimination via AIC to identify only those covariates that significantly contributed to model performance. All statistical analyses were carried out in R Statistical Software, version 2.13.0 (R Development Core Team, Vienna, Austria).

RESULTS

Patient Population

After exclusion of patients whose samples failed genotyping, 288 Caucasian paclitaxel-treated patients were included in the primary analysis and 124 non-Caucasians were evaluable in the replication cohort. Demographic data including patient and treatment characteristics for the primary and replication cohorts can be found in **Table 3**. Overall 71 patients experienced grade 2+ neuropathy during paclitaxel treatment (71/412=18%) which is consistent with other studies of paclitaxel treatment in breast cancer(146).

DMET™ Markers

Of the 1,936 genetic markers on the DMET™ Plus chip, a total of 1,372 were excluded from analysis. 1,275 markers were excluded for minor allele frequency <0.05, which is consistent with previously reported DMET™ marker allele frequencies in primarily Caucasian cohorts(177). 30 markers were excluded from the analysis for call rate <90% and 67 were eliminated for significant deviation from Hardy-Weinberg proportions. Thus after appropriate quality control 564 markers (29.1%) were included in the analysis.

Neuropathy by Genotype

Results of the exact tests conditioned on *CYP2C8*3* for the 10 markers with the strongest association with neuropathy incidence are displayed in **Table 4**, including one marker that surpassed the exploratory significance threshold (α=0.001). This was an intronic SNP in *ABCG1* (rs492338, uncorrected p=0.0008). A contingency table of neuropathy by genotype for the 285 Caucasian

patients with genotype calls at this locus is presented in **Table 3**, exhibiting increased neuropathy risk for the minor (T) allele.

The results of the secondary analysis using the cumulative dose-at-onset of neuropathy were not meaningfully different from the primary findings (HR(per allele)=2.11, 95% CI: 1.36-3.29, p=0.0008, **Figure 7**). In the cross-race replication in non-Caucasian patients, rs492338 was not significantly associated with grade 2+ neuropathy in either the Fisher's exact (p=0.60, data not shown) or log-rank analysis (p=0.54, **Figure 8**). We attempted to adjust for covariates of interest: age, race, diabetes, taxane schedule, and supplemental neuropathy therapy in the entire patient cohort, however, none of the clinical covariates survived backward elimination when included with rs492338 in a multiple Cox proportional hazards model (data not shown). Finally, the results of this analysis were not meaningfully influenced by the conditioning for CYP2C8*3; re-running the primary analysis in the Caucasian cohort unconditioned had a negligible impact on the Fisher's Exact p-values of the top 10 hits (**Table 4**).

DISCUSSION

Paclitaxel-induced peripheral neuropathy is known to be dependent on drug exposure. Within this patient population we previously demonstrated that patients' who carry the low-activity *CYP2C8*3* variant are at increased risk of neurotoxicity. We have attempted in the present study to identify germline variants that influence risk of neuropathy through a direct effect on drug PK, beyond that of *CYP2C8*3.* In order to do so we used the Affymetrix DMET™ Plus chip to simultaneously interrogate up to 1,936 genetic variants within 225

genes that encode for the proteins responsible for Drug Metabolism, Elimination, and Transport (DMET)(176). This chip has been previously used to identify genetic variants that influence treatment outcomes from various other drugs used in cancer(178-181) and other diseases(181, 182).

Despite the use of a genotyping platform that comprehensively interrogates markers relevant to drug pharmacokinetics, the only hit that surpassed our exploratory significance threshold is located in a gene (*ABCG1*) not thought to be involved in paclitaxel PK. ABCG1 is an intracellular sterol transporter that is primarily recognized for its role in regulation of intracellular cholesterol levels, particularly in cholesterol-laden macrophages(183). ABCG1 is also expressed in peripheral neurons(184) where cholesterol is converted to pregnenolone, a reaction that is inhibited by paclitaxel *in vitro*(185). Pregnenolone is then metabolized to progesterone, 5α-dihydroprogesterone, and allopregnanolone, which are referred to as neuroactive steroids(186). Neuroactive steroids are key regulators of Schwann cell proliferation and myelin formation(187); processes initiated in response to axonal demyelination, a prominent finding in paclitaxel-induced neurotoxicity both *in vitro*(188) and *in vivo*(81). Interestingly, co-treatment with neuroactive steroids enhances recovery from docetaxel-induced neuropathy in rats(189).

Several groups have investigated whether SNPs in genes relevant to paclitaxel pharmacokinetics are associated with risk of neurotoxicity. None of the significant findings from these previous candidate studies, including SNPs in *CYP3A5*(132) and *ABCB1*(139), reached statistical significance in our study, so

their influence on paclitaxel-induced neuropathy risk could not be verified. Attempts to discover loci that modulate risk of paclitaxel-induced neuropathy on a genome-wide scale have been reported by multiple groups(108, 143, 190), yielding attractive candidates for replication. Unfortunately, the top hits from these genome-wide association studies, including *EPHA5* and *FGD4*(143), *FANCD2*(142), and *RWDD3*(108) are not found on the DMET™ Plus chip and could not be assessed in this analysis.

Our study leveraged the power of the DMET™ Plus chip to simultaneously interrogate hundreds of variants that could be relevant to paclitaxel PK. This hypothesis-directed discovery approach could be a powerful tool for discovering variants in genes not previously recognized to influence drug exposure or treatment outcome. However, our results demonstrate one of the fundamental limitations of this discovery approach. In the unconditioned analysis, *CYP2C8*3*, which has been reported in multiple independent cohorts to influence paclitaxel-induced neuropathy, was our 14th highest ranked variant (p=0.016) and did not approach our significance threshold (α=0.001). True associations do not necessarily show the strongest association in discovery studies(191), and the necessity for rigorous statistical correction may lead to many false negatives(130).

In conclusion, we have used a commercially available genotyping panel that interrogates variants in genes relevant to drug metabolism, elimination, and transport in an attempt to discover and replicate SNPs that modulate risk of paclitaxel-induced neurotoxicity through their influence on drug

pharmacokinetics. We identified a variant in a gene (*ABCG1*) that is relevant to the regulation of endogenous neuroactive steroids which has not been previously investigated in candidate SNP association studies of chemotherapy-induced neuropathy to our knowledge. The finding could not be replicated in the smaller non-Caucasian cohort, suggesting that the effect may be exclusive to Caucasian subjects. Our findings suggest that limited current understanding of biology and pharmacology may preclude successful candidate selection and supports the continued use of unbiased methods in pharmacogenetic discovery. Ultimately, it is essential that the findings from our study, and all discovery studies, are successfully replicated in independent populations of patients to validate their influence on the phenotype of interest and elucidate their potential for clinical utility.

TABLES

Table 3 Characteristics of LCCC 9830 DMET™ Patient Cohort

		Primary	Replication
Self-reported Race	Caucasian	288	0
	African-American	0	107 (86%)
	Other	0	17 (14%)
Age (Years)	Median	52	45
	Range	24-84	22-68
Grade 2+ Neuropathy	Yes	49 (17%)	28 (23%)
	No	239 (83%)	97 (78%)
Diabetes Diagnosis	Yes	24 (8%)	24 (19%)
	No	264 (92%)	100 (81%)
Paclitaxel Schedule & Dose	80-90 mg/m^2 Weekly	91 (32%)	40 (32%)
	175 mg/m^2 Every 2 weeks	163 (57%)	73 (59%)
	175 mg/m^2 Every 3 weeks	34 (12%)	11 (9%)
Supplemental Neuropathy Therapy	Glutamine	84 (29%)	28 (23%)
	Gabapentin	7 (2%)	8 (6%)
	Amitriptyline	8 (3%)	1 (1%)
	Vitamin B6	5 (2%)	2 (2%)
	Vitamin B Complex	1 (<1%)	1 (1%)
	None	183 (64%)	85 (69%)
Cumulative Paclitaxel (mg/m^2)	Median	700	700
	Range	80-1280	80-1280
Paclitaxel Cycles	Median	4	4
	Range	1-16	1-16
Treatment Prior to Paclitaxel	AC (Doxorubicin/Cyclophos.)	216 (75%)	100 (81%)
	AC + Bevacizumab	2 (1%)	0
	A (Doxorubicin)	2 (1%)	0
	AC + Docetaxel	0	1 (1%)
Treatment Concurrent to Paclitaxel	Trastuzumab	46 (16%)	25 (20%)
	Bevacizumab	4 (1%)	5 (4%)
	Carboplatin	1 (<1%)	1 (1%)
	Carboplatin + Bevacizumab	1 (<1%)	2 (2%)
	Trastuzumab + Lapatinib	4 (1%)	0
	Trastuzumab + Cyclophosphamide	1 (<1%)	0
Treatment Setting*	Neoadjuvant	137 (48%)	52 (42%)
	Adjuvant	153 (53%)	73 (59%)

Counts and percentages (in parentheses) are presented for categorical data.
Medians and ranges are presented for quantitative data.
*Three patients were treated with paclitaxel neoadjuvantly and adjuvantly

Table 4 DMET™ SNPs Most Strongly Associated with Grade 2+ Neuropathy in LCCC 9830 Caucasian Cohort

Rank	Gene Variant	rsID	CYP2C8*3 Conditioned Fisher's Exact P-Value	Unconditioned Fisher's Exact P-value
1	ABCG1 (intronic)	rs492338	0.0008*	0.0017
2	CYP4A11 (3'UTR)	rs11211402	0.0010	0.0010
3	CYP4B1_14422C>T(R173W)	rs4646487	0.0015	0.0021
4	GSTA5 (intronic)	rs4715354	0.0018	0.0053
5	ABCG1 (intronic)	rs3788007	0.0033	0.0008*
6	CBR1 (intronic)	rs998383	0.0037	0.0074
7	ABCC1_94714T>C(V275V)	rs246221	0.0039	0.0038
8	GSTA1 (5' UTR)	rs4715332	0.0049	0.0077
9	SLC16A1_15385T>A(D490E)	rs1049434	0.0056	0.0041
10	CYP17A1_195G>T(S65S)	rs6163	0.0066	0.0070

*Surpassed exploratory α=0.001

Table 5 Contingency Table of Grade 2+ Neuropathy Occurrence by Genotype for rs492338

	Genotype	Neuropathy Incidence	Odds Ratio Vs. Homozygous Wild-Type
ABCG1 rs492338 [Intronic]	C/C	6/66 (9.1%)	-
	C/T	23/157 (14.6%)	OR= 1.70, 95% CI: (0.63- 5.37) p=0.38
	T/T	20/62 (32.2%)	OR= 4.70, 95% CI: (1.64- 15.57) p=0.002

FIGURE LEGENDS

Figure 7 Incidence curve for grade 2+ neuropathy by ABCG1 (rs492338) genotype in Caucasian (n=285) patients.

Figure 8 Incidence curve for grade 2+ neuropathy by ABCG1 (rs492338) genotype in non-Caucasian (n=124) patients.

FIGURES

Figure 7 Grade 2+ Neuropathy by rs492338 Genotype for LCCC 9830 Caucasian Subjects

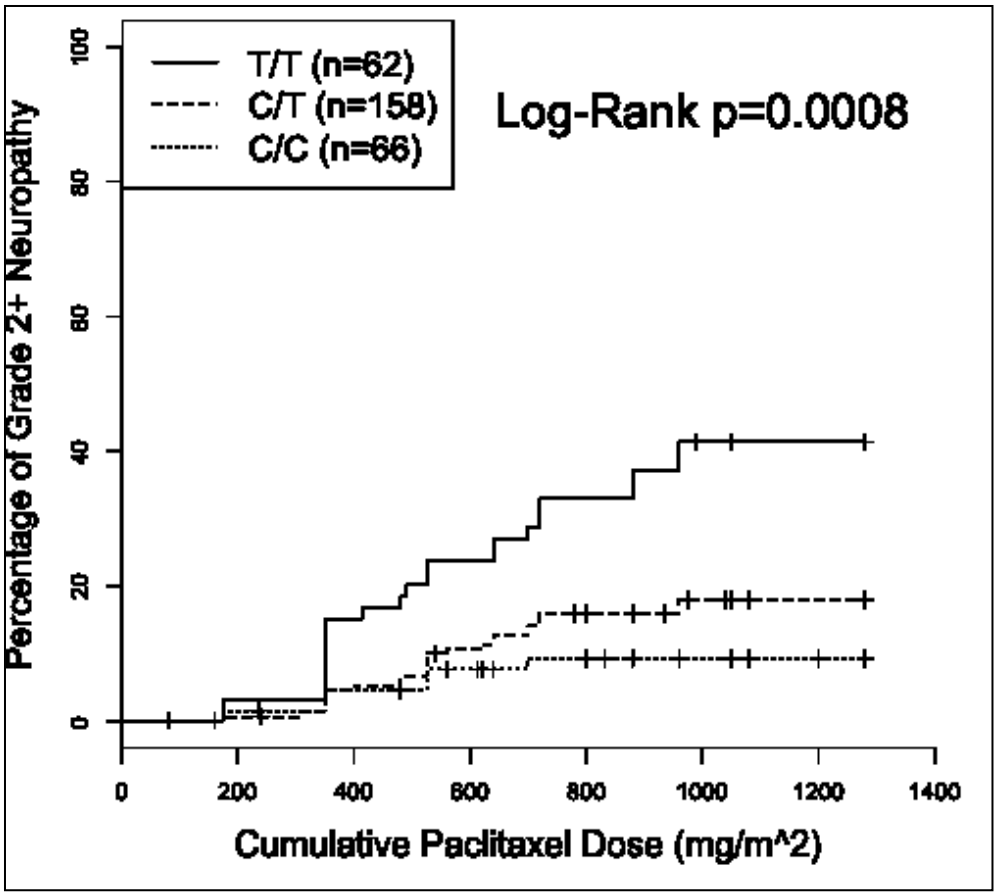
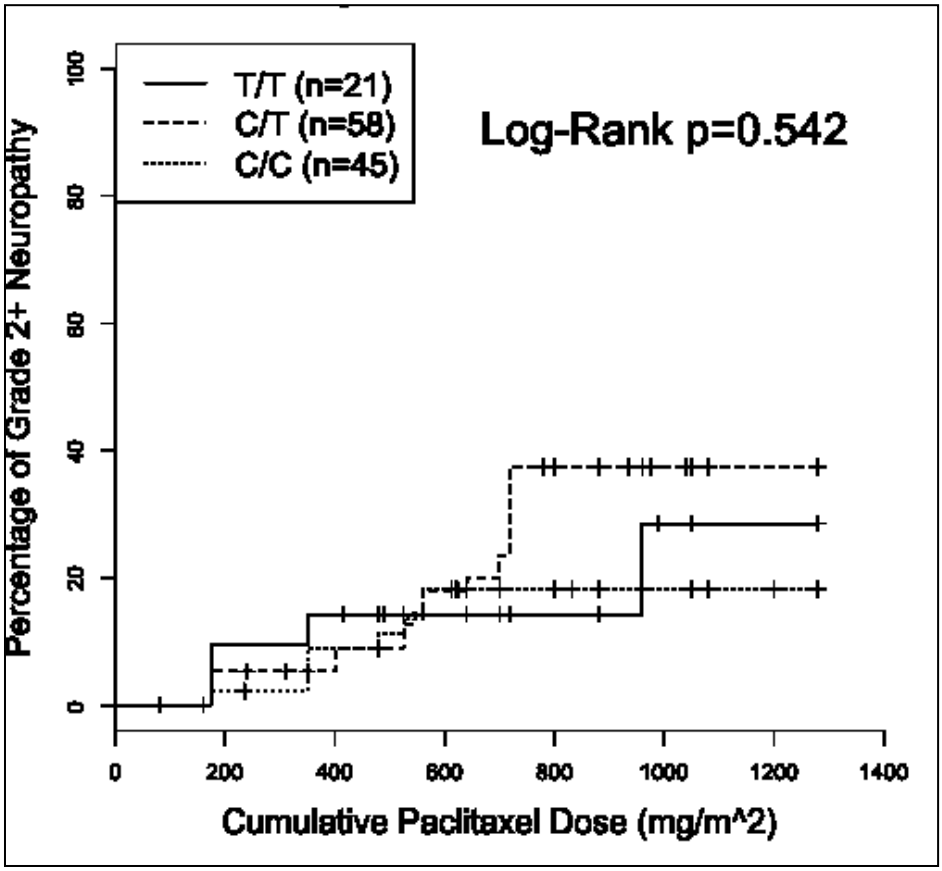


Figure 8 Grade 2+ Neuropathy by rs492338 Genotype for LCCC 9830 Non-Caucasian Subjects



CHAPTER IV

DISCOVER VARIANTS ANYWHERE IN THE GENOME THAT ARE ASSOCIATED WITH MODULATED RISK OF EXPERIENCING GRADE 3+ NEUROPATHY BY A CUMULATIVE DOSE OF DOCETAXEL THROUGH COMPETING-RISKS ANALYSIS AND GENOME-WIDE ASSOCIATION

INTRODUCTION

The taxane class includes three FDA approved chemotherapeutic agents; paclitaxel, docetaxel, and cabazitaxel(14). Taxanes work by binding to and stabilizing microtubules, ultimately inhibiting the mitotic phase of cell cycle development(50), and are approved for use in breast, gastric, lung, ovarian, and prostate cancer. The taxanes and other microtubule targeting chemotherapeutic agents such as the vinca alkaloids induce peripheral sensory neuropathy in some patients(73). Taxane-induced peripheral neuropathy often presents as a combination of paresthesia and dysesthesia, and can progress to irreversible loss of function of hands and feet with continued taxane treatment(85).

The discovery and validation of genetic loci that influence risk of taxane-induced peripheral neuropathy would be of substantial clinical benefit, enabling identification of high risk patients in whom taxane use should be avoided. This is particularly critical in tumor types in which the benefit of taxane therapy is modest, such as the use of docetaxel in castration-resistant prostate

Daniel L. Hertz, Kouros Owzar, Hitoshi Zembutsu, Chen Jiang, Jai Patel, Dorothy Watson, Mark, L. Ratain, Stefanie D. Krens, Ivo Shterev, Deanna L. Kroetz, Susan Halabi, Michiaki Kubo, William Kevin Kelly, Howard L. McLeod. Genome-wide association study of docetaxel-induced peripheral neuropathy in a hormone refractory prostate cancer clinical trial (CALGB 90401)

cancer(192). Pharmacogenetic markers that modulate risk of taxane-induced neuropathy have been reported from candidate gene studies of both paclitaxel(132, 139, 141, 142, 193) and docetaxel(140), and a genome-wide analysis of paclitaxel-induced neuropathy has been published(143), however, no study has taken a genome-wide approach to discovering genetic loci that influence docetaxel-induced neuropathy.

In this study we performed genome-wide association on a large, prospectively enrolled, chemotherapy naive cohort of hormone refractory prostate cancer patients who were treated with docetaxel with or without bevacizumab for up to 2 years. Because docetaxel therapy was discontinued prior to 2 years in many patients we utilized a competing-risks adjusted statistical model(194, 195). The primary objective of this study was to discover genetic loci that modulate risk of grade 3+ docetaxel-induced peripheral neuropathy in self-reported, genetically-defined Caucasian patients. Replication was then attempted in a genetically-defined Caucasian cohort of paclitaxel treated patients from a large prospective clinical trial.

METHODS

Patient Cohort

All patients enrolled on the Cancer and Leukemia Group B (CALGB) 90401 parent study who provided informed consent for the pharmacogenetic substudy (CALGB 60404) were eligible for this pharmacogenetic analysis. For a detailed description of the parent study see Kelly et al.(196). Briefly, patient eligibility included histologically documented adenocarcinoma of the prostate that had

progressed while on hormone deprivation therapy. Relevant exclusion criteria included prior chemotherapy or anti-angiogenesis therapy or a documented history of grade 2+ neuropathy. Subjects were randomized to receive docetaxel based treatment with bevacizumab or placebo for up to two years. Toxicity data was collected at each treatment cycle on standardized forms that mandated reporting of grade 3+ peripheral sensory neuropathies as defined by NCI CTCAE Version 3.0. Only neuropathy that occurred within 30 days of docetaxel treatment and was considered possibly, probably, or definitely related to treatment by the clinician was included in the analysis.

Docetaxel Treatment

All patients received docetaxel 75 mg/m^2 infused over 1 hour on day 1 of each 21 day cycle with 8 mg oral dexamethasone 12, 3, and 1 hour prior to docetaxel infusion. Subjects on both arms also received 5 mg oral prednisone twice daily and were randomized to 15 mg/kg bevacizumab or placebo intravenous infusion on day 1 of each cycle. Use of growth factor, aspirin, anti-emetics and luteinizing-hormone releasing hormone agonist were under the discretion of the treating physician. Docetaxel administration was held for neutropenia (absolute neutrophil count<1,500) or the dose was decreased in 10 mg/m^2 increments for hepatic dysfunction, neurotoxicity, gastrointestinal toxicity, or febrile neutropenia. The protocol mandated discontinuation of docetaxel treatment if the patient required more than two docetaxel dose decreases or in the event of specific severe toxicities or confirmed cancer progression.

Genome-Wide Genotyping

A 10 mL sample of whole blood was collected from all patients enrolling on the pharmacogenomic substudy prior to initiation of protocol treatment. Genotyping and genetic quality control were similar to that previously reported in a CALGB genome-wide association study(143). Genotyping was performed on the HumanHap610-Quad Genotyping BeadChip (Illumina, CA, USA) at the RIKEN Center for Genomic Medicine (Kanagawa, Japan). Appropriate quality control was used to eliminate subjects with call rate <95% and SNPs with call rate <99%, poor genotype clustering, or known to be unreliable (Tech Note: Infinium® Genotyping Data Analysis, 2007). SNPs with minor allele frequency <0.05 or p-value of Hardy-Weinberg distribution >1x10^-08 and all SNPs located on the sex chromosomes were also removed, leaving 498,081 SNPs for analysis. Eigensoft version 3.0 was used to define genetic ancestry for the 810 subjects and only the subjects who were self-reported and genetically-defined as Caucasian (n=623) were included in the analysis (**Figure 9**).

Statistical Analysis

The endpoint used in this study was the cumulative dose (mg/m^2) at first report of grade 3+ sensory peripheral neuropathy. Any patient who did not experience neuropathy was censored at their maximum docetaxel dose received. The primary statistical analysis utilized a competing-risks adjusted likelihood model in which any patient who did not experience neuropathy and did not complete the full two years of therapy was classified by their reason for docetaxel discontinuation: progression/death, treatment terminating adverse event, or

withdrawal/other. Using this data, a likelihood ratio test with 1 degree of freedom and 95% confidence intervals was estimated for each SNP assuming an additive genetic effect. Any SNP for which the model did not converge was excluded from analysis, bringing the final SNP total to 498,022. The top 100 hits were subjectively filtered considering the strength of association with neuropathy, biological function of the gene, and other publicly available data to create a list of priority SNPs for further analysis. The relationship between these SNPs and neuropathy was adjusted for clinical covariates with known relevance to neuropathy risk: diabetes (reported history of diabetes or current diabetes treatment vs. none), age (continuous), body mass index (BMI) (≤ 30 kg/m^2 vs. > 30 kg/m^2), and for treatment arm (bevacizumab vs. placebo). We attempted replication in an independent cohort of self-reported, genetically-defined Caucasians who received paclitaxel for adjuvant breast cancer treatment (CALGB 40101)(197). Results of the genome-wide association of the dose-at-grade 2+ neuropathy occurrence were recently published(143). The p-value of association for each SNP on our priority list was compared to a Bonferroni corrected p-value threshold.

RESULTS

The CALGB 90401 parent study enrolled 1,050 total patients, of whom 623 self-reported, genetically-defined Caucasian subjects were evaluable in the discovery analysis (**Figure 10**). Relevant demographic characteristics for patients, including patient age and relevant covariate data are displayed in **Table 6** for the entire discovery cohort and stratified by whether or not they experienced

neuropathy. The overall incidence of grade 3+ sensory neuropathy in the discovery population was 8% (50/623). The remaining 573 patients were classified as having either completed treatment without neuropathy (24/623=3.9%) or categorized based on their reason for docetaxel discontinuation: death/progression (38.4%), treatment terminating adverse event (31.9%) or withdrawal/other (17.8%) (**Table 7**). The cumulative distribution of neuropathy or competing risk event during docetaxel treatment is displayed in **Figure 11**. As expected, the risk of neuropathy was not significantly different based on patient assignment to the bevacizumab or placebo arm (p=0.24) (**Figure 12**).

The top 100 hits from the discovery analysis including rank, rsID, gene, and unadjusted p-value are reported in **Table 8**. No locus surpassed Bonferroni-corrected significance (0.05/498,022=1.004x10^-7) though multiple variants surpassed a suggestive threshold of 1x10^-6 (**Figure 13**). The top hit was an intergenic SNP (rs11017056, **Figure 14**) that increased neuropathy risk in a seemingly additive manner (HR=2.83, 95%CI: 1.89-4.25, p=5.00x10^-7, **Figure 15**). The second ranked hit is an intronic SNP in the *VAC14* gene (rs875858) which also increased neuropathy risk (HR=3.43, 95%CI: 2.11-5.62, p=7.90x10^-7, **Figure 16**).

Based on the strength of their association these two SNPs were selected for inclusion in the final list of priority SNPs (**Table 9**). This list also includes the third ranked SNP (rs10761189) which is found in *FGD3*, two intronic SNPs (rs17185211, p=1.2x10^-5 and rs478472, p=1.80x10^-5) in genes with known

relevance to neurodevelopment and neuronal connectivity in the central nervous system (*DOK6* and *NAV1*) and two SNPs from the *OPCML* gene (rs1027796, p=4.7x10^-6, rs12805206, p=7.9x10^-5) that were ranked in the top 100 hits and are not in strong LD (D'=0.12, r^2=0). These 7 SNPs were then adjusted for the clinically relevant covariates: diabetes, age, treatment arm, and BMI, and these results are also displayed in **Table 9**. Notably, our top hit rs11017056 surpassed the Bonferroni corrected genome-wide significance threshold after covariate adjustment (p=7.2x10^-08).

Each of the 7 priority SNPs was then tested, separately, in the genetically defined-Caucasian patients from the CALGB 40101 study at a Bonferroni-corrected significance threshold of p<0.0071. The results of the attempted independent replication are also displayed in **Table 9**. Though the p-value for our top hit (rs11017056, p=0.001) is smaller than the significance threshold, this association was not successfully replicated as the direction of effect was opposite of that seen in the discovery population (**Figure 17**). Thus, none of our priority SNPs were successfully replicated in the independent cohort of paclitaxel-treated patients.

DISCUSSION

Chemotherapy-induced peripheral neuropathy is a common, severe adverse effect of a variety of chemotherapeutic agents, including the taxanes. While docetaxel is somewhat less neurotoxic than paclitaxel(67-70), it does necessitate treatment discontinuation in some patients and the ability to identify individuals at increased risk of docetaxel-induced neuropathy prior to treatment

could be of major clinical benefit. Using a genome-wide pharmacogenetic discovery approach we have attempted to identify genetic loci that modulate risk of docetaxel-induced peripheral neuropathy in a cohort of 623 Caucasian patients. We then attempted to replicate this association in an independent population of paclitaxel treated Caucasian patients from the CALGB 40101 study.

The major strengths of this study are the large, prospectively enrolled, chemotherapy naïve patient cohort who received homogeneous docetaxel treatment and regular assessment for sensory neuropathy. Our genotyping platform and data processing pipeline have been previously used in published genome-wide pharmacogenetic analyses that discovered a variant that was successfully replicated in two independent populations(143). Another element of this study that differentiates it from previous pharmacogenetic analyses is the use of the competing-risks adjusted likelihood ratio test. This procedure is particularly important in the context of this trial where a large number of patients discontinued treatment due to early disease progression, death, or severe toxicity, as can be seen in **Figure 11**. By utilizing the competing-risks approach we were able to informatively censor patients who discontinued treatment early and test the specific influence of each genetic locus on the endpoint of interest, neuropathy occurrence.

Our top hit (rs11017056), which after covariate adjustment surpassed genome-wide significance, is an intergenic SNP located several hundred kilobases upstream from the nearest gene, *EBF3* (**Figure 14**). This common variant (Minor allele frequency [MAF] =0.22) increased risk of docetaxel-induced

neuropathy in this study (HR=2.83). According to the HaploReg database, rs11017056 is in complete LD with another intergenic SNP (rs34559138) which alters a binding motif for the Forkhead/winged-helix J1 (Foxj1) transcription factor(198). This transcription factor coordinates postnatal neurogenesis(199) and differentiation of neuronal-associated astrocytes through regulation of a network of genes, a large number of which are microtubule-associated proteins(200).

The second ranked hit is a rare (MAF=0.056) intronic SNP within the *VAC14* (ArPIKfyve) gene that confers increased risk of neuropathy (HR=3.43). The VAC14 protein stabilizes FIG4, creating a complex that is responsible for the regulation of PI(3,5)P2 and PI5P, which are necessary for neurogeneration(201). Interestingly, a rare mutation in the *FIG4* gene is known to cause a subtype of Charcot-Marie Tooth (CMT) disease, a hereditary neuropathy syndrome(202). The previously reported genome-wide pharmacogenetic study of paclitaxel-induced neuropathy discovered and validated an association for a SNP in *FGD4*, another gene that has been implicated in CMT(143). The third ranked hit is a common (MAF=0.389) intronic SNP in the *FGD3* gene which had a protective effect (HR=0.432). Little is known about FGD3; however, it is of interest based on its functional and structural similarity to FGD4 and their involvement in the highly-conserved cdc42 pathway(203).

This study discovered several candidates SNPs that were worth following-up in an independent cohort of taxane-treated patients. Unfortunately, none were successfully replicated. There was a statistically significant association between

our top hit (rs11017056) and paclitaxel-induced peripheral neuropathy (p=0.001), however, the direction of effect was reversed. It is possible that differences between the studies, such as the different tumor type or patient gender are the cause of our inability to replicate our findings. The most important difference between the studies may be the use of paclitaxel instead of docetaxel in CALGB 40101. It is conceivable that the genetic factors that modulate neuropathy sensitivity are different between the taxanes. Though our second highest ranked SNP is found in *FGD3*, we did not detect an association for the *FGD4* variant previously discovered and replicated in the CALGB 40101 paclitaxel-induced neuropathy GWAS (rs10771973, p=0.39), further suggesting that different genetic risk factors may exist for the two taxanes.

One other notable difference between the discovery and replication cohorts was the threshold for grade of neuropathy that was considered an event. This study did not systematically collect grade 2 peripheral neuropathy, which was the endpoint utilized in CALGB 40101. Grade 2 neuropathy is more common, improving study power, but grade 3 neuropathy is more clinically relevant and patients in this trial were not dose reduced unless they experienced grade 3 or higher neurotoxicity. A related limitation is that neuropathy assessment is subjective by nature, which may result in inconsistent or inaccurate measurement(168). Future prospective studies should seek to implement superior neuropathy collection techniques, such as those that rely on patient reporting(204).

The potential limitations of current neuropathy assessment techniques should not deter us from performing genome-wide discovery in the large cohorts of patients treated on prospective clinical studies. The SNPs discovered in this study may provide key insights into the pathways involved in the development of neurotoxicity. Based on our findings it seems that no single variant or gene is responsible for dictating patient sensitivity to taxane-induced neuropathy. This is consistent with the multifactorial nature of neuropathy which depends on both drug exposure and a variety of patient factors(91, 95, 101, 106, 109).

In conclusion, we have used a prospectively enrolled patient cohort to discover SNPs that modulate sensitivity to docetaxel-induced peripheral neuropathy. After adjustment we identified a single intergenic SNP (rs11017056) that may be acting through abrogation of a Foxj1 regulatory element. The association of this SNP could not be replicated in an independent cohort of paclitaxel-treated patients. These results suggest that it is unlikely that any single variant will be clinically useful for predicting patients' neuropathy risk when treated with docetaxel, however, replication of our results could validate the role of these genes or pathways in chemotherapy-induced neuropathy sensitivity.

TABLES

Table 6 Relevant Characteristics of CALGB 90401 Cohort and Patients Stratified by Grade 3+ Neuropathy

	90401 Cohort (n=623)	Neuropathy Yes (n=50)	Neuropathy No (N=573)
Age	69 (42-94)	73 (55-84)	69 (42-94)
Diabetes	98 (15.7%)	5 (10.0%)	93 (16.2%)
Body Mass Index	29.0 (16.1-57.2)	27.0 (16.1-41.7)	29.2 (17.3-53.3)
Bevacizumab Arm	314 (50.4%)	21 (42.0%)	293 (45.9%)
Docetaxel Dose	601 (72-2457)	604 (150-1932)	600 (72-2457)

Medians (and ranges) listed for quantitative data
Counts (and percentages) listed for categorical data

Table 7 CALGB 90401 Patient Classification for Competing-Risks Analysis

			Discovery Cohort (n=623)
Experienced Grade 2+ Neuropathy			50 (9.0%)
Completed 2 Years of Treatment			24 (3.9%)
Death or Progression			239 (38.4%)
	Death	16	
	Progression	223	
Treatment Terminating Adverse Event			199 (31.9%)
	Neutropenia	20	
	Hypertension	3	
	Thrombosis	10	
	Hemorrhage	29	
	Other	137	
Withdrawal or Other			111 (17.8%)

Table 8 100 SNPs with Strongest Association with Grade 3+ Neuropathy in 90401 Caucasian Subjects

Rank	rsID	Chr	Position	Alleles	Gene	P-value	HR
1	rs11017056	10	131720630	A/C	NA	5.00E-07	2.829
2	rs875858	16	69332956	C/T	VAC14	7.90E-07	3.430
3	rs10761189	9	94812049	A/G	FGD3	3.30E-06	2.313
4	rs2178728	2	130264445	A/G	NA	3.30E-06	2.277
5	rs8089250	18	4762807	C/T	NA	3.60E-06	2.808
6	rs1566691	5	3780179	C/T	NA	4.00E-06	3.437
7	rs1027796	11	132262880	A/C	OPCML	4.70E-06	2.293
8	rs4076630	9	94721379	C/T	NA	5.10E-06	2.282
9	rs11740657	5	135609558	A/G	TRPC7	5.20E-06	2.887
10	rs13171764	5	3783312	G/T	NA	6.40E-06	3.150
11	rs2792574	14	47746177	A/G	NA	6.40E-06	2.274
12	rs7225402	17	60810368	C/T	NA	7.50E-06	3.173
13	rs4452539	5	75795960	C/T	IQGAP2	7.90E-06	0.412
14	rs17205603	15	60252681	A/C	NA	9.30E-06	2.407
15	rs10992512	9	94692772	A/G	NA	1.20E-05	2.213
16	rs16948569	18	5633771	A/G	NA	1.20E-05	2.355
17	rs17185211	18	65299361	A/G	DOK6	1.20E-05	2.295
18	rs9604512	13	113602860	C/T	FAM70B	1.20E-05	3.142
19	rs6806193	3	181261062	C/T	NA	1.30E-05	2.502
20	rs2117152	3	85487473	A/C	NA	1.50E-05	2.162
21	rs2952605	2	130260216	G/T	NA	1.50E-05	2.328
22	rs3734124	5	135588957	C/T	TRPC7	1.60E-05	3.288
23	rs10501651	11	87205209	A/G	NA	1.70E-05	2.729
24	rs4872356	8	25695246	G/T	NA	1.70E-05	1.876
25	rs7309371	12	31772408	A/G	AMN1	1.70E-05	0.420
26	rs11051552	12	31781869	A/G	NA	1.80E-05	0.423
27	rs4438470	2	228722963	C/T	SPHKAP	1.80E-05	2.491
28	rs478472	1	199892446	A/G	NAV1	1.80E-05	3.245
29	rs9834152	3	181251225	C/T	NA	1.80E-05	2.480
30	rs6489160	12	126325609	C/T	NA	1.90E-05	2.431
31	rs11983040	7	14198205	C/T	DGKB	2.00E-05	2.472
32	rs10997287	10	68088852	C/T	CTNNA3	2.10E-05	0.247
33	rs2068434	5	75815227	C/T	IQGAP2	2.20E-05	0.420
34	rs2079459	7	14195569	C/T	DGKB	2.50E-05	2.357
35	rs13177369	5	61074267	C/T	NA	2.60E-05	2.355
36	rs6873040	5	61079065	C/T	NA	2.70E-05	2.394

Table 8 (con't)

Rank	rsID	Chr	Position	Alleles	Gene	P-value	HR
37	rs11720469	3	167963902	A/G	NA	3.30E-05	2.118
38	rs1953136	13	69974544	A/G	NA	3.30E-05	2.264
39	rs10045155	5	75793312	C/T	IQGAP2	3.60E-05	2.033
40	rs7801545	7	42266819	G/T	NA	3.60E-05	2.204
41	rs4889136	16	78928603	A/G	LOC729847	3.70E-05	2.490
42	rs1036837	18	65301877	A/C	DOK6	3.90E-05	2.229
43	rs4382847	10	52236981	G/T	A1CF A1CF	4.00E-05	2.309
44	rs17242471	5	157437693	A/G	NA	4.20E-05	2.255
45	rs4142335	13	69969424	A/C	NA	4.20E-05	2.223
46	rs10484017	14	89262294	C/T	NA	4.30E-05	2.523
47	rs388910	5	125165492	C/T	NA	4.50E-05	2.431
48	rs13392999	2	238167726	G/T	RAB17	4.60E-05	2.411
49	rs4256922	10	52238622	C/T	A1CF A1CF	4.60E-05	2.297
50	rs918462	5	151722764	C/T	NA	4.70E-05	0.285
51	rs943801	6	165912238	C/T	PDE10A	4.80E-05	2.132
52	rs11735066	4	52993145	C/T	NA	5.10E-05	3.002
53	rs1537420	13	102966614	G/T	NA	5.10E-05	2.282
54	rs7306680	12	46406222	A/G	NA	5.10E-05	2.129
55	rs17546000	1	174913623	A/G	PAPPA2	5.20E-05	2.478
56	rs6859984	5	75805324	C/T	IQGAP2	5.30E-05	2.045
57	rs13227391	7	105228545	A/G	ATXN7L1	5.40E-05	2.181
58	rs9649088	7	85221228	A/G	NA	5.70E-05	2.277
59	rs2079460	7	14195474	G/T	DGKB	5.90E-05	2.301
60	rs3008063	6	165923176	A/C	PDE10A	5.90E-05	2.120
61	rs1582329	16	51571503	A/G	NA	6.00E-05	2.353
62	rs6953042	7	85273196	A/G	NA	6.10E-05	2.273
63	rs2394279	10	68095473	C/T	CTNNA3	6.40E-05	0.418
64	rs10511298	3	112750254	C/T	CD96	6.60E-05	1.935
65	rs9529745	13	69922412	C/T	NA	6.70E-05	2.192
66	rs4332809	18	35983775	A/C	NA	6.90E-05	2.216
67	rs2339624	10	52355203	A/G	NA	7.00E-05	3.220
68	rs10229660	7	42270739	G/T	NA	7.20E-05	2.304
69	rs2827145	21	22237762	C/T	NA	7.20E-05	2.060
70	rs7242363	18	36002902	A/G	NA	7.20E-05	2.213
71	rs6003807	22	22264030	G/T	NA	7.30E-05	2.701
72	rs6769847	3	145018234	A/G	SLC9A9	7.50E-05	3.487
73	rs11051544	12	31758100	G/T	AMN1	7.70E-05	2.231
74	rs7428372	3	167966753	A/C	NA	7.70E-05	2.058
75	rs12805206	11	132295116	C/T	OPCML	7.90E-05	2.329
76	rs11658557	17	39849711	A/G	GPATCH8	8.00E-05	2.787
77	rs1646691	7	133690170	A/C	NA	8.00E-05	2.087
78	rs11216048	11	116000269	A/G	NA	8.20E-05	2.437

Table 8 (con't)

Rank	rsID	Chr	Position	Alleles	Gene	P-value	HR
79	rs4307756	0	NA	NA	NA	8.20E-05	2.437
80	rs9677476	2	231834534	A/G	ARMC9	8.20E-05	2.105
81	rs3008542	1	112322163	A/G	KCND3	8.50E-05	2.530
82	rs11223273	11	132280051	C/T	OPCML	8.60E-05	2.216
83	rs6504303	17	60823669	C/T	NA	8.60E-05	2.926
84	rs914906	13	69927917	A/G	NA	8.60E-05	2.154
85	rs1930887	13	69959984	C/T	NA	8.70E-05	2.343
86	rs1524037	2	195899339	A/G	NA	9.30E-05	2.427
87	rs16839537	2	195903999	A/C	NA	9.30E-05	2.427
88	rs377239	21	36860599	C/T	NA	9.90E-05	0.392
89	rs1086520	1	174951426	A/G	PAPPA2	1.00E-04	2.305
90	rs11612613	12	102784338	C/T	NA	1.00E-04	2.088
91	rs12904522	15	31337548	A/G	NA	1.00E-04	0.424
92	rs17151283	5	123538098	C/T	NA	1.00E-04	2.790
93	rs2339024	5	141585436	A/G	NA	1.00E-04	2.476
94	rs961909	18	65261636	A/G	DOK6	1.00E-04	2.135
95	rs11723493	4	37241658	C/T	C4orf19	0.00011	2.138
96	rs1776947	20	4874855	A/G	SLC23A2	0.00011	2.896
97	rs2196096	3	85553332	C/T	NA	0.00011	2.153
98	rs2700007	13	100832284	G/T	NALCN	0.00011	2.879
99	rs3816024	5	108463913	C/T	FER	0.00011	2.287
100	rs7505612	18	4766396	A/C	NA	0.00011	2.481

Table 9 List of priority SNPs from GWAS Discovery in CALGB 90401

Rank	rsID	Gene	MAF	HR (95% CI)	Unadj. p-value	Adj. p-value	Replication p-value
1	rs11017056	-	0.22	2.83 (1.89-4.25)	5.0E-07	7.2E-08*	0.001**
2	rs875858	VAC14	0.06	3.43 (2.11-5.62)	7.9E-07	1.6E-06	0.702
3	rs10761189	FGD3	0.40	2.32 (1.63-3.30)	3.3E-06	5.3E-06	0.987
7	rs1027796	OPCML	0.30	2.29(1.61-3.28)	4.7E-06	8.3E-06	0.305
17	rs17185211	DOK6	0.23	2.30 (1.58-3.33)	1.2E-05	3.4E-05	0.274
28	rs478472	NAV1	0.08	3.25 (1.90-5.56)	1.8E-05	2.2E-05	0.234
75	rs12805206	OPCML	0.22	2.33 (1.53-3.55)	7.9E-05	1.3E-04	0.053

*: Surpassed Bonferroni-corrected genome-wide significance threshold
**: Direction of effect was opposite of that seen in discovery cohort

FIGURE LEGENDS

Figure 9 Principal Components Analysis was used to define the genetically European subcohort for discovery. Any individual who fell more than 2 standard deviations from the median for any of the first three eigenvectors was excluded from the analysis leaving 623 subjects.

Figure 10 CONSORT Diagram specifying patient flow from enrollment on parent study to patients included in genome-wide discovery analysis.

Figure 11 Cumulative incidence of grade 3+ neuropathy and competing risks. The early rise in the death/progression and time to adverse event (TTAE) lines reflects the large number of patients discontinuing docetaxel treatment early in therapy.

Figure 12 Cumulative incidence of grade 3+ neuropathy stratified by treatment arm for the 90401 cohort demonstrating no influence of bevacizumab on neuropathy risk.

Figure 13 Manhattan plot for unadjusted association with grade 3+ neuropathy in Caucasian patients (n=623) for all SNPs that passed the QC filter (n=498,022). While no SNPs were significant at the genome-wide threshold (1x10^-8), two SNPs (rs11017056 and rs875858) surpassed a suggestive threshold of 1x10^-6.

Figure 14 SNAP plot of our top hit (rs11017056) displaying that it is an intergenic SNP that is approximately 100kb from the nearest gene (EBF3) and not in LD with any SNPs that are within any gene.

Figure 15 Cumulative incidence of grade 3+ neuropathy in CALGB 90401 genetic Europeans stratified by genotype for the top hit (rs11017056).

Figure 16 Cumulative incidence of grade 3+ neuropathy by genotype for the second ranked hit (rs875858) in the 90401 discovery cohort demonstrating an increased risk in heterozygous as compared to wild-type patients. Only three patients were homozygous for the variant allele, none of whom experienced neuropathy.

Figure 17 Attempted replication of the association between our top hit (rs11017056) and incidence of neuropathy (grade 2+) in CALGB 40101 genetic Europeans showing a significant association in the reverse direction.

FIGURES

Figure 9 Principal Components Analysis of CALGB 90401 Cohort

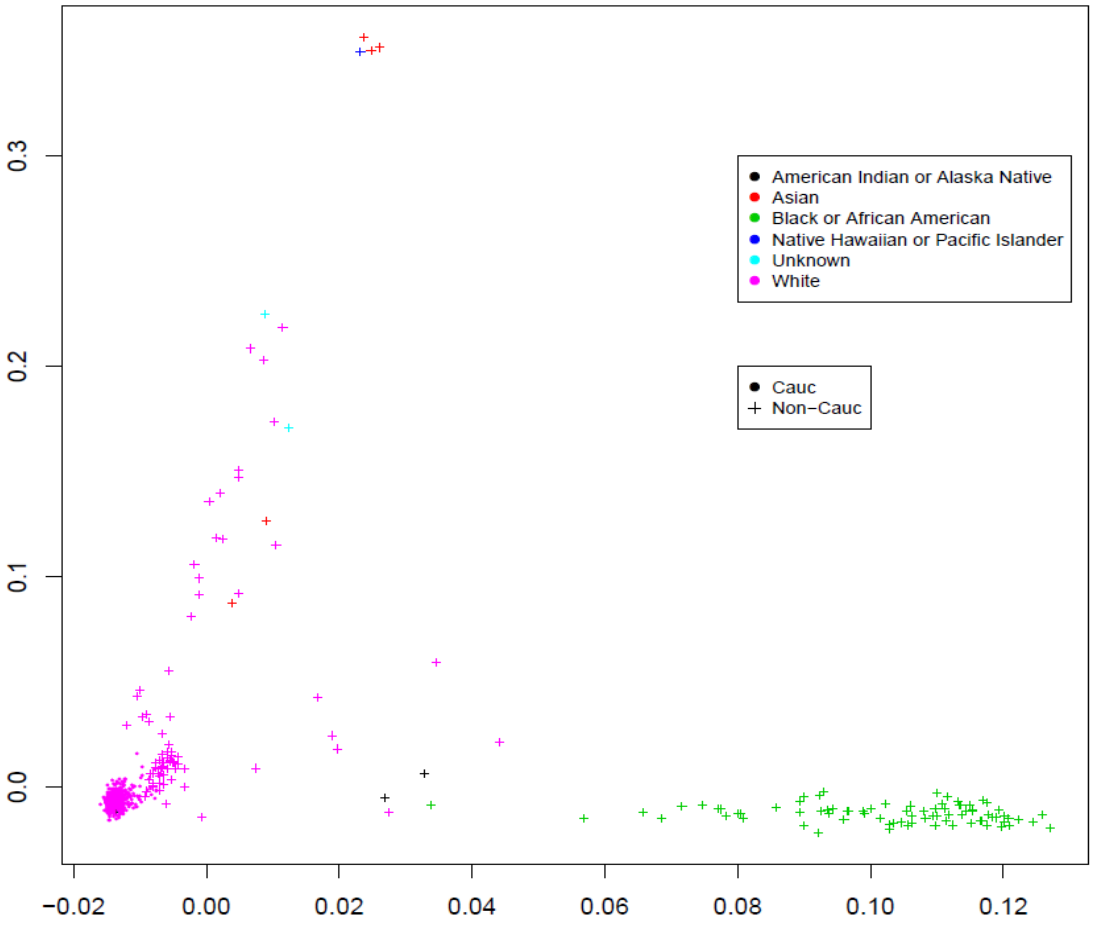


Figure 10 Consort Diagram for Patients Included in CALGB 90401 Neuropathy GWAS

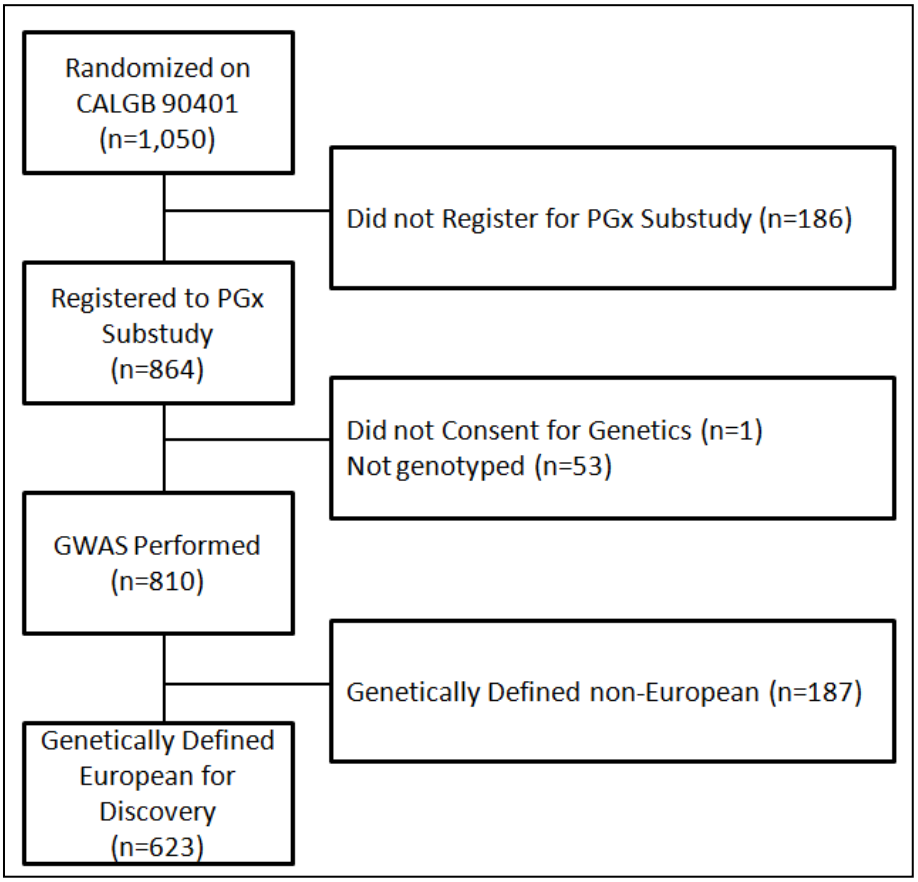


Figure 11 Cumulative Incidence of Grade 3+ Neuropathy and Competing Risks

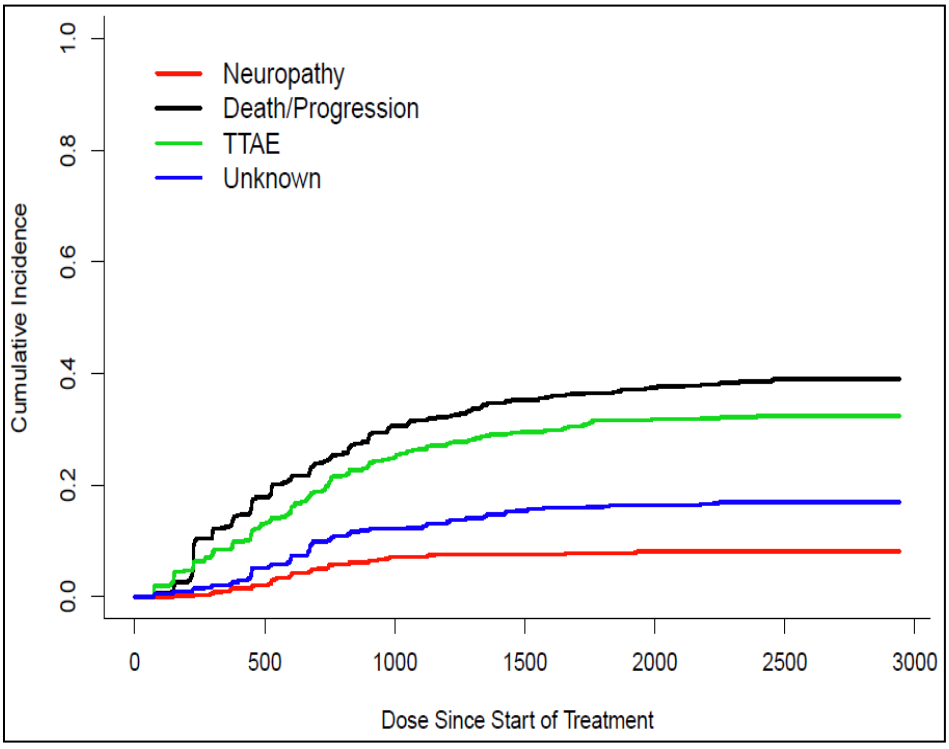


Figure 12 Cumulative Incidence of Grade 3+ Neuropathy Stratified by Treatment Arm

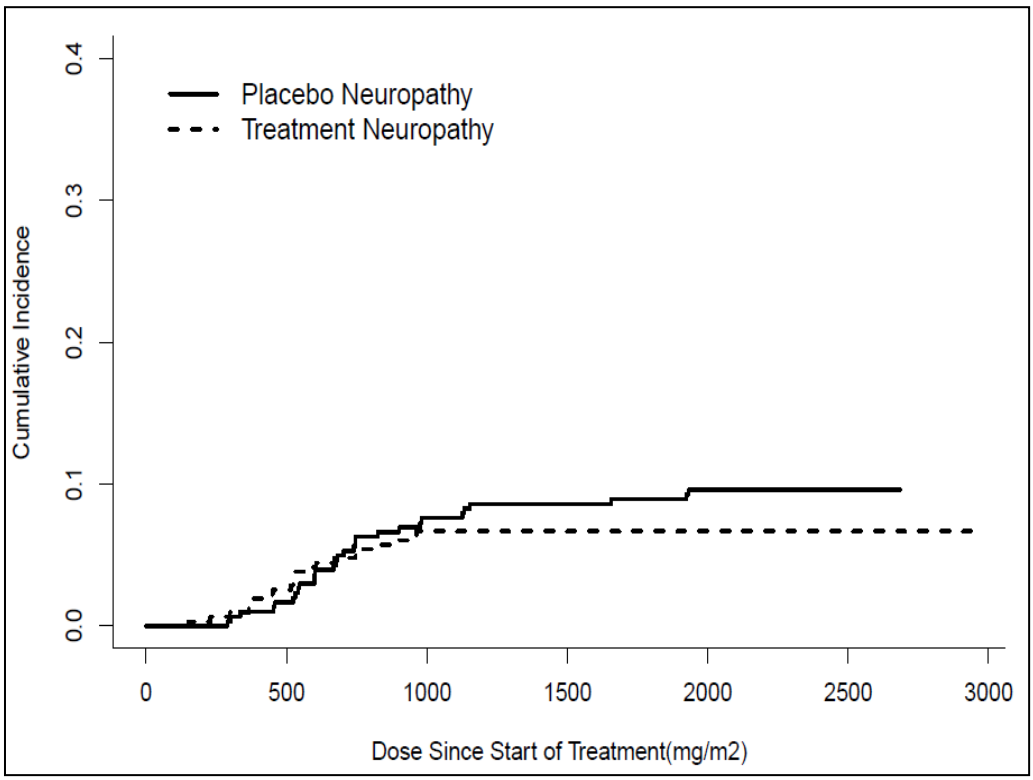


Figure 13 Manhattan Plot of Results for All SNPs Included in CALGB 90401 GWAS

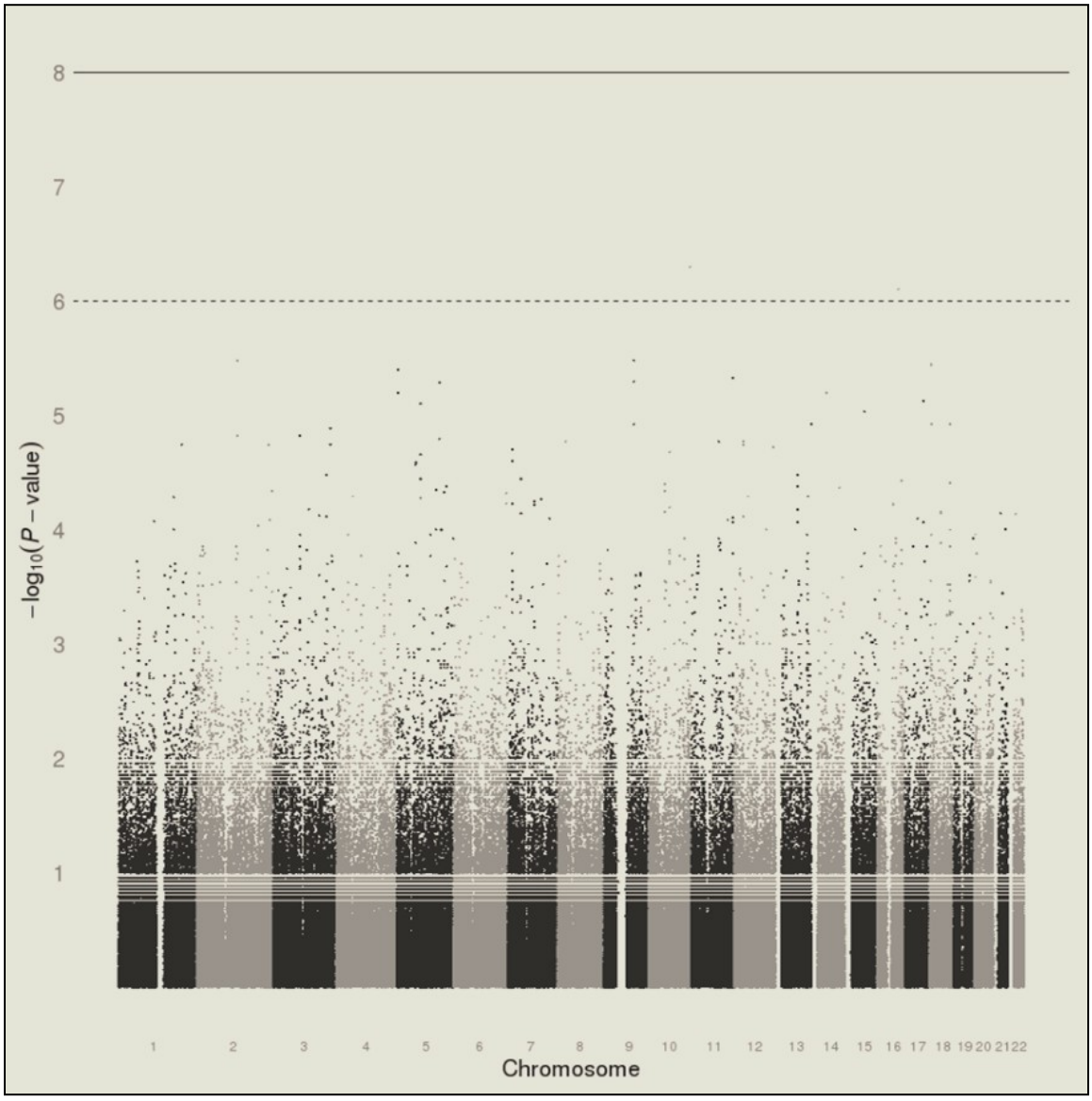


Figure 14 SNAP Plot of rs11017056

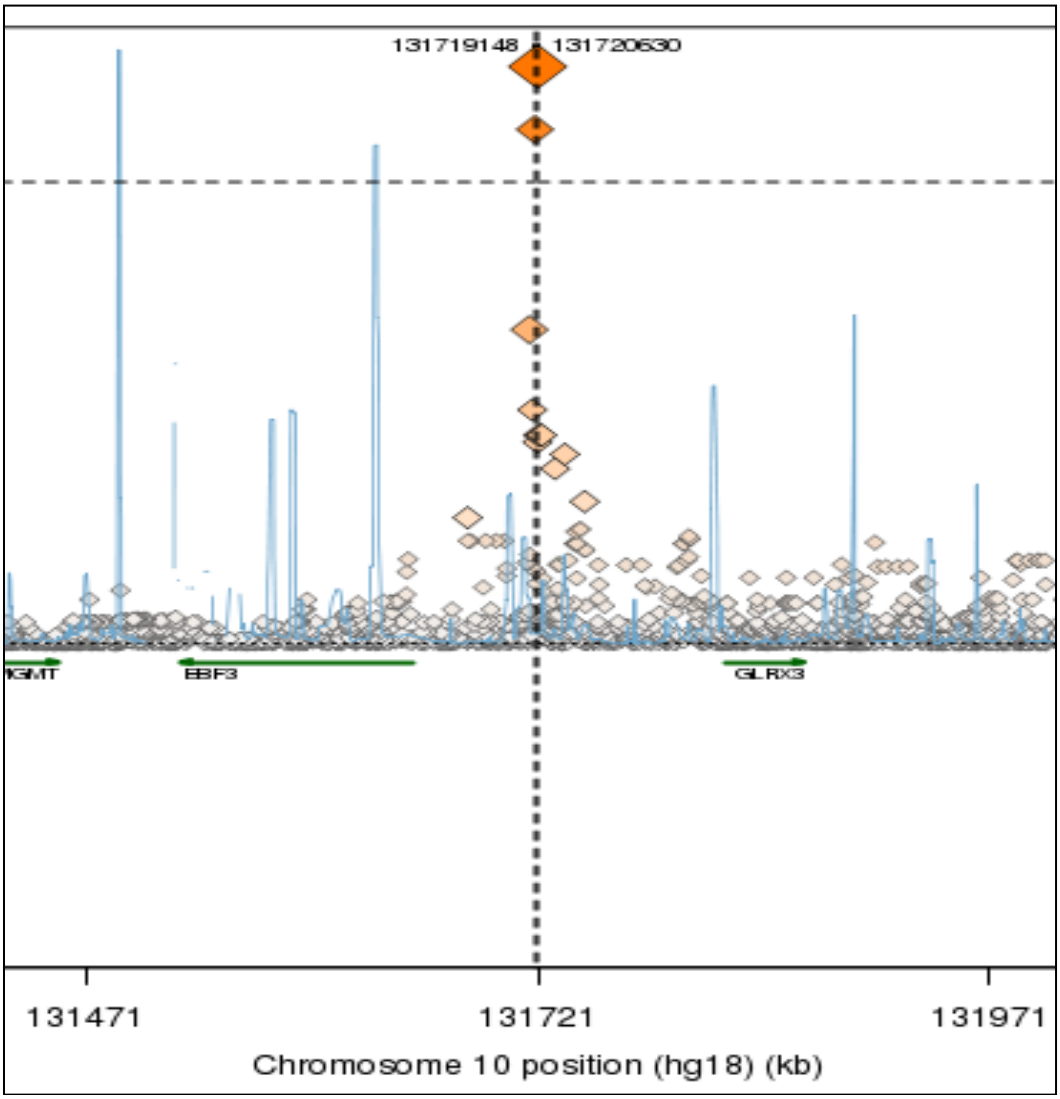


Figure 15 Incidence of Grade 3+ Neuropathy Stratified by rs11017056 Genotype in CALGB 90401

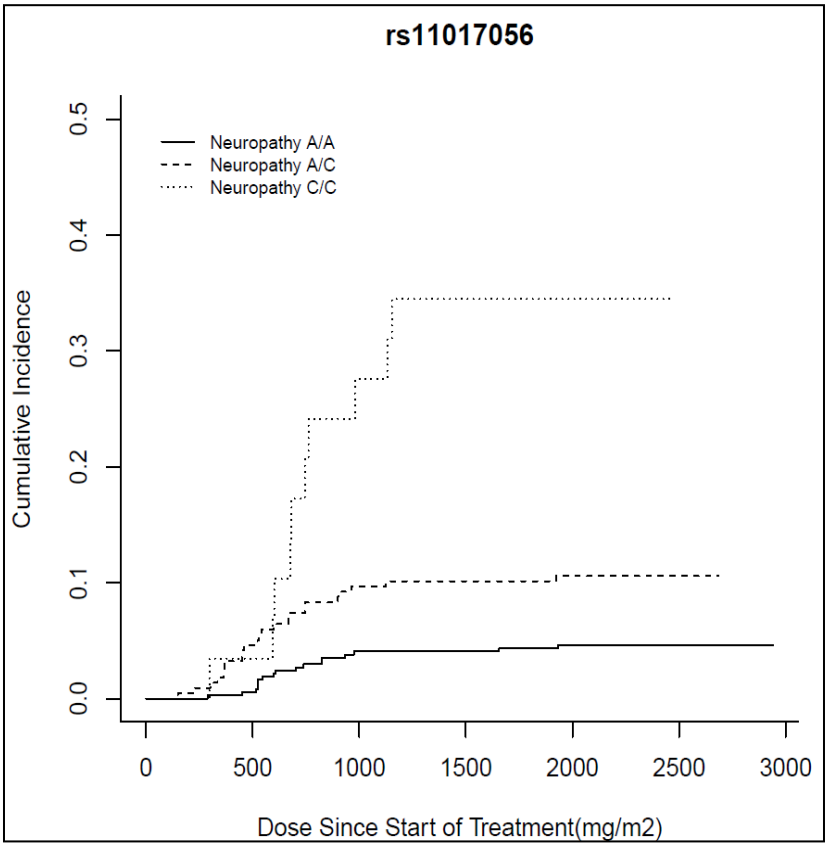


Figure 16 Incidence of Grade 3+ Neuropathy Stratified by rs875858 Genotype in CALGB 90401

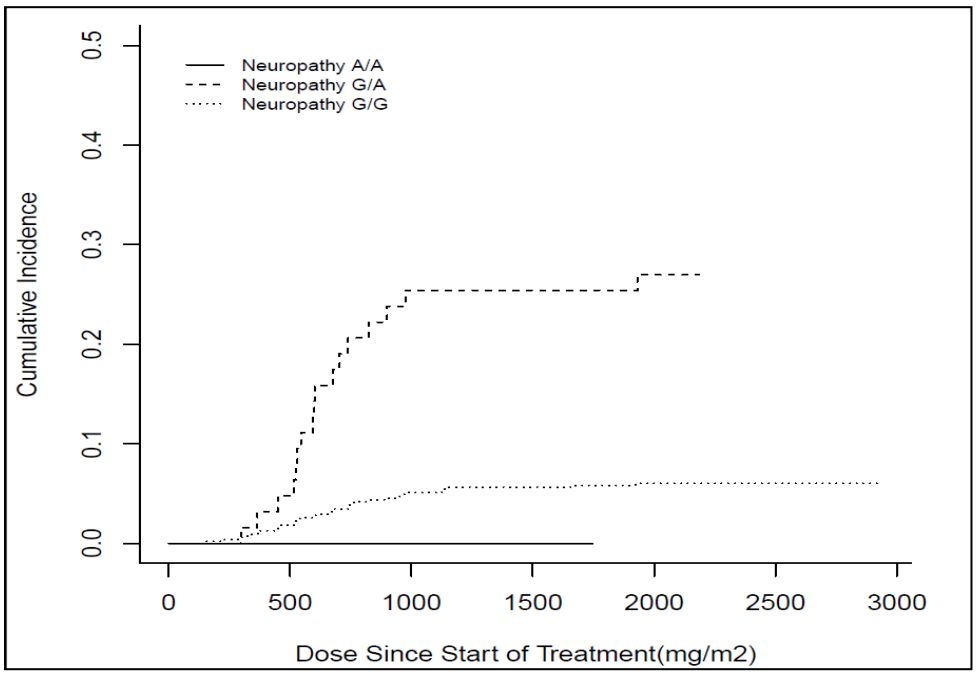
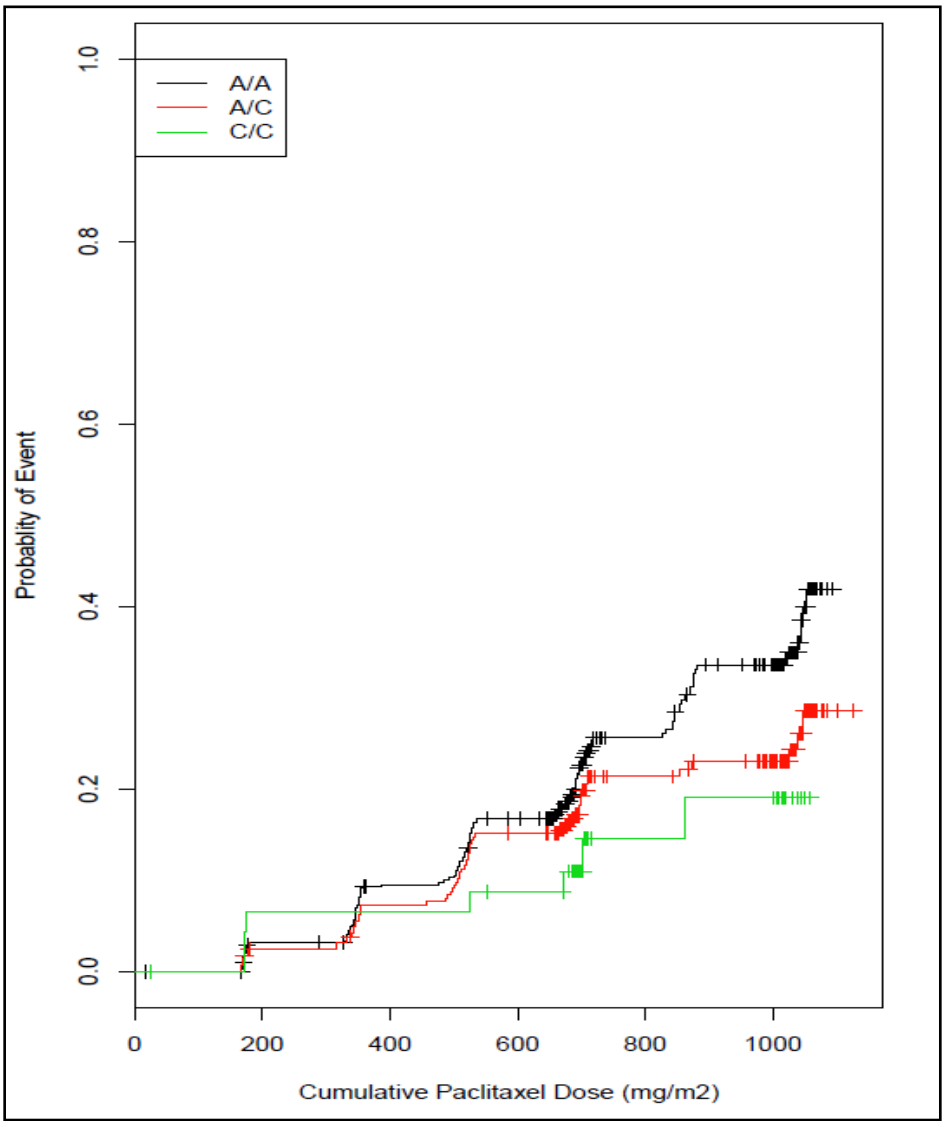


Figure 17 Incidence of Grade 2+ Neuropathy by rs11017056 Genotype in CALGB 40101



CHAPTER V

DISCUSSION AND PERSPECTIVE

The taxanes are commonly used in a variety of solid tumor types including breast, prostate, lung, and ovarian cancer. They are a highly effective class of chemotherapeutic agents, prolonging progression free and overall survival when added to the standard of care in different treatment settings. Along with this impressive efficacy, though, taxanes are associated with a spectrum of adverse effects. One of the most common severe toxicities seen with taxane treatment is sensory peripheral neuropathy. Peripheral neuropathy manifests in the hands and feet and can cause irreversible loss of balance and inability to perform tasks that require fine motor skills and dexterity. Thus, neurotoxicity appearance requires discontinuation of taxane treatment to prevent permanent damage, limiting the overall effectiveness of taxane therapy.

In head-to-head studies paclitaxel is somewhat more neurotoxic than docetaxel but it is unclear what mechanism is responsible for the disparity between the closely related agents. There are neither accurate methods to predict which patients are at elevated risk nor effective agents for neuropathy prevention or treatment. Because we lack remedies for neuropathy the ability to

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Daniel L. Hertz, Kouros Owzar, Hitoshi Zembutsu, Chen Jiang, Jai Patel, Dorothy Watson, Mark, L. Ratain, Stefanie D. Krens, Ivo Shterev, Deanna L. Kroetz, Susan Halabi, Michiaki Kubo, William Kevin Kelly, Howard L. McLeod. Genome-wide association study of docetaxel-induced peripheral neuropathy in a hormone refractory prostate cancer clinical trial (CALGB 90401)

identify patient factors that predispose an individual to neuropathy development would be of substantial clinical benefit. These patients could be monitored more closely or offered alternative treatment regimens. Prior work has suggests that increased cumulative drug exposure is one factor that determines neuropathy risk. In addition, some patients are more sensitive to neuropathy development at equivalent exposure, suggesting that there are other unknown factors that modulate a patient's risk of TIPN.

Some predictors of both drug exposure and patient sensitivity have been identified. Drug exposure is determined by the interplay of various factors, including the activity of enzymes and transporters involved in taxane distribution and elimination. Somewhat less is known about the factors that influence a patient's sensitivity to neuropathy, though advanced age, prior neuropathy, and non-Caucasian race have all been suggested to increase risk. In some cases it is unclear whether each factor is acting through a modulation of patient sensitivity or drug exposure or a combination of the two.

One likely source of inter-patient variability in drug exposure and patient sensitivity is germline genetic variation. Germline variation could influence neuropathy risk in a variety of ways including modulated expression or activity of enzymes or transporters or inability to properly respond to neurotoxicity secondary to variation in cellular signaling pathways. Thus, in order to comprehensively study the genetic predictors of neuropathy risk it is essential to utilize a variety of approaches, interrogating both candidate variants and the entire genome. The central aim of this dissertation was to discover and validate

pharmacogenetic predictors of taxane-induced peripheral neuropathy using a variety of genotyping and analysis techniques.

Chapter 2 was an attempt to validate an earlier finding that patients who carry the *CYP2C8*3* variant are at increased risk of paclitaxel induced neuropathy. Because paclitaxel is mainly eliminated through metabolic conversion by CYP2C8, this somewhat common, diminished-activity variant was a high priority candidate and has demonstrated an increase in neuropathy risk in multiple independent patient cohorts. Chapter 3 was an attempt to discover variants that modulate the risk of paclitaxel-induced neuropathy after accounting for the influence of *CYP2C8*3*. Based on the known relationship between drug exposure and neuropathy risk, a genotyping platform that interrogates much of the known variability relevant to Drug Metabolism, Elimination, and Transport (DMET™) was utilized in a hypothesis-driven discovery study. In Chapter 4 the entire genome was interrogated in an unbiased manner to discover genetic loci that modulate a patient's risk of docetaxel-induced neuropathy. The goal of this concluding chapter is to put the results of these three chapters into context and suggest future areas for investigation.

CHAPTER 2

An association between *CYP2C8*3* and increased risk of neuropathy had been previously reported in two paclitaxel studies that investigated multiple variants and phenotypes(131, 132). Within a secondary analysis of a pharmacogenetic study in breast cancer patients treated neoadjuvantly with paclitaxel I identified a trend in the same direction(133)(**Appendix 1**). This was

the prior data on which I based my hypothesis that a larger, racially stratified cohort of paclitaxel treated patients could be used to validate the association between *CYP2C8*3* and increased risk of peripheral neuropathy.

Because the *CYP2C8*3* variant is more common in European-American (CEU AF=0.14) than African-American (ASW AF=0.04) individuals, the study cohort was stratified by self-reported race. In the primary analysis of Caucasian individuals who had not been previously analyzed, the association between *CYP2C8*3* and increased risk of neuropathy was validated (HR (per allele) = 1.93 (95% CI: 1.05- 3.55), overall log-rank *p*=0.006). The association was also replicated in the African-American patients (p=0.043), despite the low allele frequency and absence of any variant homozygous individuals. Having two positive independent replications, all of the paclitaxel treated patients were combined into a single multivariable model that included clinical characteristics that are likely to be relevant to neuropathy development (age, race, diabetes, treatment schedule, neuropathy treatment). After backward elimination the final model demonstrated an additive genetic effect; each *CYP2C8*3* variant carried by a patient approximately doubled their risk of neuropathy (p=0.031). In addition, patients who are non-Caucasian were at higher risk of neuropathy as compared to Caucasians of a similar genotype (HR=1.76, 95%CI: 1.05-2.93, *p*=0.031) a finding that had been recently published by another group(109).

Many candidate gene studies interrogate multiple genotypes and phenotypes and carry out dozens of statistical associations without proper statistical correction, leading to a slew of false positives(130). Clinical validation

of an association requires replication in an independent patient cohort with an *a priori* defined analysis plan and rigorous statistical methodology. The successful replication of the *CYP2C8*3* association in this large patient cohort that analyzed a single genotype-phenotype association is compelling evidence of clinical validity. The next logical step is the translation of this finding to clinical practice.

Clinical translation of this finding could proceed in a variety of directions. One potential area for further research is to look more closely at the other variants within *CYP2C8*, specifically the *2 (rs11572103, I269F, YRI AF=0.20), *4 (rs1058930, I264M, CEU AF=0.07) and Haplotype C variants (rs1113129, CEU AF=0.19, YRI AF=0.50), all three of which have shown decreased metabolic activity(205-207). The genotyping platform that I utilized interrogated the *2 and *4 alleles and in a secondary analysis the association of these variants with increased neuropathy risk was investigated. First, any patient who carried a risk allele was collapsed into a 'low-metabolizers' group and compared with all other patients. The results of this analyses were statistically significant (HR=1.722, 95% CI 1.10-2.70, p=0.018) (**Figure 18**). Next, a likelihood ratio test was used to determine whether this association was driven entirely by the *3 variant. Removing the *3 variant significantly diminished the performance of the test (p=0.037) while removing either of the other SNPs did not (*2 p=0.172, *4 p=0.214) demonstrating that neither SNP individually contributed significant explanatory information to the overall model and the association in the low-metabolizer group was being driven primarily by the *3 variant. Unfortunately, the

DMET™ Plus chip does not interrogate the Haplotype C SNP, which has previously been reported to influence neuropathy risk(132).

Another potential area for follow-up work is the use of modeling and simulation to assist in clinical translation. Using the data from this study it is possible to model the cumulative paclitaxel dose at which patients of each genotype would have equivalent toxicity risk, as has been done previously for other SNPs(143). A similar approach would be to use data from pharmacogenetic-pharmacokinetic modeling to simulate the dose that would normalize drug exposure across patients of different genotypes. Based on data from Bergmann et al. the clearance of free paclitaxel is approximately 10% lower for *CYP2C8*3* heterozygotes as compared to wild-type homozygotes(155). Our results suggest that the effect of the *3 variant mimics an additive genetic model, implying that the decrease in paclitaxel clearance may also be linearly related to the number of variant alleles a patient carries. Interestingly, based on our data the risk of neuropathy is less than 20% in the wild-type patients suggesting that their optimal dose is higher than the doses currently used clinically. According to our neoadjuvant study (**Appendix 1**) this under-dosing is causing suboptimal treatment efficacy. Thus, one could envision a scenario where pre-treatment genotyping could lead to increased paclitaxel dosing in the 74% of Caucasian patients who are *CYP2C8* wild-type homozygotes, improving the efficacy of their treatment at an acceptable toxicity risk. One should be cautioned though that empirical treatment with 100 mg/m^2 weekly, a 25% increase over the standard 80

mg/m^2 regimen, has been reported to have unacceptable rates of peripheral neuropathy development(97).

A slightly more ambitious project would be to incorporate *CYP2C8* genotype data into models that utilize individual patient data, such as weight, age, bilirubin level, and gender, to estimate the dose-exposure relationship. Integrating clinical and genetic factors is an attractive approach to select individualized starting doses for patients who are initiating taxane therapy, and would likely attenuate some of the inter-individual variability in drug exposure seen in the initial cycles of treatment(208).

The association between *CYP2C8*3* and peripheral neuropathy was consistent in direction and magnitude in the non-Caucasian subgroup, supporting the hypothesis that the *3 variant is causative. The low frequency of the variant in non-Caucasians decreases the cost-benefit ratio of pre-treatment pharmacogenetic testing but patients who are known to be *3 carriers, regardless of race, should be considered to be at higher neuropathy risk. More interestingly, this study replicated a previously reported finding that non-Caucasians are at higher neuropathy risk than Caucasians of similar genotype, even after attempted adjustment for diabetes and other risk factors. This suggests that there are other factors at play in the etiology of neuropathy, potentially other genetic factors that are yet to be discovered. The small number of non-Caucasians enrolled in clinical trials, and particularly in pharmacogenetic studies, presents a challenge to the discovery of these loci, however, this limitation may be

surmountable within a planned meta-analysis of three large paclitaxel-induced neuropathy genome-wide association studies.

CHAPTER 3

The aim of this chapter was to discover and validate SNPs in genes relevant to paclitaxel pharmacokinetics that influence neuropathy risk. In order to do so, genotyping was carried out on the Affymetrix DMET™ Plus chip, which interrogates 1,936 variants in genes responsible for drug metabolism, elimination, and transport. This chip directly assays high priority variants in hundreds of genes, including those that code for the transporters (P-gp, MRP2, SLCO1B3) and enzymes (CYP2C8, CYP3A4) known to be involved in paclitaxel distribution and elimination. I hypothesized that taking a comprehensive approach to interrogating these variants would enable me to validate findings from previous candidate-gene studies and/or discover novel variants that influence the risk of paclitaxel-induced neuropathy through modulation of drug pharmacokinetics. Because of the previously demonstrated influence of *CYP2C8*3* on the risk of neuropathy (Chapter 2) the analysis was conditioned on this variant, enabling discovery of loci that explain the residual variability in neuropathy development.

Surprisingly, this study identified a SNP (rs492388) found within a gene that is not relevant to paclitaxel pharmacokinetics. The SNP that surpassed the exploratory significance threshold (p=0.0008) is located in an intronic region of the *ABCG1* gene. ABCG1 is an intracellular transporter that regulates cholesterol homeostasis, particularly in macrophages, but also in peripheral neurons. Within

neurons, cholesterol is metabolized to pregnenolone and progesterone, two molecules that can be converted to androgen and estrogens. Alternatively, pregnenolone and progesterone can be metabolized to “neuroactive steroids,” which are key regulators of Schwann cell function and myelin formation(186, 187). These neuroactive steroids are integral to the neurodegenerative response, providing a plausible mechanism through which these variants could modulate risk of paclitaxel-induced neuropathy.

The relationship between rs492388, or another SNP in high LD with this marker, and paclitaxel-induced neuropathy risk requires replication in an independent patient cohort, similar to that accomplished in Chapter 2 for *CYP2C8*3*. The association of the *ABCG1* SNP and peripheral neuropathy could not be replicated in 124 self-reported non-Caucasian patients from the 9830 cohort (p=0.542). There are several potential reasons that replication in a different racial cohort could be problematic. One possible reason would be differences in minor allele frequency, specifically, low allele frequency in the replication cohort. This does not seem to be the case for rs492388 which has sufficient genetic variability in the 9830 non-Caucasians (MAF=0.40). It is also possible that the association in the Caucasian patients is not directly due to this intronic variant, but is instead caused by another SNP in high LD with rs492388, and that this effect is abrogated in non-Caucasians due to differences in haplotype structure. Using the publicly available FastSNP database the SNPs in LD with our hit were interrogated to search for a likely causative variant, however, no obvious candidates could be identified(209). As discussed earlier,

the pharmacogenetics literature is full of false-positive associations that could not be replicated in additional patient cohort(210) and it may be the case that the relationship between rs492388 and paclitaxel-induced neuropathy is merely a spurious association.

One important distinction between the statistical analyses employed in Chapters 2 and 3 is the use of dose-to-neuropathy (Chapter 2) versus neuropathy occurrence (Chapter 3). Because cumulative dose is a known risk factor for neuropathy development, a dose-to-neuropathy analysis is a superior statistical methodology. This is apparent in the results from Chapter 2 in which the log-rank test (p=0.006) was more sensitive than the Fisher's test (p=0.042). In Chapter 3 the cumulative dose at neuropathy occurrence could not be used as it was extremely sensitive to neuropathies early in therapy in patients carrying rare SNPs. One SNP in particular (rs7770619) had highly significant results using a log-rank test (p=1.25x10^-9) that were driven almost entirely by a single homozygous variant subject who experienced neuropathy early in treatment (**Figure 19**). Reanalysis after exclusion of this one subject yielded a p-value that is consistent with the null hypothesis (p=0.27). Therefore, caution should be used in interpreting results from dose-to-event analyses when small numbers of patients represent entire genotype groups.

This study was unable to validate the influence of any SNP or gene relevant to paclitaxel pharmacokinetics on neuropathy risk. This could be because no other SNPs have an appreciable influence on PK, or it could be a lack of sensitivity of our phenotype (neuropathy) as a surrogate of PK. A more

sensitive approach for follow-up investigation would be to collect rich pharmacokinetic data and directly investigate the influence of genetic and clinical factors on PK variability. Work in this area is currently underway by groups utilizing population and physiologically based pharmacokinetic models(40, 45, 170). Some interesting hypotheses have been proposed based on these tools; for instance, it was recently reported that metabolite levels may be highly sensitive to changes in transport and metabolism(211). It has long been thought that the metabolites of paclitaxel are inactive and unrelated to treatment efficacy and toxicity(30) but it may be that some patients experience supertherapeutic exposure to a paclitaxel metabolite that is neurotoxic. This could explain the unexpected increase in neuropathy risk for paclitaxel treated patients who carry high-activity *CYP3A4/5* variants(132). Perhaps it would be worthwhile revisiting what we think we know about the pharmacology of paclitaxel and its metabolites using established rodent chemotherapy-induced neuropathy models.

CHAPTER 4

No previous study had utilized a genome-wide approach to discover loci that modulate docetaxel-induced peripheral neuropathy, which was the goal of Chapter 4. I then attempted to use a previously reported paclitaxel-induced neuropathy GWAS for replication.

Most PGx studies use a simple case-control approach, where patients who did not experience the event of interest (controls) are compared with patients who did experience the event (cases), as was performed in Chapter 3. For endpoints that are related to time (such as survival) or cumulative treatment

(such as neuropathy) a log-rank analysis with the time or dose-at-event is utilized(132, 143), as was performed in Chapter 2. In the log-rank procedure any patient who did not experience neuropathy is uninformatively censored at their cumulative dose received and classified as a control for analysis(212).

Patients with hormone refractory prostate cancer who are being initiated on docetaxel treatment, such as those patients enrolled on CALGB 90401, have very poor prognosis and many progress or die early in therapy. Using the standard log-rank procedure these patients would be classified as 'controls' despite discontinuation of treatment at cumulative docetaxel doses below those at which neuropathy typically develops. This censoring would lead to inflation in the estimate of neuropathy risk(213). Instead, we utilized the competing-risks technique which censors patients in an informative manner(194). Any patient who discontinued treatment prior to neuropathy was categorized by their reason for discontinuation, enabling us to accurately estimate the risk of neuropathy during docetaxel treatment and use this superior phenotype for genome-wide discovery.

Using the competing-risk model we identified several promising candidate SNPs for docetaxel-induced peripheral neuropathy. The variant with the strongest association with neuropathy (rs11017056) was an intergenic SNP that surpassed genome-wide significance after adjustment for relevant clinical covariates (p=7.2x10^-8). GWAS studies have discovered and validated many intergenic SNPs that have phenotypic consequences. Our current understanding of intergenic regions is extraordinarily limited. Using publicly available data I

determined that our intergenic SNP is in high LD with a SNP that modifies a binding site for the Foxj1 transcription factor, which is necessary for neurogenesis and neurodevelopment. Additional research that leads to improved understanding of the mechanisms by which variation in intergenic regions influences biology, such as the work done within the encode project(114), is of paramount importance in the interpretation of GWAS findings.

The SNPs with the 2nd and 3rd strongest association with docetaxel-induced neuropathy are both located within genes that are indirectly related to Charcot-Marie-Tooth disease. The first SNP is found in *VAC14* which forms a complex with FIG4, the gene responsible for CMT type4J(202). The other SNP is found in the gene encoding FGD3, a paralog of the FGD4 protein which is causative of CMT type4H(214). In retrospect it makes sense that we would discover SNPs located within genes relevant to a familial neuropathy condition, however, the complexity of human biology and our naïve understanding of pharmacology precludes successful *a priori* selection of genes and variants for association testing. These findings support continued use of genome-wide discovery methods, in parallel with candidate-gene validation, in PGx research. They also highlight the need for continued basic science research to characterize the biological functions of all human genes and the further elucidation of gene pathways(175) to enable understanding of the mechanism by which different gene variants are having similar biological consequences. This too will assist with the interpretation of genome-wide discovery studies that seek to understand genetic drivers of drug response.

I then attempted to replicate these priority SNPs in the paclitaxel-treated patients from the CALGB 40101 study, with the assumption that the genetic factors of neuropathy risk would be similar between the two taxanes. Interestingly, the top hit from the discovery study showed an association in the reverse direction in the replication cohort (p=0.010). This is most likely evidence that the original finding was a spurious association, but there remains a possibility that genetic variants that influence docetaxel- and paclitaxel-induced peripheral neuropathy are distinct. This hypothesis is supported by the lack of association in our study for the variant discovered and replicated in the CALGB 40101 PGx analysis (rs10771973, p=0.39).

None of our priority SNPs were successfully replicated in the paclitaxel-treated cohort. Until replication is attempted in additional cohorts of docetaxel treated patients it is not possible to truly determine whether these original findings were spurious or these loci are specific to docetaxel-induced neuropathy. Because peripheral neuropathy is not the dose-limiting toxicity of docetaxel, little work has been done on elucidating the clinical and genetic risk factors of docetaxel-induced peripheral neuropathy. Large populations of docetaxel-treated patients will need to be retrospectively analyzed to determine what degree of overlap exists between these risk factors.

Successful replication of the loci discovered in CALGB 90401, or those discovered in other docetaxel-induced neuropathy GWAS, should motivate additional research into the translation of these findings. Forward translation, into clinical practice, would entail pretreatment genotyping to identify patients at

increased risk of neuropathy who are candidates for modified dosing or alternative treatment regimens. However, based on the findings of this study it is unlikely that any single SNP is influential enough to be used clinically for this purpose. It is more likely that validated loci will be useful in exploring the pharmacology of docetaxel and the biological mechanisms underlying TIPN. Improved understanding of neurotoxicity etiology may help identify attractive drug targets to guide the design of therapeutics that effectively prevent or reverse taxane-induced neuropathy development.

CONCLUDING REMARKS

The overall goal of this dissertation was to discover and validate germline genetic loci that modulate risk of taxane-induced neuropathy. This was accomplished in a variety of patient cohorts using different analysis and genotyping techniques selected based on the particular hypothesis and situation. I first replicated in multiple independent patient cohorts an increase in neuropathy risk for the low-activity *CYP2C8*3* variant. Clinical translation of this association, possibly through characterizing the relationships between *CYP2C8* genotype, paclitaxel exposure and neuropathy development, could ultimately enable clinicians to individualize paclitaxel treatment to optimize therapeutic outcomes. I then used a genotyping panel that interrogates variants in genes relevant to drug pharmacokinetics to explain the residual variability in neuropathy risk in this patient cohort. The intent was to discover or validate other SNPs which, through a modulation of paclitaxel pharmacokinetics, influence neuropathy risk. The SNP that I found instead is located in a gene (*ABCG1*) relevant to the regulation of

neuroactive steroids. Finally, I used a genome-wide discovery approach in a cohort of docetaxel treated patients, leading to the discovery of a single intergenic SNP that may increase neuropathy risk in docetaxel-treated patients. Successful replication of the influence of either of these SNPs on taxane-induced neuropathy could enable pretreatment identification of patients at high risk of neuropathy, expand our understanding of the underlying mechanism of TIPN, and/or suggest novel targets for therapeutic strategies that prevent or treat neuropathy. Any of these outcomes would major breakthroughs in cancer treatment.

FIGURE LEGENDS

Figure 18 Incidence of grade 2+ neuropathy by metabolizer status collapsing all patients carrying a CYP2C8*2, *3 or *4 allele into a low-metabolizer phenotype for the 9830 cohort (n=412).

Figure 19 Incidence curve of dose-to-grade 2+ neuropathy for rs7770619 (PPARD, p=1.25x10^-9). Without the single homozygous variant subject the p-value of association is 0.27, demonstrating the sensitivity of this analysis method.

FIGURES

Figure 18 Incidence of Grade 2+ Neuropathy by Metabolizer Status in LCCC 9830

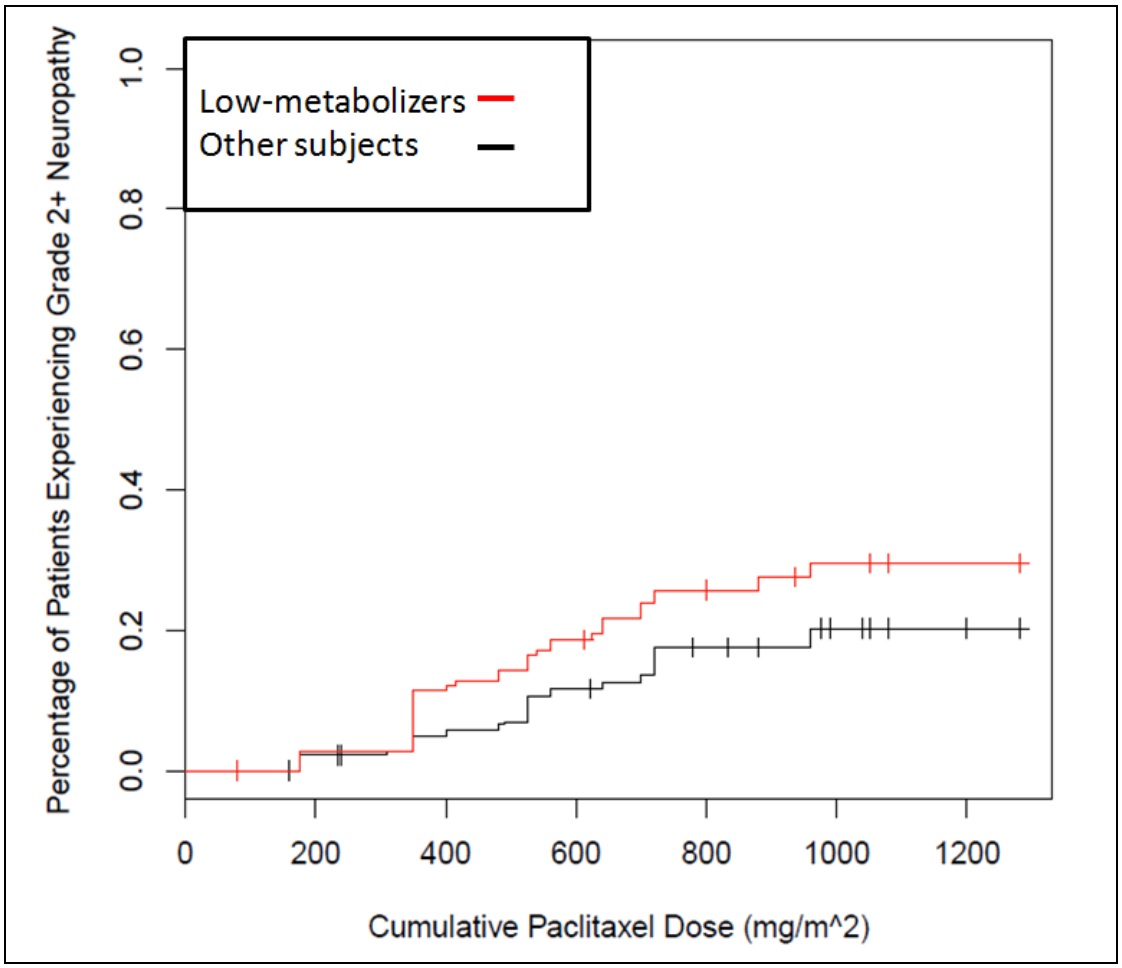
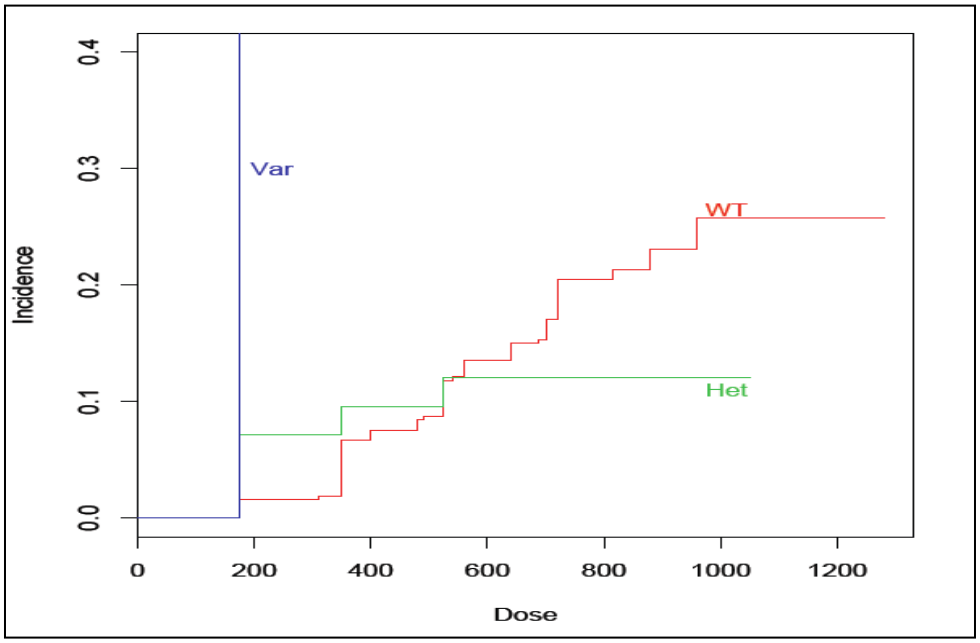


Figure 19 Incidence of Dose-at-Grade 2+ Neuropathy for rs7770619



APPENDIX 1

*CYP2C8*3* PREDICTS BENEFIT/RISK PROFILE IN BREAST CANCER PATIENTS RECEIVING NEOADJUVANT PACLITAXEL

INTRODUCTION

Interpatient variability in toxicity and response are important problems in the use of cancer chemotherapy. For example, paclitaxel, one of the most commonly used therapies for breast cancer and other cancers, has interpatient variability of 19-26% in (unbound) drug clearance(45), causes grade 3 or higher neuropathy and neutropenia in 5-8% and 2-4% of patients, respectively(148) and a response rate as first line, single agent treatment in metastatic breast cancer of 20-30%(215). Studying host factors responsible for the variability in chemotherapeutic outcomes and developing strategies to individualize therapy in order to maximize response and minimize toxicity is an active area of research. Pharmacogenomics, study of the interplay of genetics and drug therapy outcomes, is one promising approach to achieving individualized therapy(110). Genetic variation can influence therapy by a number of mechanisms. Variants in genes relevant to drug disposition or metabolism can modulate the patient's exposure to the drug, whereas variation in genes that are involved in drug action can influence the patient's sensitivity. For example, germline genetic polymorphisms have been discovered which increase the likelihood of a patient

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experiencing severe toxicity to irinotecan(216, 217) or modulate the optimal dose of a patient's warfarin therapy(122).

A number of putative pharmacogenetic markers for paclitaxel outcomes, in breast cancer and in other solid tumors, have been evaluated(108, 132, 135, 139, 143, 172-174, 190, 218, 219). Most of these studies focused on known mutations in biologically relevant candidate genes such as *CYP2C8*, *CYP3A4*, and *ABCB1*, which code for the enzymes involved in paclitaxel metabolism and the transporters that influence paclitaxel disposition. More recent genome-wide association studies used an unbiased approach to examine the entire genome to address this question, and have reported intriguing candidate SNPs in genes not previously investigated(108, 143, 190). The clinical phenotypes most often studied were severe toxicities, such as neuropathy, or measures of survival. Some of these retrospective pharmacogenetic studies suggest that genetic variability may be associated with clinical outcome to paclitaxel therapy, and others do not. However, the toxicity endpoints are often confounded by prior or combination therapy, while the survival endpoint is confounded by a multitude of factors including ER status or tumor subtype(220) and stage at diagnosis(221). To our knowledge, no published pharmacogenomic study has exclusively utilized a neoadjuvantly treated population, in which toxicity and tumor response to paclitaxel therapy can be assessed in the absence of these confounding factors.

In the current study, we genotyped a cohort of patients treated with neoadjuvant paclitaxel for polymorphisms that have previously demonstrated significant associations with efficacy or toxicity. These subjects are uniquely

informative since tumor response and toxicity data were collected exclusively for the taxane treatment phase. We hypothesized that polymorphisms in genes relevant to paclitaxel metabolism (*CYP2C8* & *CYP3A4*/*3A5*), transport (*ABCB1*), or mechanism (*CYP1B1*) would influence the likelihood that a patient would respond to, or experience severe toxicity from, paclitaxel therapy.

MATERIALS AND METHODS

Patients and Treatments

Relevant candidate SNPs were evaluated in a cohort of patients treated between 2005 and 2009 and derived from the University of North Carolina Lineberger Comprehensive Cancer Center (UNC LCCC) Breast Cancer Neoadjuvant Database, which includes prospective annotation of clinical data, treatment details, toxicity and outcome. Eligible women received neoadjuvant paclitaxel-containing regimens and enrolled in both the UNC neoadjuvant database and a concurrent IRB approved clinical trial that collected genomic DNA from all newly diagnosed patients. All patients received paclitaxel (T) treatment guided by standard neoadjuvant protocols which had a defined and conventional treatment dose, schedule, and duration. In most cases patients received neoadjuvant doxorubicin and cyclophosphamide (AC) either before or after the paclitaxel; however, the tumors were measured before and after each phase of therapy so that clinical response to the anthracycline component and the taxane component could be identified separately. Some patients with HER2 over-expressing tumors received trastuzumab concurrent with the paclitaxel. Tumor size was measured clinically by the patient's medical oncologist and

percent change in tumor size was calculated from these measurements. Clinical response was defined as: complete response (cCR; 100% reduction in tumor size), partial response (cPR; 30%-99% reduction), stable disease (cSD; 29% reduction-20% enlargement of tumor) or progressive disease (cPD; >20% enlargement) according to RECIST criteria (222). Patients who achieved complete response to AC before the start of taxane therapy were excluded from the efficacy analysis because they were not evaluable for response to taxane treatment. Pathological response, which could not be evaluated between treatment regimens, was not used as an endpoint in this study due to the inability to separate the confounding effects of other chemotherapy treatments. Toxicities were evaluated during paclitaxel treatment, recorded prospectively, and coded by NCI CTC AE V4.0 based on the physician's description(149). Patients who were treated at outside institutions did not have toxicity data and were excluded from that part of the analysis. All patients signed informed consent to participate and agreed to allow DNA to be collected for additional pharmacogenetic studies. The study protocol was approved by the UNC Institutional Review Board.

SNP Genotyping

A 30 mL blood sample was collected from each subject at the time of study enrollment. DNA used for genotyping was extracted by the UNC Biospecimen Processing Facility and plated at 5 ng/uL. Target gene region amplification was carried out by PCR in a 20 μL reaction including 2 μL genomic DNA and polymorphisms were genotyped on a Pyromark Pyrosequencer Q96 MD as previously described (223, 224). The polymorphisms genotyped were as

follows: *CYP1B1*3* (rs1056836, 4326C>G), *CYP2C8*3* (rs11572080, 416G>A and rs10509681, 1196A>G), *CYP3A4*1B* (rs2740574, -392A>G), *CYP3A5*3C* (rs776746, 6986A>G), and *ABCB1*2* (rs1045642, 3435C>T, rs2032582, 2677G>T/A, and rs1128503, 1236C>T). Genotyping was carried out blinded to clinical data with negative controls included in each run and at least 5% of samples were repeated for quality control to ensure accuracy of assay results. Any assay with call rate or concordance with repeated samples <95% was excluded from analysis.

*CYP3A4*1B/CYP3A5*3C* and *ABCB1* 1236C>T, 2677G>T/A, and 3435C>T were included in haplotype analyses. Haplotypes were inferred using PHASE Version 2(225, 226) for polymorphisms with LD>0.7. Any subject with missing genotype information at any locus of the haplotype was considered to have an unknown haplotype and excluded from analysis. *CYP3A4/3A5* haplotypes were grouped according to Baker et al. (*1: *CYP3A4**1A/*CYP3A5**3C, *2: *CYP3A4**1B/*CYP3A5**1A, *3: *CYP3A4**1A/*CYP3A5**1A, *4: *CYP3A4**1B/*CYP3A5**3C)(19). After *ABCB1* haplotype inference (Wild-type: C-G-C, Variant: T-T(A)-T, Mixed: other) each patient was assigned a diplotype number (1-5) in order of increasing genetic variation as described in Sissung et al.(140).

Statistical Analysis

Genotype calls were assessed for concordance with Hardy-Weinberg Equilibrium (HWE) using a Pearson chi-square test with D.f.=1. Assays with HWE p-value <0.05 in the cohort were then tested in the Caucasian subcohort as

population admixture violates key assumptions of Hardy-Weinberg Equilibrium(157). Each genotype or haplotype was individually tested for an association with efficacy or toxicity using logistic regression modeling. In the haplotype analysis, the “variant” group was defined by grouping diplotypes. For *CYP3A4/3A5* any individual carrying the *2 haplotype (CYP3A4*1B/CYP3A5*1A) was considered a variant carrier. For *ABCB1* any individual with diplotype 4 or 5 was considered a variant carrier and was compared to non-carrier diplotypes 1-3. For the genotype analyses, variant carriers were compared to homozygotic wild-type individuals (dominant genetic model). The primary efficacy endpoint was clinical complete response (cCR) to taxane therapy (Yes vs. No). The primary toxicity endpoint was any grade 3 or higher adverse event during taxane therapy (Yes vs. No). Secondary endpoints of efficacy and toxicity were clinical response rate (cRR; cCR+cPR=cRR) and grade 3 or higher neuropathy during paclitaxel therapy, respectively. Following univariate testing, additional covariates for efficacy (estrogen receptor (ER) status, tumor grade, concurrent trastuzumab treatment, whether paclitaxel treatment was preceded by other chemotherapy phase) were included in a multivariable model to adjust for their prognostic importance. Backward selection was used to eliminate covariates that did not significantly contribute to the model using AIC as a selection criterion. Self-reported race was used as stratification factor for significant associations to account for racial heterogeneity in the cohort. In order to correct the primary efficacy analysis for multiple comparisons the p-values were multiplied by 7, the number of independent statistical associations performed, (a Bonferroni

correction for multiple comparisons) so that the p-value to be compared to the standard significance threshold of α=0.05 is valid. P-values of all secondary and sub-analyses are uncorrected as these are exploratory in nature and should be interpreted as such. All statistical analyses were performed in R Statistical Software, version 2.13.0 (R Development Core Team, Vienna, Austria).

RESULTS

Patient Population

111 patients treated neoadjuvantly with paclitaxel-containing regimens were eligible for analysis. After excluding subjects missing efficacy or toxicity data, 103 subjects were included in the efficacy and 109 subjects in the toxicity analysis. Demographic data including patient, treatment, and tumor characteristics for the whole population are presented in **Table 10**.

Allele Frequencies

The two highly linked SNPs in *CYP2C8**3 (rs11572080, 416G>A and rs10509681, 1196A>G) were completely concordant in this population. *CYP3A4* and *CYP3A5* were out of Hardy Weinberg Equilibrium prior to accounting for race, as expected given the large difference in allele frequencies among Caucasian and African-American individuals(227), but no significant deviations were seen in stratified samples. Allele frequencies in Caucasian subjects for all variants were consistent with those reported in The International HapMap Project(159) or the NCBI EntrezSNP database(228) and replicated samples were 100% concordant with the original genotype calls, thus no assays were excluded from analysis (**Table 11**). As expected, significant LD was seen between

*CYP3A4**1B and *CYP3A5**3C (r=0.93) and the three polymorphisms in *ABCB1* (r>0.7), which were then grouped into haplotypes as planned (**Table 12**).

Response by Genotype

Clinical complete response to paclitaxel for the efficacy cohort was 30.1% and the mean change in tumor size was a 49% reduction (**Table 13**). Response by genotype is presented in **Table 14**, demonstrating significance only for *CYP2C8*; the odds ratio for an individual carrying *CYP2C8**3 to achieve clinical complete response was 3.92 with a 95% confidence interval of 1.46-10.48 (corrected p=0.046) (**Figure 20**). Of the 22 subjects carrying the *CYP2C8**3 variant, 12 achieved clinical complete response (55%) as compared to only 19 out of the 81 wild-type subjects (23%). In order to ascertain whether this association was independent of other prognostic factors a multivariable model that included tumor grade, ER status, concomitant trastuzumab, and whether paclitaxel was preceded by another phase of chemotherapy was tested. After backward elimination of covariates that were not significant, the final model included tumor grade and whether paclitaxel was preceded by another phase of chemotherapy. After controlling for these prognostic factors the association of *CYP2C8**3 status remained significant in the final model (uncorrected p=0.003, corrected p=0.022) while the other covariates were not significantly associated with achievement of clinical complete response (**Table 15**). Next, the association of *CYP2C8**3 and clinical complete response was stratified by race to ensure that racial heterogeneity was not falsely inflating our results. In the self-reported Caucasian subjects the magnitude of effect was marginally greater and the

significance similar to that seen in the entire efficacy cohort (OR=5.31, 95% CI: 1.59-17.67, corrected p=0.049). Only two non-Caucasians carried the *3 variant in our cohort so the association in non-Caucasians was not analyzed.

To evaluate the robustness of our finding, a secondary efficacy analysis was carried out with clinical response rate (cRR=cCR+cPR). The clinical response rate in the cohort was 63% (65/103). In the *CYP2C8**3 carriers the response rate was 82% (18/22) vs. 58% (47/81) in the *CYP2C8* wild-type subjects. In the univariate logistic regression model this association showed a strong trend in the same direction with an odds ratio of 3.16 (95% CI: 0.98-10.19, uncorrected p=0.054), supporting our primary findings.

In order to examine whether, as hypothesized, this finding was specific to paclitaxel, we then tested the association between *CYP2C8**3 status and clinical complete response to the doxorubicin-cyclophosphamide (AC) phase of therapy in patients who received the combination. Out of 100 subjects who received the AC combination who had evaluable response, the rate of clinical complete response was not significantly different between *3 carriers (2/22=9.0%) and wild-type homozygotes (8/78=10.3%) (OR=1.14, 95% CI: 0.62-15.96, uncorrected p=0.872).

Toxicity by Genotype

Of the 109 subjects included in the toxicity analysis, 34 experienced at least one grade 3 or higher toxicity (31.2%) (**Table 16**), however, none of the genetic markers were associated with this cumulative endpoint (data not shown). Analysis of the secondary toxicity endpoint, grade 3 or higher peripheral

neuropathy, revealed a trend toward increased neuropathy in subjects carrying the *CYP2C8**3 variant (5/23=22%) vs. wild-type individuals (7/86=8%) (OR=3.13, 95% CI: 0.89-11.01, p=0.075) (**Figure 21, Table 17**).

DISCUSSION

This study investigated pharmacogenetic predictors of breast cancer treatment outcomes following neoadjuvant paclitaxel. By measuring the tumor before and after each phase of sequential chemotherapy, and collecting toxicity during each phase separately, we were able to isolate the taxane-specific outcomes from the sequential therapy. We employed a candidate polymorphism replicate strategy based on reported associations with clinical outcomes in previous pharmacogenetic studies in taxane treated cancer patients. These candidate genes covered the major metabolic pathways of paclitaxel, *CYP3A4/3A5* and *CYP2C8*, the efflux transporter *ABCB1*, and *CYP1B1* which has been shown to influence taxane treatment efficacy.

Our results indicate that patients carrying the *CYP2C8*3* polymorphism are more likely to achieve clinical complete response than patients homozygous for the wild-type isozyme. This finding is supported by the strong trend in the same direction for clinical response rate. The association with tumor response remained significant after adjustment for covariates and stratification by self-reported race. There was no association between *CYP2C8* genotype and clinical complete response to the AC phase of sequential therapy, dismissing the possibility that patients carrying *CYP2C8*3* had more chemosensitive tumors. Thus, there seems to be a true pharmacogenetic association between the

*CYP2C8*3* polymorphism and clinical response to neoadjuvant paclitaxel therapy.

Clinical response, instead of the more accepted pathological response, which has prognostic implications for future survival(229-231), was selected due to the collection of tumor size data between phases of sequential therapy, which enables us to isolate the response to the paclitaxel phase of treatment from that of the other administered therapy. Although clinical measurement is not a component of RECIST classification, that methodology was designed with radiographic measurements in the metastatic setting in mind. Conversely, pathologic response, a more conventional endpoint for neoadjuvant studies, cannot differentiate among drugs given preoperatively so would have introduced considerable noise from the inclusion of anthracycline and antimetabolite effect in the efficacy estimates. Moreover, the ability to use response to the AC component of therapy as an internal control for the specificity of the findings for paclitaxel would have been lost. There is a documented relationship between clinical measurement and pathologic response(232) supporting the use of easily obtained serial clinical measurements in the palpable lesions relevant in this setting. The reported relationship between clinical and pathological response indicates that our finding may have an important influence on survival; however, it is essential that these findings are confirmed with pathological or radiographic tumor measurements before and after taxane therapy in independent patient cohorts.

Two previous groups have reported that patients carrying *CYP2C8*3* are at a higher risk of paclitaxel-induced peripheral neuropathy(131, 132), but this finding was not observed in other studies(134, 135, 137). All of these studies primarily included patients who were on combination therapy or who had been previously treated with chemotherapy. Our results, in previously untreated patients not receiving combination therapy with other neurotoxins, are consistent with those of Leskela et al. and Gréen et al. and suggest that there may be a true association between *CYP2C8*3* and risk of peripheral neuropathy. The difference between our study and that of Leskela et al. is the event rate. Their study included grade 2+ neurotoxicity, a substantially more common phenotype than our grade 3+ endpoint, but one that does not require a change in therapy, unlike higher grades of neuropathy as were measured in this study. Additionally, they used a cumulative dose-to-event analysis, which is consistent with the cumulative nature of neurotoxicity. This was not feasible in our study given the relatively small number of patients (12) who experienced grade 3 or higher neuropathy. In fact, it is important to note that a general limitation of the current study is the modest sample size.

The paclitaxel parent compound, not its metabolites, is thought to be responsible for the drug's efficacy and toxicity(30). Paclitaxel clinical outcomes are related to the amount of time the total drug concentration remains above a threshold level(95, 233) and the cumulative exposure may determine the extent of neuropathy development(91, 172). The *CYP2C8*3* variant has diminished *in vitro* metabolic activity for paclitaxel(153, 205, 234, 235) and carriers of this

variant have decreased clearance of free paclitaxel, and a corresponding increase in drug exposure(155). These findings provide a rational mechanism for the increased paclitaxel treatment response and toxicity seen in *CYP2C8*3* carriers in this study.

It is not possible to distinguish the influence of each of the two non-synonymous polymorphisms in *CYP2C8*3* in this population due to complete concordance, and it will be difficult to do this in any clinical study based on their high linkage disequilibrium. However, *in vitro* data suggests that the causative SNP is the K399R variant (rs10509681) which, unlike the R139K variant, has diminished paclitaxel metabolic activity when each is tested in isolation(153, 154). If this were true, then it would be important for future researchers to focus their analyses specifically on the K399R variant which is sometimes present in patients without the R139K variant(152).

These patients were treated according to standard neoadjuvant protocols, which specify an appropriate treatment dose, schedule, and duration. Recent data demonstrates that the 3-weekly regimen received by 20% of these patients is inferior to the weekly or every-2 week regimen(97). In follow-up analyses, multivariable models that included treatment schedule were analyzed to see if schedule had a significant influence on the achievement of clinical complete response, which it did not (uncorrected p=0.100, data not shown). The pre-defined treatment duration also ensures that the assessment of response and neuropathy are not confounded by dramatic differences in cumulative paclitaxel received. Only 12 patients discontinued paclitaxel before receiving the full course

of therapy, 11 for toxicity and 1 due to disease progression during treatment. Indeed, carriers of *the CYP2C8*3* variant received a similar number of cycles (median=4) and weeks of therapy (mean= 9.99 vs. 9.60) compared with wild-type patients so it is unlikely that differences in response or neurotoxicity are attributable to differences in cumulative paclitaxel administered.

Numerous groups have investigated polymorphisms in other genes relevant to paclitaxel exposure or mechanism. The polymorphisms in *CYP3A4* and *CYP3A5* have been interrogated independently and typically do not show associations with outcome(134, 219, 236), though associations have been reported(175). However, recent data suggests that looking at the *CYP3A4*1B/3A5*1A* variants as a high metabolic activity haplotype may be a superior strategy(19). We were unable to identify a statistically significant association with paclitaxel treatment outcomes for either the *CYP3A4*1B* variant alone or the two variants in combination.

The *ABCB1* variants have also been the focus of a number of retrospective pharmacogenetic studies, with inconsistent results. Variants at the 3435 position have been associated with shorter overall survival and worse progression free survival in paclitaxel treated cancer patients(173, 174). Variants at the 3435 and 2677 position have been implicated in higher risk of paclitaxel-induced neutropenia(139) and docetaxel treatment outcomes(140). In our study the variants of *ABCB1* tested individually or in haplotypes did not have a statistically significant effect on paclitaxel treatment outcome.

CYP1B1 is not involved in taxane metabolism(237), yet an association between taxane efficacy and the *CYP1B1*3* variant has been demonstrated repeatedly(219, 238-240). *In vitro* studies by Sissung et al. reveal that *CYP1B1*3* enhances estrogen metabolism to compounds that antagonize the mechanism of action of docetaxel and paclitaxel, and covalently bind docetaxel, providing two plausible mechanisms for the decreased efficacy seen in patients with this genotype. We found no evidence of a link between *CYP1B1* genotype and paclitaxel efficacy.

In conclusion, we report evidence that *CYP2C8*3* carriers are more likely to achieve complete clinical response to neoadjuvant paclitaxel. This association was independent of other important clinical covariates. The odds of an individual who carried the *3 variant achieving clinical complete response were nearly 4 times higher than those for an individual carrying two wild-type alleles. Additionally, our results support the previously reported possibility that individuals carrying this variant are at increased risk of experiencing paclitaxel related neuropathy. Our data suggests a potential biomarker for identifying patients before treatment who are more likely to benefit from therapy, but may be at an increased risk of experiencing certain adverse events. The results of this small study warrant further investigation of this association in larger neoadjuvantly treated patient cohorts, and if confirmed may prompt studies of dose individualization based on host genotype.

TABLES

Table 10 Patient Characteristics of LCCC9830 Neoadjuvant Cohort

Self-reported Race	Caucasian	79 (71%)
	African-American	27 (24%)
	Other	5 (5%)
Age (Years)	Median	50 (11.2)
	Range	27-78
Menopausal Status	Pre-menopausal	57 (51%)
	Post-menopausal	54 (49%)
Grade at Diagnosis	1	7 (6%)
	2	29 (26%)
	3	60 (54%)
	Unknown	15 (14%)
Receptor Status	ER+	56 (50%)
	ER or PR+	63 (57%)
	HER2+	31 (28%)
Stage at Diagnosis	IIA-IIB	42 (38%)
	IIIA-IIIC	59 (53%)
	IV	10 (9%)
Taxane Regimen[a]	T	16(14%)
	TC	2 (2%)
	TCH	1 (1%)
	TH	1 (1%)
	AC-T	73 (66%)
	AC-TH	18(16%)
Taxane Schedule and Dose	Weekly (80-90mg/m^2)	36 (32%)
	Q2 Weeks (175mg/m^2)	52 (47%)
	Q3 Weeks (175mg/m^2)	22 (20%)
	Q2.5 Weeks (175mg/m^2)	1 (1%)
Early Paclitaxel Discontinuation	Toxicity	11 (10%)
	Disease Progression	1 (1%)
Total Weeks of Taxane	Median	9.0 (2.9)
	Range	1-23

Abbreviations: A, Doxorubicin (Adriamycin); C, Cyclophosphamide (Cytoxan); T, Paclitaxel (Taxol); H, Trastuzumab (Herceptin)

[a]: Regimen includes all drugs taken before or during paclitaxel treatment

'-' indicates these are sequential treatments

Table 11 LCCC 9830 Neoadjuvant PGx Study Genotyping Results

Gene Variant	Amino Acid or Base Change	Call Rate	Variant Allele Frequency	Hardy-Weinberg Equilibrium P-value	Variant AF in Caucasians	Expected AF in Caucasians[a]
CYP1B1*3		99.1%	0.53	0.136	0.45	0.45
CYP2C8*3	R139K	100%	0.11	0.574	0.14	0.13
	K399R	100%	0.11	0.574	0.14	0.14
CYP3A4*1B		100%	0.17	<0.001	0.04	0.03[b]
CYP3A5*3C		99.1%	0.80	<0.001	0.94	0.96
ABCB1*2	1236C>T	100%	0.38	0.913	0.43	0.45
	2677G>T/A	99.1%	0.34	0.140	0.44	0.47
	3435C>T	98.2%	0.42	0.533	0.51	0.57

[a]Reported from International HapMap Project for Caucasian (CEU) population
[b]Estimated from Caucasian/CEPH populations in dbSNP

Table 12: Haplotype Grouping for CYP3A4/3A5 and ABCB1

Gene	Diplotype	Number
CYP3A4/3A5	*1/*1	76
	*1/*3	5
	*1/*4	3
	*3/*3	1
	*1/*2	11
	*2/*2	8
	*2/*3	5
	*2/*4	1
	Unknown	1
	***2 Carrier**	**25**
	***2 Non-Carrier**	**85**
ABCB1	1	30
	2	21
	3	31
	4	13
	5	11
	6	1
	7	1
	8	0
	9	0
	10	0
	Unknown	3
	1-3	**82**
	4-5	**24**
	Excluded	5

Complete breakdown of number of individuals who fall into each haplotype category as defined for CYP3A4/3A5 in Baker et al. (19) and ABCB1 in Sissung et al. (140) followed by the grouping for the association study. (CYP3A4/3A5 *2 carriers vs. other and ABCB1 Diplotype 1-3 vs. 4&5).

Table 13 Response to Paclitaxel Therapy in LCCC 9830 Neoadjuvant PGx Efficacy Cohort

% Change in Tumor Size	Mean	-49%
	Median	-43%
	Maximum Decrease	100%
	Maximum Increase	29%
Clinical Response	Clinical Complete Response	31 (30.1%)
	Clinical Partial Response	34 (33.0%)
	Clinical Stable Disease	36 (35.0%)
	Clinical Progressive Disease	2 (1.9%)
	Excluded (Unevaluable)	8

Table 14 Association of Genotype and Haplotype with Clinical Complete Response in LCCC 9830 Efficacy Cohort

Gene Variant	Odds Ratio (95% CI)	Uncorrected p-value	Bonferroni Corrected p-value
CYP1B1*3	0.53 (0.21-1.33)	0.1761	1.0000
CYP2C8*3	3.92 (1.46-10.48)	0.0066	0.0459
CYP3A4*1B	0.88 (0.32-2.37)	0.7929	1.0000
CYP3A4/3A5 Haplotype	1.16 (0.42-3.21)	0.7798	1.0000
ABCB1 3435	1.23 (0.50-3.01)	0.6548	1.0000
ABCB1 2677	1.98 (0.83-4.73)	0.1245	0.8716
ABCB1 Haplotype	0.49 (0.15-1.61)	0.2389	1.0000

Table 15 Final Multivariable Model of Clinical Complete Response in LCCC 9830 Neoadjuvant PGx Efficacy Cohort

	Odds Ratio	95% Confidence Interval	Uncorrected P-value
CYP2C8	5.11	1.73-15.12	0.003
Tumor grade	1.08	0.50-2.35	0.840
Preceded by another phase of chemotherapy	0.59	0.19-1.79	0.350

Table 16 Grade 3+ Toxicities During Paclitaxel Treatment in LCCC 9830 Toxicity Cohort

Any Toxicity	Any	34 (31%)
	None	75 (69%)
	Excluded	2
Specific Toxicities	Neuropathy	12 (11%)
	Neutropenia	10 (9%)
	Myalgia	9 (8%)
	Hypersensitivity	7 (6%)
	Fatigue	4 (4%)
	Gastrointestinal	3 (3%)
	Anemia	2 (2%)
	Other	4 (4%)

Table 17 Association of Genotype and Haplotype with Grade 3+ Neuropathy in LCCC 9830 Neoadjuvant PGx Toxicity Cohort

Gene	Odds Ratio (95% Confidence Interval)	Variant Carrier vs. Wild-type uncorrected p-value
CYP1B1*3	0.76 (0.18-2.41)	0.537
CYP2C8*3	3.13 (0.89-11.01)	0.075
CYP3A4*1B	1.01 (0.25-4.05)	0.984
CYP3A4/3A5 Haplotype	1.19 (0.30-4.80)	0.806
ABCB1 3435	6.71 (0.83-54.19)	0.074
ABCB1 2677	2.41 (0.60-9.61)	0.214
ABCB1 Haplotype	0.76 (0.15-3.80)	0.740

FIGURE LEGENDS

Figure 20 Percentage of patients carrying *CYP2C8*3* vs. *CYP2C8*1* wild-type homozygotes achieving clinical complete response. Patients carrying *CYP2C8*3* were more likely to achieve clinical complete response (OR=3.92, 95% CI: 1.46-10.48, corrected p=0.046).

Figure 21 Percentage of patients carrying *CYP2C8*3* vs. *CYP2C8*1* wild-type homozygotes experiencing severe peripheral neuropathy. There was a trend toward greater risk of severe neuropathy in patients carrying the *3 variant, though it did not achieve statistical significance (OR=3.13, 95% CI: 0.89-11.01, uncorrected p=0.075)

FIGURES

Figure 20 Clinical Complete Response by CYP2C8 Genotype in LCCC 9830 Neoadjuvant PGx Study Cohort

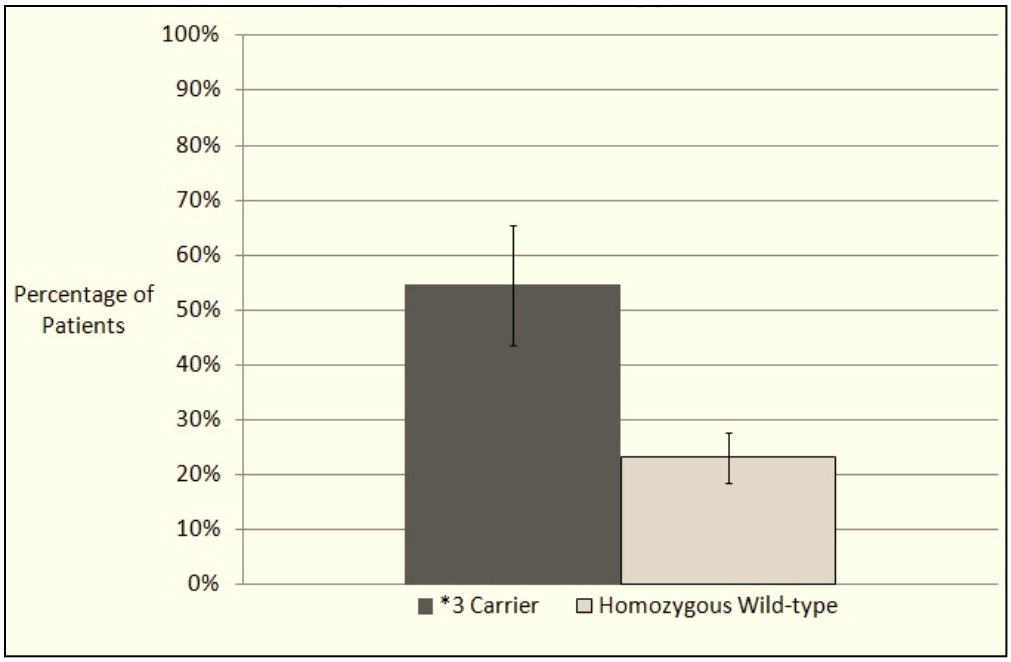
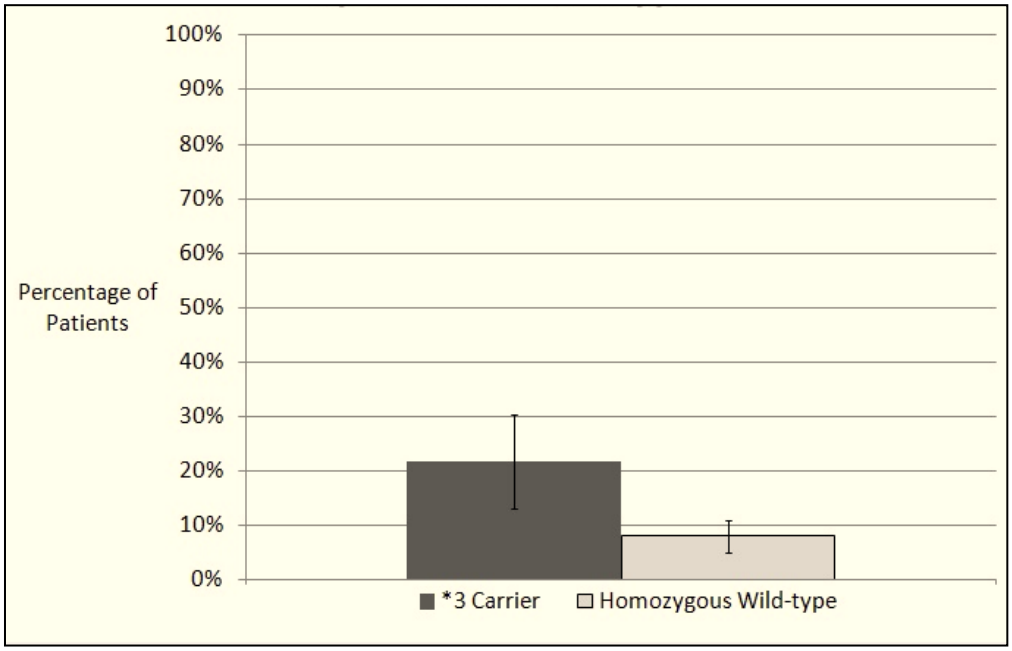


Figure 21 Grade 3+ Neuropathy by CYP2C8 Genotype in LCCC 9830 Neoadjuvant PGx Cohort



APPENDIX 2

PILOT STUDY OF ROSIGLITAZONE AS AN IN VIVO PROBE OF PACLITAXEL EXPOSURE

INTRODUCTION

Interpatient variability in toxic and therapeutic response to chemotherapy, partially caused by differences in the activity of the CYP450 enzyme system, is a substantial problem in cancer treatment. One emerging approach to characterize a drug's metabolism is through the use of a probe; a marker agent that shares the drug's metabolic pathway. Probe-based tests to measure CYP450 phenotype that are safe, easy to administer, and quickly interpretable have been developed for some enzymes(241) relevant to anti-cancer therapy but not all.

Paclitaxel is one of the most effective chemotherapeutic agents used in the treatment of solid tumor malignancies. It is metabolized to inactive metabolites primarily by CYP2C8 with a contribution from CYP3A4(30). A probe assay that could explain the significant interpatient variability in paclitaxel exposure might help clinicians choose a more appropriate dosage for an individual patient to optimize efficacy and limit toxicity.

The erythromycin breath test (ERMBT) has been widely implemented to measure hepatic CYP3A4 activity(242), however, there is no such probe for CYP2C8. Interestingly, the rates of rosiglitazone metabolism and paclitaxel hydroxylation have been shown to correlate in human liver microsomes

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expressing CYP2C8(243). In healthy subjects, a 2 mg oral dose of rosiglitazone is safe, >99% bioavailable, and the plasma concentration at 3 hours is highly predictive of $AUC_{0\text{-}inf}$ (r^2=0.98) (Clarke, S.: GlaxoSmithKline, Personal communication), making rosiglitazone an attractive probe candidate.

Despite the known *in vitro* correlation, rosiglitazone has not been previously used as a probe for CYP2C8 to predict paclitaxel exposure in patients. In this study we used rosiglitazone and ERMBT as surrogates of CYP2C8 and CYP3A4 activity, respectively, and hypothesized that the combination of these probes would explain the variability in paclitaxel exposure in cancer patients.

METHODS

This study was conducted at the General Clinical Research Center (GCRC) and approved by the Institutional Review Board at UNC Chapel Hill. Eligible patients were > 18 years old with solid tumor malignancies, were scheduled to receive weekly paclitaxel, and provided written informed consent.

On study day 1, Erythromycin Breath Test (ERMBT) was carried out as previously described(244). The percentage of the dose exhaled as ^{14}C over 1 hour ($AUC_{0\text{-}1}$) was $\log_e$ (Ln) transformed and used as a measure of CYP3A4 activity. Each subject (all of whom fasted for > 6 hours) was then administered a 2 mg oral dose of rosiglitazone. A blood sample was collected 3 hours after dosing and plasma concentration analysis was performed as previously described(245). Rosiglitazone concentrations at 3 hours ($rosi_3$) were adjusted to a standard dose per median body surface area (BSA) [1.80 mg/m^2], Ln transformed, and used as a measure of CYP2C8 activity.

Approximately 6 hours after the rosiglitazone dose, each subject was administered a one-hour infusion of paclitaxel (75 mg/m^2 to 90 mg/m^2) as per their standard treatment. Blood samples (7 mL) were collected prior to infusion and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, and 6 hours after the start of infusion. Limited sampling, which enables reasonably accurate estimation of paclitaxel exposure(246), was employed for patient convenience. An additional sample was collected 18-24 hours post infusion for estimation of $AUC_{0\text{-}inf}$. Plasma was separated and stored at -80°C before undergoing analysis via LC/MS/MS as previously described to measure total paclitaxel concentration(247).

Paclitaxel pharmacokinetic analysis was performed in WinNonlin Pro Version 5.2.1 (Pharsight Corp., Mountain View, CA) using non-compartmental methods. Concentration values were adjusted to a standard dose per median BSA [79.7 mg/m^2]. $AUC_{0\text{-}6}$ was calculated using the linear trapezoidal method and Ln transformed for use in the primary analysis. All later discussion refers to normalized concentrations.

Statistical analysis was conducted in SAS 9.2 (SAS Institute Inc, Cary, NC) using a multiple regression model with $rosi_3$ and ERMBT $AUC_{0\text{-}1}$ the independent variables and paclitaxel $AUC_{0\text{-}6}$ the dependent variable.

RESULTS

Twenty (20) patients with planned or ongoing weekly paclitaxel treatment were enrolled. Demographic data for the 14 subjects who had evaluable samples for paclitaxel pharmacokinetic analysis are displayed in **Table 18**. The mean (±SD) paclitaxel $AUC_{0\text{-}6}$ was 6,646 (±2,454) ng*hr/ml, ERMBT $AUC_{0\text{-}1}$ was 2.76

%/hr (±1.2) and $rosi_3$ was 81.8 (± 25.9) ng/mL. In the two-variable regression model $rosi_3$ was a statistically significant predictor of paclitaxel AUC_{0-6} (p=0.019); however, ERMBT was not (p=0.47). After exclusion of ERMBT, $rosi_3$ alone explained about 38% of the variability in paclitaxel AUC_{0-6} (r^2=0.38, p=0.018, **Figure 22**). In follow-up exploratory analyses no other relevant covariates (e.g., age, albumin, cancer type, and smoking status) significantly contributed to the model (data not shown).

DISCUSSION

In this pilot study, a 3-hour rosiglitazone plasma concentration after a 2 mg oral dose explained 38% of the variability in paclitaxel exposure. As expected, higher rosiglitazone concentration was associated with increased paclitaxel exposure. This is the first report of an *in vivo* association between rosiglitazone and paclitaxel exposure and supports the hypothesis that rosiglitazone may be a reasonable probe for *in vivo* exposure to CYP2C8 substrates.

Because this was a small pilot study, additional secondary analyses of the data were conducted to investigate the robustness of our findings; with results consistent with the primary finding. Using paclitaxel AUC_{0-6} and $rosi_3$ without dose normalization had little impact on our primary finding (r^2=0.33, p=0.029). The correlation between paclitaxel AUC_{0-inf} and AUC_{0-6} was strong (r^2=0.92) and use of Ln AUC_{0-inf} instead of Ln AUC_{0-6} did not meaningfully change the results (r^2=0.32, p=0.034). A slightly stronger relationship was found (r^2=0.51, p=0.004) when paclitaxel AUC_{0-6} and $rosi_3$ were transformed to ranks prior to regression,

suggesting that the findings did not result from the impact of a particularly influential value.

The highest rosiglitazone concentration seen was 120 ng/mL while rosiglitazone's reported Ki is 1,998 ng/mL(248), thus same-day administration of rosiglitazone and paclitaxel are unlikely to have resulted in competitive enzyme inhibition. In a similar study ERMBT explained 67% of the variability in docetaxel clearance(242), which is metabolized exclusively by CYP3A4. Our finding that ERMBT was not predictive of paclitaxel clearance is consistent with other evidence that CYP2C8 is the primary enzyme responsible for paclitaxel metabolism in most patients. It is possible that in a larger study a CYP3A4 probe may explain some of the residual variability in paclitaxel exposure unexplained by rosiglitazone. It is also likely that a portion of our unexplained variability can be attributed to the role of drug transporters in the pharmacokinetics of ERMBT and paclitaxel(249).

In conclusion, this report supports further study of rosiglitazone as an *in vivo* probe of CYP2C8 activity in humans. In particular, rosiglitazone may have a role in guiding dosing of CYP2C8 substrates that have a narrow therapeutic index, such as paclitaxel, particularly in elderly patients and others who historically have been empirically dose-reduced.

TABLES

Table 18 Demographic and Clinical Characteristics of Rosiglitazone Probe Study Patients

		Study Population (n=14)
Gender	Female	11 (79%)
	Male	3 (21%)
Ethnicity	Caucasian	12 (86%)
	African-American	1 (7%)
	Other	1 (7%)
Age	Median (Range)	47 (25-64)
BSA (m^2)	Median (Range)	1.80 (1.51-2.33)
Cancer Type	Breast	4 (29%)
	Lung	6 (43%)
	Melanoma	2 (14%)
	Ovarian	1 (7%)
	Other	1 (7%)
Paclitaxel Dose (mg/m^2/week)	Median (Range)	79.7 (75-90)
Concomitant Therapy*	Herceptin	4 (29%)
	Carboplatin	6 (43%)
	None	4 (29%)

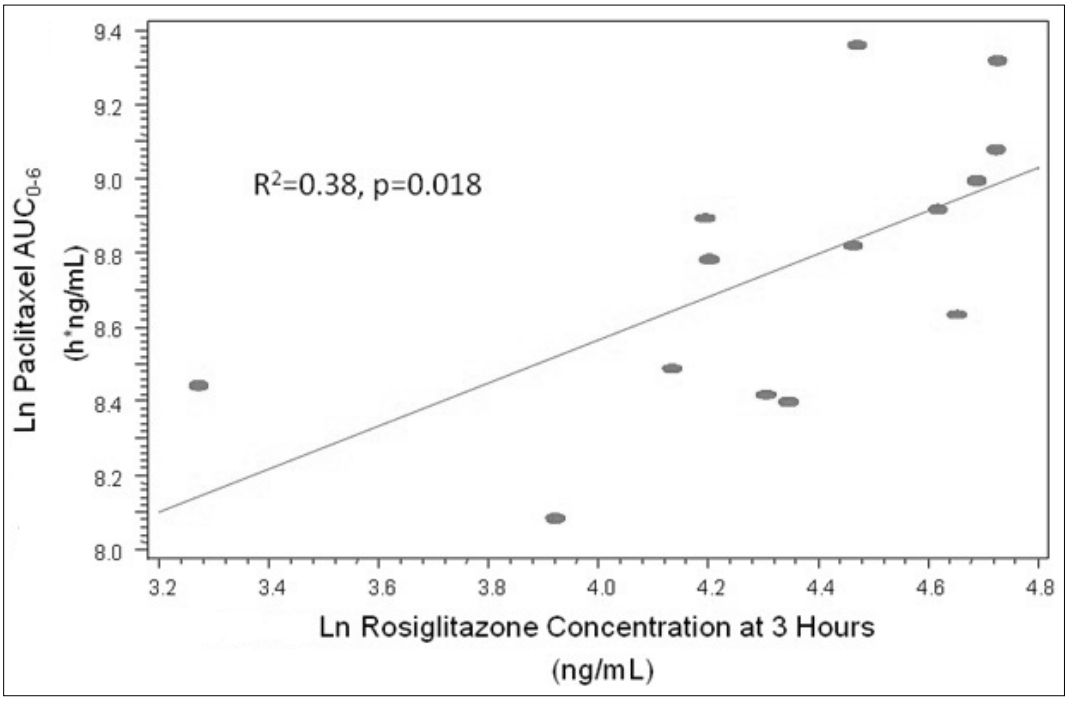
*Neither agent is known to modulate CYP2C8 or CYP3A4 activity.

FIGURE LEGENDS

Figure 22 Relationship between Ln rosiglitazone 3-hour concentration and Ln paclitaxel AUC_{0-6} (n=14). Concentrations of both drugs were adjusted to a standard (median) dose per BSA.

FIGURES

Figure 22 In Vivo Correlation Between Paclitaxel and Rosiglitazone Exposure



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