

Neurofilament light chain as a biomarker of axonal damage in sensory neurons and paclitaxel-induced peripheral neuropathy in ovarian cancer patients

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Abstract

Paclitaxel-induced peripheral neuropathy (PIPN) is a barrier to effective cancer treatment and impacts quality of life among cancer patients. We used a translational approach to assess the utility of neurofilament light chain (NFL) as a biomarker of PIPN in a human cell model and in ovarian

cancer patients. We measured NFL in medium from human induced pluripotent stem-cell derived sensory neurons (iPSC-SNs) treated with paclitaxel. Serum NFL (sNFL) levels were quantified in 190 ovarian cancer patients receiving paclitaxel/carboplatin chemotherapy at baseline and after each of the following 2 or 6 cycles. PIPN-related adverse outcomes were retrospectively obtained, and Cox regression model was performed with different sNFL cut-offs after first cycle. The apparent elimination half-life of sNFL was estimated in patients that discontinued paclitaxel. Paclitaxel neurotoxicity in iPSC-SNs was accompanied by NFL release in a concentration-dependent manner ($P < 0.001$, ANOVA). sNFL levels increased substantially in patients during paclitaxel/carboplatin chemotherapy with considerable interindividual variability. Patients with sNFL > 150 pg/mL after first cycle had increased risk to discontinue paclitaxel early (unadjusted HR: 2.47 (95% CI 1.16-5.22), adjusted HR: 2.25 (95% CI: 0.88-5.79)). Similar trends were shown for risk of severe PIPN and paclitaxel dose reduction due to PIPN. The median elimination half-life of sNFL was 43 days (IQR 27-82 days). NFL constitutes an objective biomarker of neurotoxicity in iPSC-SNs and in ovarian cancer patients with high sNFL predicting PIPN-related adverse outcomes. If prospectively validated, sNFL can be utilized to study PIPN and may guide clinical decision making and personalize treatment with paclitaxel.

Keywords: Chemotherapy-induced peripheral neuropathy, paclitaxel, neurofilament light chain, induced pluripotent stem cells, sensory neurons, dorsal root ganglia, translational research

Introduction

Paclitaxel is one of the most widely used chemotherapeutic agents against a broad range of solid tumors, including breast, ovarian, and lung cancers [8]. While essential for cancer treatment,

paclitaxel is frequently associated with adverse effects, such as peripheral neuropathy. Paclitaxel-induced peripheral neuropathy (PIPNe) predominantly manifests as sensory symptoms which occur in up to 70% of cancer patients [25]. PIPNe is a growing problem in oncology as the proportion of long-term survivors is increasing due to improvements in early diagnosis and treatment of cancers [7]. The lack of effective treatment and prevention of PIPNe leaves dose reductions, treatment delays and discontinuation as the only countermeasures, which is suboptimal because it may limit treatment success and potentially survival [25]. Duloxetine is the only agent with moderate evidence for treatment of established PIPNe [17]. Current methods for diagnosis and classification of PIPNe are based on patient-reported outcomes and clinician-based grading. Several grading tools are available to assess severity of symptoms and functional consequences, including the Common Terminology Criteria for Adverse Events (CTCAE), and the Total Neuropathy Score (TNS) [26]. Although valuable, current grading tools are limited by their subjective nature and are too burdensome to obtain clinical utility. The grading tools rely on patients' ability to describe symptoms and functional consequences, and they display considerable inter- and intraobserver variability [9]. Consequently, there is a risk of misestimating patients' symptoms which might lead to inappropriate treatment decisions [20]. This highlights an urgent need for an objective and sensitive biomarker to classify PIPNe and to predict an individual patient's risk of PIPNe.

Neurofilament light chain (NFL) is a major structural component of neurons in the central and peripheral nervous system (CNS, PNS). Damage to axons leads to release of NFL into the interstitial fluid and eventually into peripheral blood. Recently developed technologies enables detection of very low concentrations of NFL [31]. Elevated NFL levels are believed to represent axonal degeneration which is the key event of neurodegenerative disorders of the CNS and PNS [16]. Animal studies showed that serum NFL (sNFL) levels increased during repeated treatment

with paclitaxel, cisplatin and vincristine and correlated to neurotoxicity as shown by morphological and functional alterations [18, 19].

Human induced pluripotent stem cells (iPSC) have revolutionized the field of human disease modeling as they provide an unlimited resource of almost any cell type of the human body, such as sensory neurons. This is a considerable advantage as sensory neurons are not a renewable resource and limited by organ donor availability and technical challenges [6]. The purpose of this study was to use a translational approach to assess the utility of NFL as a biomarker of PIPN by performing i) a cell-based study in human iPSC-derived sensory neurons (iPSC-SNs) and ii) a clinical study in ovarian cancer patients (Figure S1, available as supplemental digital content at <http://links.lww.com/PAIN/B760>). Specifically, our objectives were to: a) assess neurotoxicity and the related release of NFL after paclitaxel exposure in iPSC-SNs, b) investigate NFL increases in patients during paclitaxel/carboplatin chemotherapy, and c) evaluate sNFL as a predictor of PIPN-related adverse outcomes.

Methods

Sensory neuron differentiation

Two commercially available human iPSC lines (A18945, ThermoFisher, Roskilde, Denmark, hpscereg.eu/cell-line/TMOi001-A; WTC-11, Gladstone Institute of Cardiovascular Disease, UCSF, hpscereg.eu/cell-line/UCSFi001-A) were differentiated into sensory neurons following a published protocol [4]. An overview of the procedure is shown in **Figure S2** (available as supplemental digital content at <http://links.lww.com/PAIN/B760>). Human iPSCs were cultured in mTeSR1 medium (85850, StemCell Technologies, Vancouver, BC, Canada) on Matrigel (354277, Corning, NY,

USA) at a minimum density of 50,000 cells/cm². When cells reached 70-80% confluency, cells were enzymatically passaged as aggregates using Accutase (00455556, ThermoFisher). mTeSR1 medium was supplemented with 10 μM ROCK inhibitor (S1049, Selleck Chemicals, Houston, TX, USA) upon thawing and passaging. Importantly, medium was replaced with mTeSR1 medium without ROCK inhibitor within 20 hours. Cells were maintained by daily medium change. Sensory neuron differentiation was initiated at ~90% confluency using KSR medium which contained 82% KnockOut DMEM (10829018, ThermoFisher), 15% KnockOut Serum Replacement (10828028, ThermoFisher), 1% GlutaMAX (35050038, ThermoFisher, Paisley, Scotland, UK), 1% MEM non-essential amino acids (11140035, ThermoFisher, Paisley, Scotland, UK), 1% Penicillin-Streptomycin (P4333, Sigma-Aldrich, Søborg, Denmark) and 0.1 mM β-mercaptoethanol (31350010, ThermoFisher, Paisley, Scotland, UK). On days 0-5, SMAD signaling was inhibited by using 0.1 μM LDN193189 (S7507, Selleck Chemicals) and 10 μM SB431542 (S1067, Selleck Chemicals). Medium was changed daily, and N2 medium was added with 25% increments every other day starting on day 4. N2 medium consists of 50% DMEM/F-12 (11320033, ThermoFisher) and 50% Neurobasal (21103049, ThermoFisher) with 1% N2 Supplement (17502048, ThermoFisher), 1% B27 Supplement (17504001, ThermoFisher) and 1% Penicillin-Streptomycin. On days 2-10, three additional inhibitors were added to direct differentiation into the sensory neuronal lineage: 3 μM CHIR99021 (S1263, Selleck Chemicals), 10 μM SU5402 (S7667, Selleck Chemicals) and 10 μM DAPT (S2215, Selleck Chemicals). On day 12, immature iPSC-SNs were enzymatically dissociated with Accutase followed by gentle trituration and filtration through a 40 μm strainer (431750, Corning). Single cells were seeded onto 24-well plates (VWR) and 12-well plates (VWR) at a density of 150,000 viable cells/cm². Plates were triple coated with 20 μg/mL poly-L-ornithine hydrobromide (P3655, Sigma-Aldrich), 10 μg/mL laminin (23017015, ThermoFisher) and 2 μg/mL fibronectin (F1141, Sigma-Aldrich). Immature iPSC-SNs were

maintained in N2 medium supplemented with human growth factors (25 ng/mL NGF- β , 450-01; BDNF, 450-02; GDNF, 450-10; NT-3, 450-03; Peprotech, Cranbury, NJ, USA) and 0.2 mM L-ascorbic acid (A4403, Sigma-Aldrich). On day 14, cells were treated with freshly prepared Mitomycin-C (1 μ g/mL, M4287, Sigma-Aldrich) for 2 hours to eliminate non-neuronal cells. On day 16, the medium was changed completely to remove dead cells. The subsequent 50% medium changes were performed every 3-4 days. Laminin (1 μ g/mL) was added to the medium once a week to maintain cell attachment. The mature iPSC-SNs were used for experiments between day 35 and day 45.

Compound preparations and considerations

Paclitaxel (T7402, Sigma-Aldrich) and 5-fluorouracil (5-FU, FF2627, Sigma-Aldrich) were dissolved and serially diluted in dimethyl sulfoxide (D8418, DMSO, Sigma-Aldrich). The stock solutions were diluted 1:500 to ensure equal exposure of 0.2% DMSO in all conditions. iPSC-SNs were treated with 0.1, 1 and 10 μ M paclitaxel or 10, 100 and 1000 μ M 5-FU for 48 hours. The clinically relevant concentrations of paclitaxel and 5-FU were selected based on their clinical pharmacokinetic profile and accounts for their distinct plasma protein binding as well as interindividual variability in pharmacokinetic profiles [21]. 5-FU was included as a negative control as it is known not to cause peripheral neuropathy [29].

Immunocytochemistry

iPSC-SNs were fixed by diluting 16% paraformaldehyde (28906, ThermoFisher) 1:4 into the cell medium for 10 minutes. Cells were permeabilized with 0.25% Triton X-100 for 15 minutes, and non-specific binding was blocked using 1% bovine serum albumin for 1 hour. Cells were incubated with primary NFL monoclonal antibody (1:100, MA5-14981, ThermoFisher) overnight at 4°C and

labeled with secondary Alexa Fluor 488-conjugated anti-rabbit (1:400, A11008, ThermoFisher) for 1 hour at room temperature. Stained cells were stored in phosphate-buffered saline, and images were acquired using ImageXpress Pico (Molecular Devices, San Jose, CA, USA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from iPSC-SNs seeded in 12-well plates (VWR) using RNeasy Mini Kit (74106, Qiagen AB, Stockholm, Sweden) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (4374966, Applied Biosystems, Foster City, CA, USA) with genomic DNA removal. Quantitative real-time polymerase chain reaction (qPCR) was performed in 96-well reaction plates using 10 ng cDNA template, 20X TaqMan Gene Expression Assay (**Table S1**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>), 2X TaqMan Gene Expression Master Mix and RNase-free water. All assays contained a no reverse transcriptase control and a no template control as negative controls. qPCR was performed using StepOne Plus RT-PCR equipment (ThermoFisher, Carlsbad, CA, USA). The relative changes in mRNA expression were calculated using the comparative C_t method [24] and involved normalization to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH).

Patient data

The clinical study is a retrospective cohort study with review of medical records and analysis of blood samples from an already established biobank (Department of Oncology, Lillebaelt University Hospital of Southern Denmark). The included participants were scheduled for treatment in the period from October 2013 to September 2021. All participants fulfilled the following inclusion criteria: (1) histologically verified epithelial ovarian cancer, (2) indication for first-line combination chemotherapy with carboplatin (AUC 5 mg·min/mL) and paclitaxel (175 mg/cm²), and (3) age

above 18 years. The exclusion criteria were as follows: (1) patients with other known cancers except from a) basal cell carcinoma, b) adequate and cured carcinoma in situ cervicis uteri or c) other cancers with low risk of recurrence, (2) pregnant or breastfeeding women and (3) patients receiving experimental treatment.

A total of 200 patients were included in our study where 190 patients received paclitaxel/carboplatin chemotherapy. A patient flow diagram is summarized in **Figure S3** (available as supplemental digital content at <http://links.lww.com/PAIN/B760>). As a part of combination chemotherapy, Cremophor EL-diluted paclitaxel were scheduled as 3-h infusion every 3 weeks for a total of 6 cycles. Due to poor general condition or concerns of adverse effects, 10 patients did not receive paclitaxel, but were treated with carboplatin as monotherapy (AUC 5 mg·min/mL). This group of patients was used as comparison controls as the occurrence of peripheral neuropathy with carboplatin is less frequent at approximately 5% [30]. Therefore, this study only focuses on modifications of paclitaxel dose and schedule i.e., early discontinuation and dose reduction. Early discontinuation was defined as the inability to complete the 6 scheduled cycles of paclitaxel. Dose reduction was defined as adjustment of paclitaxel dose to less than standard dose (175 mg/m²). Study data were collected and stored using REDCap hosted by OPEN [10]. Medical records were reviewed manually and systematically for the following baseline patient characteristics: demographics, cancer stage, ECOG (Eastern Cooperative Oncology Group) performance status, diabetes, alcohol consumption and smoking. The absolute dose (mg) of paclitaxel and the type of chemotherapy regimen were obtained, and it was noted if modification of the scheduled paclitaxel treatment was due to PIPN or other reasons. The following reasons for deviation from scheduled paclitaxel treatment were recorded: hematological toxicity, renal toxicity, febrile neutropenia, poor health condition, allergic reaction, wishes of the patient or limited treatment response. Furthermore,

baseline liver (aminotransferase (ALAT)) and kidney function (standard glomerular filtration rate (GFR) measured by chrom-EDTA clearance) were obtained to allow adjustment of statistical analyses since they may have unknown effects on paclitaxel exposure. Hepatic clearance was calculated using a previously described model [3]. Adjustment of diabetes was not possible due to limited number of patients with diabetes. PIPN severity was graded using CTCAE (version 2.0) and performed systematically and face-to-face with the patient by the treating physician 3 weeks after each cycle and always on the day when the next cycle of chemotherapy was given. These data were retrospectively obtained from a research database. If the chemotherapy was delayed due to hospitalization or toxicity, CTCAE was evaluated at the following cycle. CTCAE grades were defined as: 0) no symptoms, 1) mild symptoms such as paresthesia and loss of reflexes, 2) moderate symptoms limiting instrumental activities of daily living, 3) severe symptoms limiting self-care, mobility, and activity, and 4) life-threatening symptoms. Importantly, CTCAE gradings and information from medical records were obtained prior to measuring sNFL levels.

When this study was designed, NFL had never been measured in cancer patients treated with paclitaxel. Therefore, it was not possible to perform a power calculation to estimate the size of the study population. The study was originally divided into two parts i) 100 patients with NFL measurements at baseline (no chemotherapy) and after each cycle of chemotherapy (7 blood samples/patient), and ii) 100 patients with NFL measurements at baseline and after cycle 1 and 2 only (3 blood samples/patient). All patient data were combined to ensure a large cohort of patients with sNFL levels at all the defined sNFL cut-offs.

Measurement of NFL levels

iPSC-SN medium was collected for NFL measurements 48 hours after treatment, and the respective cells were lysed using radioimmunoprecipitation assay buffer (89900, ThermoFisher). NFL measurements of iPSC-SN medium and patient serum were carried out at the Department of Biochemistry and Immunology, Lillebaelt University Hospital of Southern Denmark. Measurements were performed blinded to experimental and clinical data using a commercial NFL assay on the Simoa HD-1 Analyzer (Quanterix, Lexington, MA, USA). NFL measurements of iPSC-SN medium were initially validated and required serial dilutions due to very high NFL levels. The serial dilutions might introduce small but acceptable uncertainty. Quality control was performed using two controls prepared from commercially available control material provided by the manufacturer in addition to an in-house prepared serum pool. The in-house serum pool was used as an internal control and included in each run for evaluating and monitoring assay performance over time. The total analytical variation for the included controls were <12 %. The lower limit of detection and quantification was 0.038 pg/mL and 0.174 pg/mL, respectively.

Study approval

The clinical study was approved by the Regional Committees on Health Research Ethics for Southern Denmark (J. no. VF-20050050, S-20210003), the Danish Data Protection Agency (J.no. 2005-41-5127, 2021-522-0310), OPEN (no. OP_1331), and University of Southern Denmark Research and Innovation (J.no. 11.289).

Analysis of patient data

All analyses were conducted in R (version 4.0.2; R Statistical Foundation for Statistical Computing). Kaplan-Meier curves and Cox regression were performed using the survminer package. The elimination half-life was estimated with non-compartmental analysis using the ncappc

package under the assumption of linear kinetics and elimination from a systemic pool. All other graphs were created using the ggplot2 package.

We defined the following three endpoints to evaluate sNFL as a predictor of adverse outcomes: i) the first occurrence of grade 2 PIPN or higher, ii) the cycle where paclitaxel was discontinued due to PIPN, and iii) the first occurrence of a dose less than standard dose due to PIPN. We used a time-to-event approach using Kaplan-Meier curves and Cox regression. The approach was modified by substituting time with cumulative paclitaxel dose. Body surface area (BSA) was calculated using the Monsteller equation to adjust individual paclitaxel doses (mg/m^2). If patients did not experience any events, the total cumulative paclitaxel dose (cycle 6) was used in the analysis. Cox regression was adjusted for the following confounders obtained at baseline as previously described: age, BSA, performance status, alcohol consumption, smoking, liver function (ALAT and hepatic clearance) and kidney function (standard GFR) [23]. Since the optimal cut-offs for NFL are unknown, we exploratively defined 4 different cut-offs (<50, 50-99, 100-149, >150 pg/mL) that stratified patients into subgroups with similar sizes.

We assessed interrater reliability of data extraction from the medical records using Cohen's kappa. This was performed for a group of 30 randomly selected medical records. Kappa was calculated for each of the included variables that were related to CTCAE grading of PIPN and modifications to paclitaxel dose and schedule. Descriptive variables were not double extracted and therefore not included in the calculation.

The elimination half-life of sNFL was determined in patients who discontinued paclitaxel and had at least three blood samples analyzed after paclitaxel discontinuation. We identified patients with

substantially increased sNFL and thus included patients with sNFL levels above 100 pg/mL at the day of paclitaxel discontinuation in the analysis.

Analysis of cell data

iPSC-SN experiments were performed with two individual donors. For each experiment, all conditions had three technical replicates. NFL measurements were performed with three independent iPSC-SN differentiations for A18945 and one for WTC-11. NFL measurements were adjusted for protein concentration (as a surrogate of differences in cell density) as determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (23227, ThermoFisher). Fold changes in sNFL levels was calculated relative to the mean value of the DMSO control for each individual differentiation. QQ-plot was used to check normality of iPSC-SN data, and ordinary one-way analysis of variance (ANOVA) were used to test for statistical significance.

Results

Paclitaxel causes concentration-dependent release of NFL from iPSC-derived sensory neurons

Human iPSCs from two different, healthy donors were successfully differentiated into sensory neurons using an established protocol [4]. iPSC-SNs displayed a characteristic dorsal root ganglia morphology where cell bodies are organized in large ganglia with numerous of axons emanating. After 35 days of differentiation, a relatively pure culture of iPSC-SNs was observed (**Figure S2**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>). iPSC-SNs expressed important genes involved in the perception of pain (TRPV1, TRPM8, SCN9A, TAC1, PIEZO2), and lacked expression of pluripotency (NANOG, POU5F1, **Figure S4**, available as supplemental

digital content at <http://links.lww.com/PAIN/B760>). Immunolabeled images indicate that NFL is highly expressed in the cell bodies and along the axons. Treatment with clinically relevant concentrations of paclitaxel caused a concentration-dependent reduction in the complexity of the neuronal network and led to thickening of fascicles and axonal swellings (**Figure 1**). We also observed that the neuronal network becomes disorganized compared to the DMSO control where the axons are highly organized. The observed phenotype of paclitaxel-treated iPSC-SNs reflects axonal retraction which is an early sign of neuronal damage. In line with the observed neurotoxicity, NFL is released from iPSC-SNs to cell medium upon paclitaxel exposure in a concentration-dependent manner ($P < 0.001$, ANOVA; **Figure 2**). NFL levels varied substantially at 10 μM paclitaxel. Absolute NFL levels and protein concentrations used for normalization can be found in **Table S2** (available as supplemental digital content at <http://links.lww.com/PAIN/B760>). 5-FU (100 μM) did not affect the neuronal network, and NFL was not released to cell medium after treatment with up to 1000 μM 5-FU (**Figure S5, Table S3**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>).

Clinical characteristics and adverse outcomes of ovarian cancer patients scheduled for paclitaxel/carboplatin chemotherapy

To determine the clinical relevance of our in vitro findings, we investigated if increased sNFL levels were found in patients treated with paclitaxel. Furthermore, we evaluated if high sNFL levels after first cycle of paclitaxel were associated with increased risk of severe PIPN and PIPN-related adverse outcomes. The cohort included 200 women with ovarian cancer who were scheduled for 1st line paclitaxel/carboplatin chemotherapy with carboplatin and paclitaxel. A total of 10 patients were not treated with paclitaxel and primarily received carboplatin monotherapy. Patient characteristics are listed in **Table 1**. We calculated a mean kappa value of 0.95 across variables which indicate an

almost perfect interrater reliability of medical record reviews. The individual kappa values are listed in **Table S4** (available as supplemental digital content at <http://links.lww.com/PAIN/B760>). PIPN severity and PIPN-related adverse outcomes were evaluated at each cycle during paclitaxel/carboplatin chemotherapy. Severe PIPN defined as a PIPN grade of 2 or higher was observed in 82 (43%) of 190 patients over the total course of treatment. Discontinuation of paclitaxel was observed for 65 (34%) patients due to PIPN. Dose reduction of paclitaxel occurred in 68 (36%) patients due to PIPN (**Table 2**).

sNFL levels increase in ovarian cancer patients during paclitaxel/carboplatin chemotherapy

sNFL levels in the 190 patients generally increased over the course of paclitaxel/carboplatin chemotherapy (**Figure 3**). Substantial interindividual differences were observed with regards to the development and course of increased sNFL levels with cumulative paclitaxel dose. For instance, some patients had markedly and rapidly increased sNFL levels while other patients had relatively stable or steadily increasing sNFL levels. The difference in sNFL levels from baseline to cycle 1 varied between individual patients. Approximately 35% of patients had no change (<25 pg/mL) while approximately 44% had moderate to substantial increased sNFL levels (≥ 50 pg/mL, **Figure S6**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>). Among the 10 ovarian cancer patients not receiving paclitaxel, sNFL levels remained relatively stable during treatment primarily with carboplatin monotherapy (**Figure S7**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>).

sNFL levels after first cycle of paclitaxel/carboplatin chemotherapy might predict adverse outcomes

Figure 4 shows that increased sNFL after first cycle of paclitaxel/carboplatin chemotherapy can identify low- and high-risk groups for paclitaxel discontinuation. Similar patterns exist for severe PIPN (grade 2 or higher) and paclitaxel dose reduction (**Figure S8, Figure S9**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>). Accordingly, Cox regressions show that increasing sNFL cut-offs after first cycle of paclitaxel were associated with increased cumulative risk of severe PIPN, paclitaxel discontinuation and paclitaxel dose reduction (**Table 3**). The hazard ratios (HRs) of covariates included in the adjusted Cox regression are shown in **Table S5** (available as supplemental digital content at <http://links.lww.com/PAIN/B760>). We also tested sNFL fold changes from baseline after first cycle as cut-offs (data not shown), however, absolute sNFL values provided better discrimination between risk-groups. We found that 14% of patients had sNFL levels higher than the lower cut-off of 50 pg/mL at baseline, and this was associated with a small increased risk of paclitaxel discontinuation but not severe PIPN and paclitaxel dose reduction (data not shown). Additionally, we observed a significant association between baseline sNFL levels and age in this cohort ($R^2=0.22$, $P<0.01$). The use of cumulative dose instead of the planned six cycles as the time parameter in the Kaplan-Meier plot created similar looking curves but with greater discrimination between risk-groups (data not shown), indicating that it is necessary to account for individual cumulative paclitaxel exposures.

Increase of sNFL levels and severity of PIPN

Baseline sNFL levels were not different among patients with different PIPN grades (**Table S6**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>). Median sNFL levels after cycle 1 and cycle 2 were higher for patients with PIPN grade 2 or 3 compared to PIPN grade 1. The highest median sNFL level was observed for PIPN grade 3 after cycle 3. Surprisingly, high NFL levels was observed for patients with asymptomatic or mild PIPN (grade 0-1) after cycle 5 and

6, and patients with severe PIPN (grade 3) had low NFL levels after cycle 4 and 5. Patient NFL levels were censored upon paclitaxel discontinuation and thus, NFL values at later cycles are from fewer patients. These patients experience serious PIPN symptoms, but do not have substantially increased NFL values.

sNFL elimination half-life

The elimination half-life of sNFL was estimated in 9 patients who had sNFL levels above 100 pg/mL and at least three blood samples analyzed after paclitaxel discontinuation. We determined the median apparent elimination half-life of sNFL to 43 days (interquartile range (IQR) 27-82 days). The time curve of sNFL indicates substantial interindividual differences in sNFL apparent elimination half-life (**Figure S10**, available as supplemental digital content at <http://links.lww.com/PAIN/B760>).

Discussion

We studied the utility of NFL as a biomarker of PIPN using a human cell model and patient data. We demonstrate that neurotoxicity after paclitaxel exposure was accompanied by the release of NFL from iPSC-SNs. Accordingly, sNFL levels increased substantially during treatment with paclitaxel/carboplatin chemotherapy in ovarian cancer patients. High sNFL levels after first cycle predicted increased risk of severe PIPN and PIPN-related adverse outcomes during later cycles. Finally, we showed that sNFL has an apparent elimination half-life of approximately 43 days.

The evidence for the utility of sNFL as a biomarker of PIPN is scarce. Our *in vitro* findings are in agreement with a recent study showing that NFL is released from iPSC-SNs upon paclitaxel treatment [11]. This study show that prolonged treatment for 72 hours reduced viability which was

associated with increased NFL levels. In contrast, we utilized a time course of 48 hours that allowed us to assess early neurotoxicity without affecting viability [22, 32, 33]. While we observed significant increased NFL levels in iPSC-SN medium after 48 hours of treatment, the study showed increased NFL levels only after 72 hours exposure. We observed that paclitaxel led to axonal blebbing similar to the mentioned study but our iPSC-SNs do not appear to fragment. However, we see fewer but thicker neurons upon paclitaxel treatment. The discrepancies between the studies may be due to donor-specific effects and the use of different time courses. Additionally, the study showed that sNFL levels correlated with PIPN severity as assessed by TNS 28 weeks after treatment in a small, mixed cohort (n=31) of breast and ovarian cancer patients. In another pilot study (n=20), sNFL levels increased during paclitaxel treatment and likely reflected the axonal damage underlying PIPN [1]. The latter study measured sNFL at two time points during paclitaxel treatment i.e., after 3 cycles and after treatment completion. A recent prospective study in breast cancer patients (n=59) showed an early increase in sNFL after 2 weekly cycles of paclitaxel [13]. In contrast, we measured sNFL levels already after cycle 1 with markedly increased sNFL, indicating that initial cycles might be optimal for early risk prediction of PIPN. Serial measurements of sNFL during oxaliplatin treatment in colorectal cancer patients (n=43) showed that sNFL levels increased with cumulative dosing and correlated to the severity of oxaliplatin-induced peripheral neuropathy [14]. This might suggest sNFL as a general biomarker of peripheral neuropathy caused by any neurotoxic chemotherapy, but this needs to be validated in future studies.

We observe substantial interindividual variation in sNFL increases during paclitaxel/carboplatin chemotherapy. Some patients present with dramatic and rapidly increased sNFL, while others experience only slightly elevated sNFL levels even after 6 cycles of paclitaxel/carboplatin. This corresponds to the clinical observations that some patients can tolerate high doses of paclitaxel

without developing PIPN while other patients are sensitive to even low doses of paclitaxel. The different clinical presentations of PIPN might be explained by genetic susceptibility to paclitaxel neurotoxicity and heterogeneity of the molecular mechanisms underlying PIPN. We observe that some patients who discontinued paclitaxel due to severe PIPN had low sNFL levels, and other patients had increased NFL levels while experiencing mild or asymptomatic PIPN. Considering that NFL reflects axonal damage, these observations might indicate that several PIPN phenotypes exist. Some of which might be caused by functional impairment rather than structural damage of sensory neurons. This has never previously been reported and thus, further studies are needed to verify our findings.

The primary weakness of our clinical study is that it was retrospectively performed and dependent on medical records. The use of CTCAE to assess PIPN severity has several limitations, including low interrater reliability, and inadequate evaluation of neuropathic pain. The high burden of grade 2 PIPN or higher (43%) in our cohort was not comparable to the prevalence of 18% estimated in a similar cohort [5]. The difference in PIPN prevalence might be due to difference between patient-reported outcomes and clinician-based grading, or difference in age distribution and number of patients with comorbidities. Future studies should aim to more carefully phenotype PIPN prospectively by utilizing additional grading tools of PIPN, such as TNS or patient-reported outcomes (PRO) [15, 27]. Our study lacked information of pre-existing peripheral neuropathy before initiation of chemotherapy. Although the included patients were chemo-naïve, 10 patients had diabetes and may have been predisposed to PIPN. The included patients has a median age of 66.5 years and thus, we cannot exclude that co-morbidities might have influenced CTCAE gradings and NFL levels. sNFL levels have been shown to correlate with age [12] and we demonstrate similar association between age and baseline sNFL levels. However, age did not impact risk of

PIP-N-related adverse outcomes in this cohort, likely due to the rather narrow age distribution in our cohort. Despite the substantial variability introduced by the study design, we still observe statistically significant associations between NFL and PIP-N-related adverse outcomes. In the Kaplan-Meier plot, we defined an event if paclitaxel dose was reduced or discontinued specifically due to PIP-N. For some patients, PIP-N is not the sole reason, and we cannot exclude that the results can be biased by other reasons. Another limitation to our study is the lack of an external validation cohort. Unfortunately, we were unable to identify a suitable cohort. However, as sNFL levels were self-controlled i.e., each patient acts as their own control, we are confident to conclude that sNFL increases in patients during paclitaxel/carboplatin chemotherapy. For patients treated with carboplatin monotherapy, we observed that one out of ten patients had increased NFL levels. This may indicate that carboplatin neurotoxicity is not fully negligible. Future studies should aim to prospectively assess sNFL as a biomarker of PIP-N preferably in patients with curative intended treatment. These studies should ideally adjust for concomitant medications known to exacerbate PIP-N symptoms and PIP-N-related adverse outcomes as previously reported [23, 28]. For our *in vitro* work, a limitation is the lack of replications for the iPSC donor WTC-11. The clinical background of the included iPSC donors is unknown and thus future *in vitro* studies should aim to generate iPSCs from patients known to be tolerant or susceptible to neurotoxic chemotherapy. Combination chemotherapy is utilized for most cancers, and thus future *in vitro* studies could be improved by treating cells with combination chemotherapy. This may uncover additive or synergistic effects that are not detected when treating cells with monotherapy. This will more closely mimic clinical conditions and may improve translation.

The main strength of our study is the translational approach that combines *in vitro* findings with clinical data. We assess paclitaxel neurotoxicity in the cell type affected in patients using iPSC-

derived sensory neurons. Our clinical study is the most comprehensive longitudinal study of sNFL as a biomarker of peripheral neuropathy caused by a neurotoxic chemotherapeutic agent to date. We measured sNFL at baseline and after cycle 1 through cycle 6 with simultaneous evaluation of PIPN severity and PIPN-related adverse outcomes. Additional strengths of our clinical study are the sample size, the inclusion of a negative control group of ovarian cancer patients not receiving paclitaxel and the homogeneity of our study population in terms of cancer diagnosis and first-line chemotherapy treatment in newly diagnosed chemo-naïve patients.

To date, no study has estimated the apparent elimination half-life of sNFL in humans. A clinical study suggests that the elimination half-life of sNFL in multiple sclerosis patients is between several weeks and months, depending on the extent of axonal damage [2]. Our median estimate of 43 days falls within this interval, and we observe similar variation in elimination half-lives among ovarian cancer patients. The wide IQR of 27 to 82 days might be explained by differences in clearance or continued release of NFL from damaged sensory neurons. Understanding elimination half-life is important as it defines the optimal sample collection time points and indicates the clinical relevance of a certain biomarker. The long elimination half-life of sNFL makes it a good candidate for a biomarker as it allows flexibility in serial sampling and monitoring. We determined the elimination half-life of sNFL in PIPN patients who discontinued paclitaxel. Hereby, the intermittent exposure causing NFL release is removed, which allows estimation of elimination half-life. This is difficult for neurodegenerative diseases within the CNS and PNS.

In conclusion, we show that paclitaxel causes axonal damage, which leads to release of NFL from iPSC-SNs. Ovarian cancer patients had substantially increased sNFL levels during paclitaxel/carboplatin chemotherapy, and high NFL levels after first cycle were associated with an

increased risk of severe PIPN and PIPN-related adverse outcomes during later cycles. Thus, sNFL might be a useful biomarker to predict patients at risk for severe PIPN, paclitaxel discontinuation and paclitaxel dose reduction due to PIPN. From a research perspective, sNFL might serve as an important tool in PIPN research within pharmacogenomics and PIPN risk factors but also in the assessment of efficacy and toxicity of novel drug candidates. From a clinical perspective, sNFL may help evaluating benefit-to-risk balance and guide clinical decision making early in the treatment course and ultimately individualize treatment with paclitaxel.

Conflicts of interest

T.B.S. has given paid lectures for Pfizer and Eisai and done consulting for Pfizer. All unrelated to this work. All other authors declared no competing interests for this work.

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The clinical data and R code that support the findings of this study are available on reasonable request from the corresponding author. The clinical data are not publicly available due to General Data Protection Regulation (GDPR), and ethical restrictions.

Supplemental video content

A video abstract associated with this article can be found at <http://links.lww.com/PAIN/B761>.

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Figure legends

Figure 1. Paclitaxel reduces the complexity of the neuronal network in a concentration-dependent manner. iPSC-derived sensory neurons were treated with indicated concentrations of paclitaxel for 48 hours. The control was treated with vehicle (0.2% DMSO). Cells were fixed, immunolabeled for

NFL, and images were captured using ImageXpress Pico with the 10X objective. Representative images are shown for the indicated concentrations. The experiment was performed with iPSC donor A18945.

Figure 2. NFL is significantly released from sensory neurons derived from two distinct iPSC donors upon paclitaxel exposure ($P < 0.001$, one-way analysis of variance (ANOVA)). Donor 1 and 2 refers to A18945 ($n=3$) and WTC-11 ($n=1$) respectively. Differences in relative fold changes were tested for significance using ANOVA. Abbreviations: DMSO, dimethyl sulfoxide; NFL, neurofilament light chain.

Figure 3. Serum neurofilament light chain (sNFL) levels increase in ovarian cancer patients during paclitaxel treatment with considerable interindividual variability ($n=190$). Data are visualized with (A) a linear y-axis with exclusion of one data point at 4000 pg/mL and (B) a logarithmic y-axis. Data indicate 100 patients with blood samples analyzed from cycle 0-6 and 100 patients from cycle 0-2 only with exclusion of the 10 negative controls. Patient data were censored after paclitaxel discontinuation and thus, data exclusively show sNFL levels for each patient after paclitaxel treatment.

Figure 4. Kaplan-Meier plot shows that increasing sNFL cut-offs after first cycle of paclitaxel are associated with increased cumulative risk of paclitaxel discontinuation due to PIPN. Negative controls and one patient with a missing blood sample were excluded from the analysis. Abbreviations: PIPN, paclitaxel-induced peripheral neuropathy; sNFL, serum neurofilament light chain.

TABELS

Table 1. Characteristics of 200 female patients included in this cohort.

Characteristics	Median [IQR]
Age (years)	66.5 [57.0, 72.0]
BSA (m ²)	1.8 [1.6, 1.9]
Baseline measurements	
ALAT (U/L)	23.0 [16.0, 31.0]
Standard GFR (mL/min/1.73m ²)	81.0 [68.0, 91.0]
ECOG performance status	n (%)
0	148 (74.0%)
1	35 (17.5%)
2	14 (7.0%)
Unknown	3 (1.5%)
FIGO stage	
I	26 (13.0%)
II	16 (8.0%)
III	60 (30.0%)
IV	98 (49.0%)
Chemotherapy regimen^a	

Adjuvant	84 (42.0%)
Neoadjuvant and adjuvant	33 (16.5%)
Neoadjuvant intended and converted to palliative	38 (19.0%)
Palliative	45 (22.5%)
Miscellaneous conditions	
Diabetes mellitus	10 (5.0%)
Alcohol consumption	
Above recommended limit ^b	5 (2.5%)
Below recommended limit	194 (97.0%)
Unknown	1 (0.5%)
Smoking	
Never smoker	103 (51.5%)
Previous smoker	60 (30.0%)
Smoker ^c	35 (17.5%)
Unknown	2 (1.0%)

Abbreviations: ALAT, alanine aminotransferase; BMI, body mass index; BSA, body surface area; ECOG; Eastern Cooperative Oncology Group; FIGO, International Federation of Gynecology and Obstetrics; GFR, glomerular filtration rate.

^aPatients receiving adjuvant and/or neoadjuvant chemotherapy had surgery prior to baseline NFL measurements. ^bGrouping was based on the high-risk alcohol consumption limit i.e., 14 units per week for women according to guidelines by the Danish Health Authority. ^cSmokers were defined as patients that have been smoking within the last 6 months at the day of inclusion.

Table 2. Cumulative incidence of adverse outcomes in ovarian cancer patients at each cycle of chemotherapy.

Adverse outcome	Cycle 1 (n)	Cycle 2 (n)	Cycle 3 (n)	Cycle 4 (n)	Cycle 5 (n)	Cycle 6 (n)	Overall incidence ^a (%)
Severe PIPN ^b	12	30	48	64	78	82	43%
Discontinuation due to PIPN ^c	0	5	13	25	40	65	34%
Discontinuation due to other reasons ^d	0	1	1	1	5	8	4%
Dose reduction due to PIPN ^c	0	15	35	54	65	68	36%
Dose reduction due to other reasons ^d	0	2	7	10	11	14	7%

Abbreviations: PIPN, paclitaxel-induced peripheral neuropathy.

^aOverall incidence of adverse outcomes from 190 patients treated with paclitaxel/carboplatin chemotherapy. ^bSevere PIPN is defined as a Common Terminology Criteria for Adverse Events (CTCAE) grade of 2 or higher. ^cPIPN was either the sole reason for discontinuation and dose reduction, or in combination with other toxicities. ^dOther reasons for discontinuation and dose reduction include hematological toxicity, febrile neutropenia, and nephrotoxicity.

Table 3. Unadjusted and adjusted Cox regression highlights that higher sNFL levels after first cycle of paclitaxel/carboplatin chemotherapy is correlated to PIPN-related adverse outcomes. The different sNFL cut-offs were compared to the reference group with sNFL <50 pg/mL.

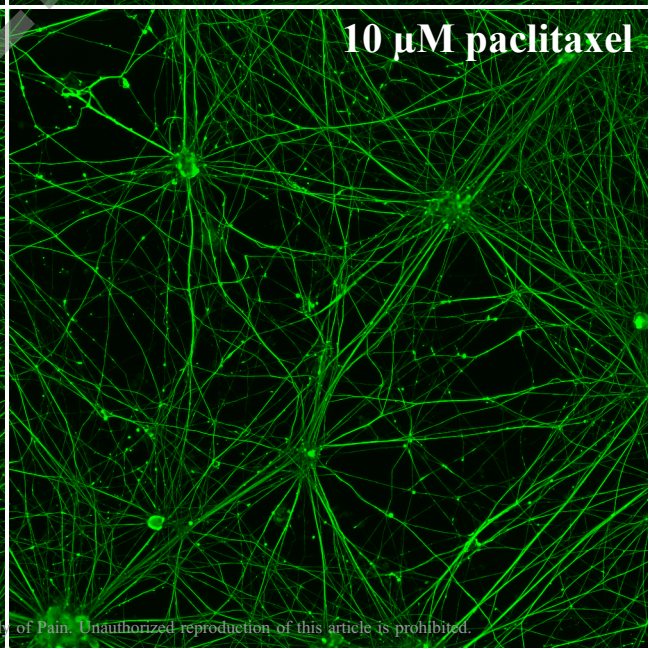
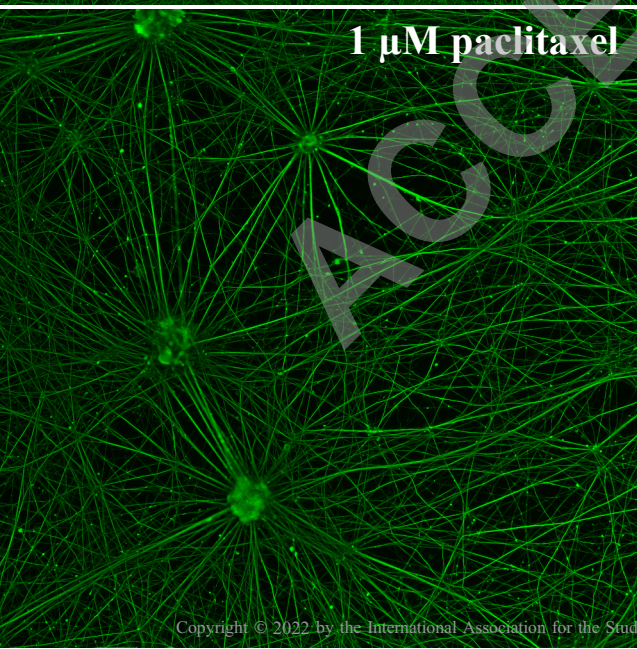
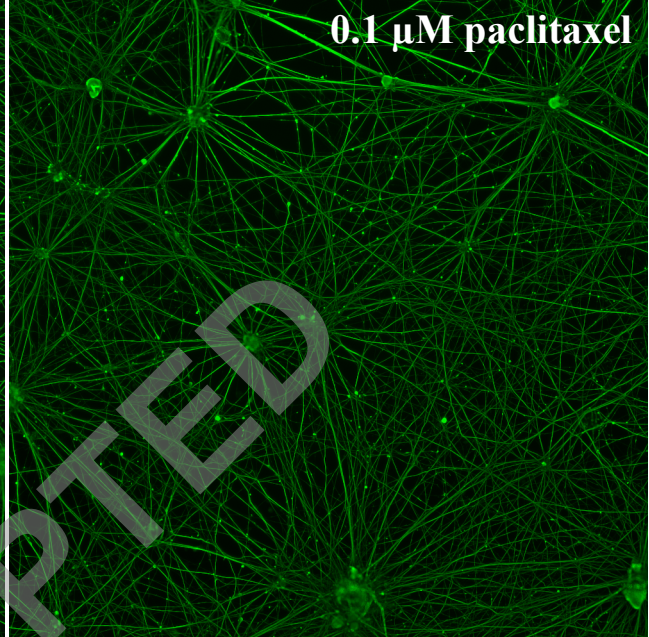
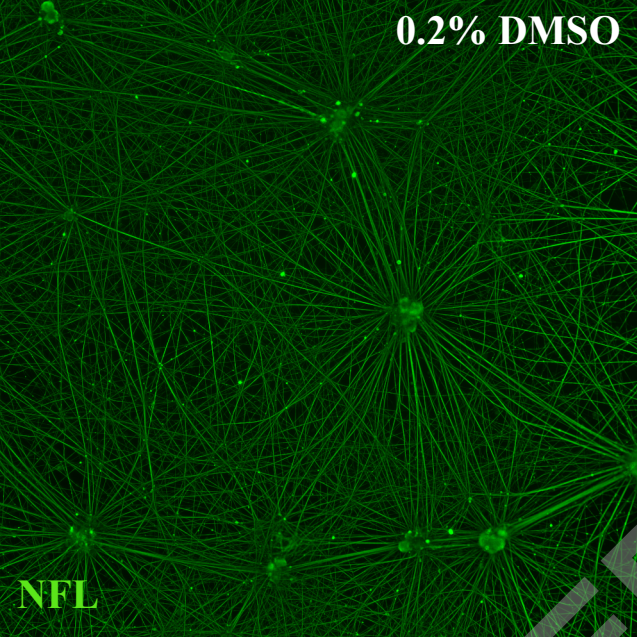
Adverse outcome	sNFL cut-off after first cycle of paclitaxel (pg/mL)	Unadjusted		Adjusted ^a
		n	HR [95% CI]	HR [95% CI]
Severe PIPN ^b	<50	69	Reference	Reference
	50-99	54	1.30 [0.74- 2.29]	1.05 [0.55-2.02]
	100-149	37	1.46 [0.79- 2.70]	1.26 [0.59-2.69]
	>150	29	1.98 [1.03-3.79]	1.66 [0.72-3.84]
Discontinuation due to PIPN ^c	<50	69	Reference	Reference
	50-99	54	1.56 [0.81- 3.01]	1.24 [0.59-2.59]
	100-149	37	1.84 [0.92-3.67]	1.76 [0.76-4.06]
	>150	29	2.47 [1.16- 5.22]	2.25 [0.88-5.79]
Dose reduction due to PIPN ^c	<50	69	Reference	Reference
	50-99	54	1.37 [0.74-2.54]	1.51 [0.72-3.14]

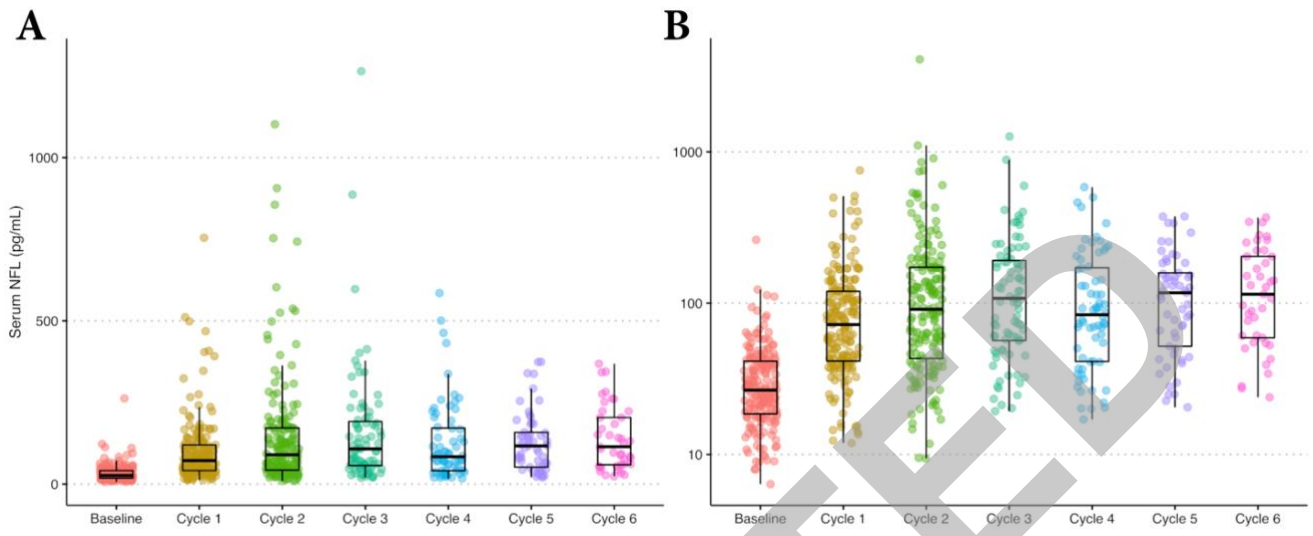
	100-149	37	1.60 [0.85-3.03]	2.03 [0.88-4.70]
	>150	29	1.54 [0.70-3.38]	1.80 [0.66-4.90]

^aCox regression was adjusted for age, body surface area, performance status, alcohol consumption, smoking, liver function (ALAT, hepatic clearance) and kidney function (standard GFR). ^bSevere PIPN is defined as Common Terminology Criteria for Adverse Events (CTCAE) grade of 2 or higher. ^cPIPN was either the sole reason for discontinuation and dose reduction, or in combination with other toxicities.

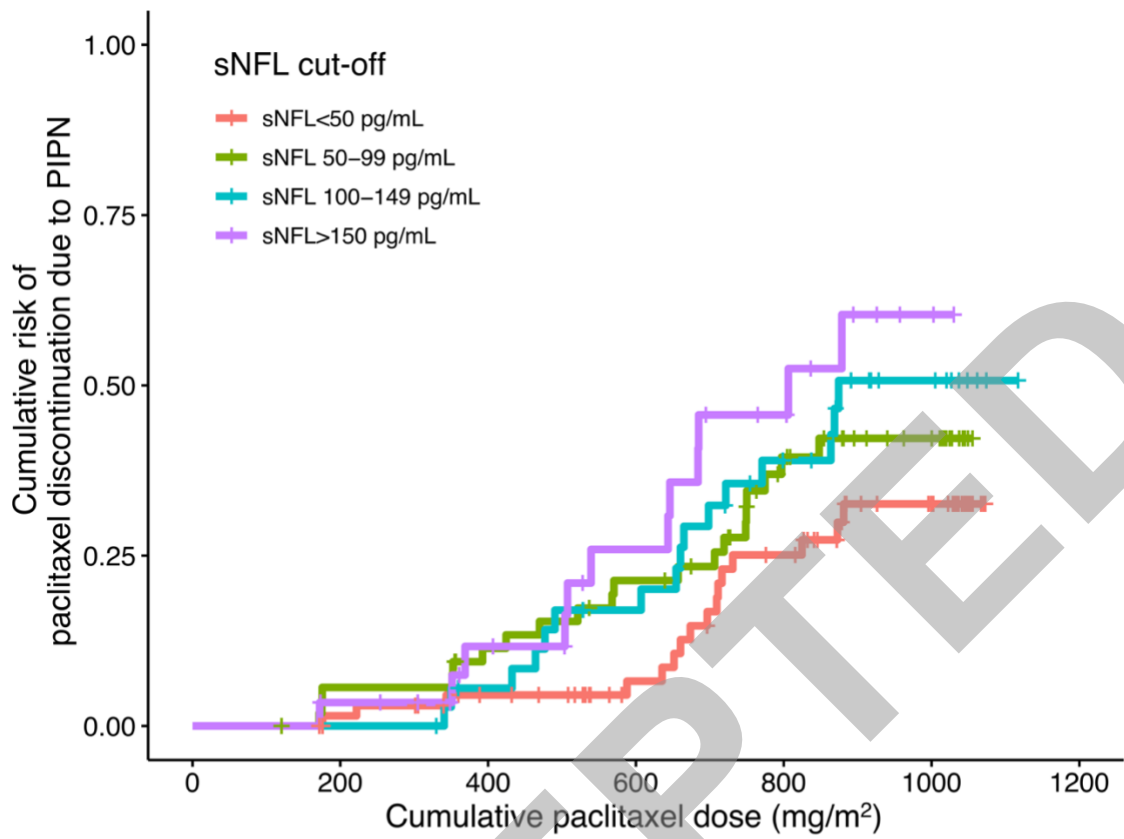
Abbreviations: ALAT, alanine aminotransferase; CI, confidence interval; GFR, glomerular filtration rate; HR, hazard ratio; PIPN, paclitaxel-induced peripheral neuropathy.

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Number at risk

69	66	59	46	35	18	0
54	50	45	39	24	14	0
37	37	33	27	17	8	0
29	27	21	15	9	2	0

NFL level relative to control

