Plasma levels of platinum-induced fatty acid [16:4n-3] do not affect response to platinum-based chemotherapy: A pilot study in non-small cell lung cancer patients

Van der Meij, Barbara S; Teleni, Laisa; Stanislaus, Avalyn E.; Murphy, Rachel A.; Robinson, Lindsay; Damaraju, Vijaya L.; Chu, Quincy; Sawyer, Michael B.; Mazurak, Vera

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Title Page

Title

Plasma levels of Platinum-Induced Fatty Acid [16:4n-3] do not affect response to platinum-based chemotherapy: a pilot study in non-small cell lung cancer patients

Authors

Barbara S. van der Meij

Bond University Nutrition and Dietetics Research Group, Faculty of Health Sciences and Medicine, Bond University, Australia; and

Department of Dietetics and Foodservices, Mater Health Services, Mater Hospital, Australia

Highest academic degree: PhD

e: bvanderm@bond.edu.au

Laisa Teleni

Bond University Nutrition and Dietetics Research Group, Faculty of Health Sciences and Medicine, Bond University, Australia

Highest academic degree: Master of Nutrition and Dietetics (MND)

e:laisa.teleni@gmail.com

Avalyn E. Stanislaus

Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Canada

Highest academic degree: PhD

e:avalyn@ualberta.ca

Rachel A Murphy

School of Population and Public Health, University of British Columbia, Canada

Highest academic degree: PhD

e:rachel.murphy@ubc.ca

Lindsay Robinson

Human Health and Nutritional Science, University of Guelph

Guelph, Ontario, Canada, N1G 2W1

Highest academic degree: PhD

e: lrobinso@uoguelph.ca

Highest academic degree: PhD

Vijaya L. Damaraju

Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Canada

Highest academic degree: PhD

e: Vijaya.Damaraju@albertahealthservices.ca

Quincy Chu

Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Canada

Highest academic degree: MD

e: qschu@ualberta.ca

Michael B. Sawyer

Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Canada

Highest academic degree: MD PhD

e: Michael.Sawyer@albertahealthservices.ca

Vera Mazurak

Faculty of Agriculture, Life and Environmental Sciences, University of Alberta, Canada

Highest academic degree: PhD

e: vmazurak@ualberta.ca

Keywords (max 6)

Fish oil, chemotherapy resistance, treatment response, 16:4(n-3), NSCLC

Abbreviations

AI	adequate intake
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ESPEN	European Society of Parenteral and Enteral Nutrition

FAME	fatty acid methyl esters
FO	fish oil
MSCs	mesenchymal stem cells
NSCLC	non-small cell lung cancer
REF	Reference group of non-cancer controls
SC	standard care

Abstract

Background&Aims:

Pre-clinical studies suggest that 16:4(n-3) in purified form or as a component of fish oil might induce platinum-based chemotherapy resistance. Our aim was to determine plasma total and free 16:4(n-3) before and during platinum-based chemotherapy in non-small cell lung cancer (NSCLC) patients supplemented with fish oil or provided standard care, and to explore relationships between plasma 16:4(n-3) levels and tumor response to treatment.

Methods:

In a retrospective, secondary data analysis of a prior clinical trial, plasma from patients with NSCLC (n = 21) who underwent platinum-based chemotherapy and were assigned to 2.2 g/day of eicosapentaenoic (EPA) plus 1.1 g DHA/day as fish oil (FO; n = 12) or received no intervention (standard care; SC; n = 9). Plasma 16:4(n-3) was quantified as free and esterified (total) fatty acid using HPLC-MS/MS. Plasma 16:4(n-3) levels were evaluated over time in relation to fish oil supplementation and response to platinum-based therapy.

Results:

Plasma 16:4(n-3) was detected in all samples. The percentage change/day in plasma esterified (total) 16:4(n-3) was higher for FO versus SC group (2.7 versus -1.8%/d, U = 20, p= .02), but change in plasma free 16:4(n-3) was not different between FO and SC. Median plasma free and esterified 16:4(n-3) were similar between responders and non-responders to platinum-based chemotherapy. Total and free plasma 16:4(n-3) fatty acids were similar between NSCLC patients and REF (NSCLC vs REF: total 16:4(n-3): 122.9 vs. 95.2 nM and free 16:4(n-3) 23.9 vs. 27.6 nM).

Conclusions:

This first of its kind study that evaluated plasma 16:4(n-3) in NSCLC patients showed that 16:4 (n-3) was elevated during FO supplementation, independent of fish oil supplementation or platinum-based chemotherapy.

Introduction

The question of whether or not to consume fish and their oils during chemotherapy treatment for cancer is of interest to oncologists as well as patients and their families. The majority of evidence derived from experimental models of cancer treatment as well as human studies, suggests that provision of fish oil, as a source of the long chain EPA and DHA, concurrent with chemotherapy treatments, attenuates inflammation and improves the tumor effectiveness of anti-neoplastic drugs without imposing additional toxicity on the host (1–3). Ongoing discussions on controversial findings from a series of studies by a single research group (4–7) recently implicated the n-3 fatty acid hexadeca-4,7,10,13-tetraenoic acid [16:4(n-3)] in the development of chemoresistance. In tumor-bearing mice, circulating mesenchymal stem cells (MSCs) injected either at the site of or distant to the tumor were recruited to the tumor site (4). Platinum chemotherapy (cisplatin) stimulated MSCs to secrete the n-3 fatty acid, 16:4(n-3), determined to be responsible for inducing resistance to a number of anti-neoplastic agents (4). MSCs, as well as 16:4n-3 production might be activated by platinum-based chemotherapy. In tumor-bearing mice, 2.5 pmol of purified 16:4(n-3) orally administered induced chemotherapy resistance; cisplatin did not shrink tumors and tumor size was not different from those of a control group receiving vehicle alone. These findings when tested in experimental models by other groups remain uncorroborated and hence are the only line of research to question the use of 16:4n-3 in experimental models of cancer.

Commercially available fish oils may be a source of 16:4(n-3) in addition to providing the precursor for endogenous synthesis of 16:4(n-3)(5). In a series of follow-up experiments, Daenen *et al* (5) detected 16:4(n-3) in unspecified commercially available fish oil supplements to reveal a range of 16:4(n-3) concentrations from 0.2 to 5.7 μM. In healthy humans, consumption of both 10 and 50 ml of these fish oils, resulted in detection of plasma 16:4(n-3) during the postprandial period (8 hours), which was higher than that following oral intake of oily fish (5). As little as 1 μL of fish oil [containing 5.4 μM 16:4(n-3)] induced chemotherapy resistance at the same level as purified 16:4n-3 in BALC/c mice bearing the C26 colon carcinoma.

The controversy that stemmed from the publication of a series of work from Daenen et al. was fueled in part by the lack of recognition of the studies that had reported improved response of tumors to a variety of chemotherapy treatments including platinum-containing therapies during supplementation with n-3 fatty acids such as those found in fish oil. Since the time of the publication, there have been even more studies that have evaluated a variety of drug and tumor combinations in both human and preclinical experimental models, to show that fish oils are safe, and often beneficial to the host while enhancing drug efficacy to the tumor (2). Therefore, results of this experimental series are not aligned with the context of other work in this area and the importance of 16:4n-3 in humans is not yet resolved (8,9), thereby prompting the current study. This retrospective analysis uses a prospectively collected sample set from a previous clinical trial in patients with non-small cell lung cancer

(NSCLC) undergoing platinum-based chemotherapy. Patients were assigned to fish oil supplements or standard of care to answer questions related to safety of fish oil supplementation during cisplatin treatment and to determine its relevance to chemoresistance in humans with cancer. The objectives were to: determine plasma levels of 16:4(n-3) before and during administration of platinum-based therapy; explore whether plasma 16:4(n-3) concentrations increase with intake of fish oil supplements; and explore potential relationships between plasma 16:4(n-3) concentrations and response to chemotherapy.

Materials and Methods

Study design

Plasma samples from NSCLC patients were available from the '*Murphy trial*' (10,11) for which patients were accrued at the Cross Cancer Institute (Edmonton, Canada) between 2007 to 2009 for evaluating the effects of non-randomized intervention of fish oil supplementation compared to standard care during platinum-based chemotherapy (cisplatin and/or carboplatin doublet therapy) in NSCLC patients. The *Murphy trial* (10,11) enrolled 40 NSCLC patients who underwent at least 6 weeks of first line platinum-based doublet chemotherapy. Patients opted to receive either standard care (SC) or fish oil (FO) in an open label study design. Based on previous phase I and II studies of Fearon, Barber and Wigmore (12,13), fish oil supplementation was dosed to meet 2.2 g EPA/day plus 1.1 g DHA/day as either 7.5 ml liquid FO or 4 x 1g capsules of FO per day depending on patient preference to achieve the desired dose. The fish oil dose was determined from the literature and supported a minimum effective dose of 2.0 grams of EPA in attenuating muscle loss in cancer cachexia (12). Compliance was reported to be at least 90% across the FO group as determined by capsule/liquid counts, patient records, and an increase in plasma EPA and DHA concentrations. Baseline characteristics of these groups were compared to a standard care group of patients with the same diagnosis and treatment plan to confirm lack of bias in the patient allocation in each arm of this open label study. Outcomes included muscle mass and tumor response to therapy (defined as complete response, partial response, or stable disease from CT scans of tumor and/or metastases). Plasma was collected from each group at the time of diagnosis (baseline) and one day before each of four cycles of chemotherapy coinciding with blood draws as part of standard clinical care and stored at -80°C until analysis. Written consent to use samples banked from the Murphy Trial was obtained during enrollment. This study was approved by the Health Research Ethics Committee of Alberta (ETH23340 and ETH24078). Plasma from a reference group of non-cancer controls (REF) was obtained from 11 adult men [median age 57y (47-68y)] participating in a study assessing responses to an oral fat tolerance test of a variety of fatty acid compositions(14) and stored at -80°C until analysis.

Sample selection

From the remaining stored plasma samples, patients who provided both a baseline blood sample and at least one sample during 2-4 cycles (approximately 6-12 weeks) of platinum-based chemotherapy were selected for analysis of 16:4(n-3), EPA and DHA content.

Plasma samples were matched to data on patient characteristics and treatment protocols were collected (10,11). Treatment response was collected retrospectively from medical records. Treatment response was determined by oncologists through physical exam accompanied by CT imaging.

Fatty acid analysis

Analysis of plasma EPA and DHA in plasma were quantified in plasma phospholipids (PL) as described earlier (10) and expressed as the total amount (µg/ml) and proportion (% w/w, 'free') of total fatty acids. Briefly, the Folch method was used to extract plasma lipids and phospholipids were isolated using G plates. Bands corresponding to the PL band were scraped and directly methylated prior to analyzing fatty acid methyl esters (FAME) using gas liquid chromatography.

Protocols for quantifying free and total 16:4(n-3) levels in human plasma were established based on the methods from Bollinger (15). Plasma levels of 16:4(n-3) were evaluated using derivatization coupled with LC-MS/MS. Validation methods and results of fatty acid analysis are outlined in the supplementary material.

To determine free 16:4(n-3) concentration, plasma (80 µl) was added to glass tubes and an internal standard (final concentration 25 nM) was added; volume was adjusted to 125 µl with water (HPLC grade). Methanol (250 µL) was added and tubes were gently mixed prior to adjusting the pH < 2 with 1M HCl. Isooctane (750 µl) was added and the mixture was gently vortexed for 2 minutes. Tubes were centrifuged at 1500 g for 2 minutes at room temperature (RT). The upper organic phase was collected, and the mixture re-extracted with another 750 µL of isooctane. Tubes were then centrifuged at 1500 g for 2 minutes at RT and the upper phase added to the previous extract. Extracts were evaporated under nitrogen. Dried extracts were derivatized using a commercially available AMP+ Derivatization kit (Cayman Chemicals). To the dried extract, 20 µL of cold 4:1 Acetonitrile/DMF, 20 µL of cold EDC solution, 10 µL of HOBt solution and 30 µL of the AMP+ solution was added, then vortexed and heated (30 minutes, 60 °C). After cooling, the sample was immediately stored at -30 °C until analysis.

To determine total 16:4(n-3) concentration in plasma, 10 µL methanol and 12.5 µL NaOH were added to 10 µL plasma and mixed. The mixture was heated to 60 °C on a heating block for 30 minutes. The pH was adjusted to pH < 2 with 5M HCl, internal standard (final concentration 25 nM) added and the

volume adjusted to 125 µL with water. To the tubes, 250 µL methanol and 750 µL isooctane were added and extracted and derivatized as described for free 16:4(n-3) determination.

LC-MS/MS analysis was performed on an Agilent 1260 Infinity system coupled to an AB Sciex 3200 QTRAP®. Fatty acid separation was performed on a Phenomenex Luna Omega C18 column (50 x 2.1mm, 1.6 µm) with a SecurityGuard ULTRA C18 guard column. Column oven and autosampler temperatures were set at 40 °C and 10 °C, respectively. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient was 15.5 minutes and was performed as follows: 30-70% B in 8 minutes, increased to 100% B in 0.5 min and held at 100% B for 2 minutes, then 30% B in 0.5 minute and equilibrated at 30% B for 4.5 min. The flowrate was 0.5 mL/min and the injection volume was 5 µL. The mass spectrometer was operated in positive mode using multiple reaction monitoring scans.

Data Analysis

Since 16:4(n-3) values were not normally distributed, data was analyzed using non-parametric tests. Descriptive statistics were presented as median (range) for continuous data or count (%) for categorical data, stratified for treatment group. For categorical data, between-group differences were explored using Fisher's exact test. To test for correlations between plasma fatty acids, we used Spearman's Rho, and changes in fatty acid concentrations from pre- to post-chemotherapy administration were evaluated within group change using the Sign test. Change rates (i.e., % change per day to account for differences in timing between the samples) were assessed using the total change in 16:4(n-3) between two blood draws divided by the number of days between blood draws and compared between groups using the Mann-Whitney U test. Treatment response was collapsed into 'response' (complete response, partial response, stable disease) and 'no response' (progressive disease) categories. Differences in follow-up 16:4(n-3) concentrations between 'response' and 'no response' categories were tested using Mann Whitney U test.

Results

Of the 40 patients in the *Murphy trial* (10,11), 23 patients at baseline (FO = 12, SC = 11) and 21 patients during chemotherapy (FO = 12, SC = 9) had sufficient plasma samples available for additional fatty acid analyses of 16:4 (n-3). There were no significant differences in baseline characteristics between FO and SC groups (**Table 1**).

Plasma 16:4(n-3) levels and fish oil supplementation

The fatty acid 16:4(n-3) was detected in both the free and esterified (total) fractions in all patient and reference samples. In the FO group, total 16:4(n-3) significantly increased from baseline to follow-up ($p = 0.007$; **Table 2**). In the SC group, total 16:4(n-3), free 16:4(n-3), EPA and DHA did not change during chemotherapy. Compared to baseline, FO supplementation was associated with a greater increase in total plasma 16:4(n-3) and plasma EPA when compared with the SC groups ($p = 0.02$ and $p = 0.02$) (Table 2). To account for differences in the number of days between blood draws, a %change/day was calculated. Plasma total 16:4(n-3) was higher for FO versus SC group (2.65%/d versus -1.81%/d, $U = 20$, $p = 0.02$), but change/day in plasma free 16:4(n-3) was not different between FO and SC. There was a strong, positive correlation between the amount of plasma EPA and both total 16:4(n-3) ($r_s = 0.76$, $p < 0.001$) and free 16:4(n-3) $r_s = 0.696$, $p < 0.001$). There was a moderate positive correlation between DHA and total, but not free 16:4(n-3) ($r_s = 0.478$, $p = 0.03$).

Plasma 16:4(n-3) levels and treatment response

Of the patients who received chemotherapy ($n = 21$), seven had progressive disease and 14 responded to treatment [stable disease ($n = 8$), partial response ($n = 3$), and complete response ($n = 3$)]. There was no significant difference in median total plasma 16:4(n-3) in those who responded to treatment versus those with progressive disease (respectively 109.5 (64.1-476.3) versus 94.9 nM (36.1-663.8), $p = 0.37$) or the free plasma fatty acid fraction (respectively 22.4 (2.4-160) versus 23.6 nM (3.9-74.6), $p = 0.88$) (**Figures 1** & **2**).

Plasma 16:4(n-3) levels in cancer versus non-cancer controls

To explore the expected range of 16:4(n-3) in plasma of people without cancer, we evaluated plasma free and total 16:4(n-3) in free living participants. There was no significant difference between pooled baseline NSCLC patients (FO + SC) versus REF subjects: median plasma total 16:4(n-3) [122.93 (37.2-567.4) versus 95.21 nM (40.5-214.1), respectively ($p = 0.73$)] or free 16:4(n-3) [26.18 (4.5-93) versus 27.57 nM (17.5-86.2), respectively ($p = 0.47$)].

Discussion

The most important finding of this study is that plasma 16:4(n-3) does not affect the tumor response to platinum-based therapies in humans. The data aligns with many human and experimental studies but contrasts with a series of preclinical studies that implied fish oil supplementation and consumption of oily fish could be harmful when taken concurrently with platinum-based chemotherapy. To our knowledge, this is the only available biological dataset with the patient group, supplementation, and treatment protocol to evaluate questions related to the relevance of 16:4(n-3) during platinum-based

treatment for cancer in humans. Although this study was limited to NSCLC and did not investigate the full spectrum of platinum-based chemotherapies, cisplatin, and carboplatin are two widely used chemotherapy drugs in standard care, including in countries where fish is often consumed. The data do not support the notion that 16:4(n-3) levels associate with a reduced treatment response.

The most recent publication by Daenen et al. concluded that "The use of [fish oil] products during chemotherapy treatment should be avoided," which gathered considerable media attention and subsequently it was recommended that "Fish oil and fish containing high levels of 16:4(n-3) [one of the fatty acids mentioned before] may best be avoided on days surrounding chemotherapy" (5). This recommendation collides with current health policies, clinical practice guidelines as well as daily clinical oncological practice. Notably, dietary recommendations are made by National health agencies (such as the Food and Drug Administration in the United States) based on best available evidence as well as information on minimum required amounts of essential nutrients and upper limit of safety to set standards and regulations using specific standards of evidence. While there are currently no formal recommendations for fish oil there are recommendations for intake of omega-3 fatty acids, for alpha-linolenic acid (18:3n-3), and for EPA+DHA among the healthy population in some countries and regions. In the oncology setting, the European Society of Parenteral and Enteral Nutrition (ESPEN, 2017) is the leading agency that sets nutritional guidelines based on best available evidence and recommends use of n-3 PUFA, in the form of fish oils, for advanced cancer patients undergoing chemotherapy who are at risk of weight loss or muscle wasting (16).

Our data indicate that 16:4(n-3) is present in total and, to a lesser amount, free fatty acid fractions of plasma, before chemotherapy has commenced at a level similar to that observed in a reference group of non-cancer controls. Levels of plasma total 16:4(n-3) detected in all groups were variable and higher than previously reported in healthy volunteers (5). Total fatty acids represent the collection of fatty acids in the lipoproteins including phospholipid, triglyceride, and cholesterol esters. Lipoproteins are derived from the liver and intestine during the postprandial period. Therefore, the presence of 16:4(n-3) at baseline and in the reference group suggests that 16:4(n-3) is present naturally in those consuming North American diets. Majority of plasma free fatty acids are derived from stored lipids in peripheral tissue and our results suggest storage of 16:4n-3 in adipose tissue. As anticipated, plasma total 16:4(n-3) positively correlated with EPA and increased after fish oil supplementation, a result consistent with Deanen et al. who reported elevated plasma total 16:4(n-3) levels for at least 8 hours following ingestion of a fish oil supplement in healthy humans (n=30) (5). Unlike the experimental series of Roodhardt et al, we did not detect a plasma free or total 16:4(n-3) response following platinum therapy in NSCLC patients. This may relate to timing of measurement in our study; subjects were on daily fish oil supplement which could have been consumed at any time within the 24 hours around their chemotherapy infusion. Blood samples analyzed for 16:4(n-3) levels were drawn just prior to the second, third, or fourth cycle coinciding with blood draws for standard care at this

institution (for example to assess hematological toxicities from chemotherapy). Therefore, whether the concentration of 16:4n-3 would relate to timing of cisplatin delivery cannot be established by the current study and this is recognized as a limitation given the retrospective nature of the analysis. Although this resulted in variable follow-up time points, we standardized baseline and follow-up differences by testing for change over time. Further, we had access to a small reference group of non-cancer subjects that were collected during the same time period for comparison. We also acknowledge the small sample size. In spite of these limitations, we were able to address the question of whether 16:4n-3 relates to tumor response which is revealed with the current study design. We also aimed to correct for number of days on chemotherapy and fish oil by standardizing to a daily unit. An important note is that the sensitivity of detection of 16:4(n-3) is greater than that used in the experimental series of Daenen et al.

Given the recognized importance of consuming fish and their oils in our diet as a source of essential n-3 fatty acids, and potential benefits of fish oil supplementation during chemotherapy, our data provides additional evidence supporting ESPEN guidelines, which actively recommends the consumption of fish oil during cancer treatment. An adequate intake (AI) of omega-3 fatty acids in humans is 1.1 g for females and 1.6 g for males based on best evidence from human studies (17). Platinum-based chemotherapy treatment did not influence plasma 16:4(n-3). While fish oil supplementation increases the amount of 16:4(n-3) in plasma, this fatty acid was not associated with tumor response to platinum-based therapies in humans.

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N/A

Author Contributions

Study conception and design: Teleni, Sawyer, van der Meij, Mazurak

Acquisition of data: Stanislaus, Murphy, Damaraju, Chu, Sawyer, Robinson, Mazurak

Analysis and interpretation of data: Teleni, Stanislaus, Damaraju, Sawyer, van der Meij, Mazurak

Drafting of manuscript: Teleni, van der Meij, Mazurak

Critical revision: Teleni, Stanislaus, Murphy, Damaraju, Chu, Sawyer, van der Meij, Mazurak

Conflicts of Interest Disclosure

The authors declare no conflicts of interest.

Table 1 Participant characteristics at baseline

	REF (n=11)	NSCLC FO (n=12)	NSCLC SC (n=11)
Age, median (range), years	57 (47-68)	63 (45-72)	67 (57-76)
Sex, No. (%)			
Male	11 (100)	8 (67)	5 (46)
Female	0	4 (33)	6 (54)
Cancer Stage, No. (%)	-		
I		-	1(9)
II		1 (8)	1 (9)
IIIA		1 (8)	1 (9)
IIIB		2 (17)	3 (27)
IV		8 (67)	5 (46)
Chemotherapy, No. (%)	-		
Carboplatin		9 (75)	7 (64)
Cisplatin		3 (25)	4 (36)

REF, Reference group of non-cancer controls; NSCLC, non-small cell lung cancer; FO, fish oil group; SC, standard care group

Table 2. Within group differences of plasma PUFAs during platinum-based chemotherapy treatment

| | REF (n=11) | NSCLC FO (n=12) | | NSCLC SC (n=11) | |
	med (range)	Baseline med (range)	Follow-up med (range)	Baseline med (range)	Follow-up[b] med (range)
Total 16:4(n-3)	95.2 nM (40.5-214.1)	128.32 nM (40.5-567.5)	221.47 nM (62.7-663.9)[a*]	122.93 nM (37.2-254.8)	67.13 nM (36.1-146.1)
Free 16:4(n-3)	27.57 nM (17.5-86.2)	40.27 nM (7.9-93)	36.21 nM (11.3-160)	23.89 nM (4.5-59)	15.33 nM (2.4-35.6)
EPA	3.9 (0.4-12.4)	6.94 µg/ml (4-13.7)	152.94 µg/ml (1.7-41.7)[a*]	7.55 µg/ml (1.1-14.3)	6.85 µg/ml (1.3-14.4)
DHA	6.35 (1.6-15.2)	14.83 µg/ml (4.5-27)	15.22 µg/ml (2.0-33.3)	10.93 µg/ml (6.9-23.7)	13.29 µg/ml (3.6-21.4)

REF, Reference group of non-cancer controls; NSCLC, non-small cell lung cancer; FO, fish oil group; SC, standard care group; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid.

[a]Sign test (exact significance, 2-tailed) using binomial distribution comparing absolute median values at baseline and follow-up within each group where *p<0.05, **p<0.001

[b]missing values = 2.

Figure legends

Figure 1. Follow-up total 16:4(n-3) by treatment response in non-small cell lung cancer patients.

Figure 2. Follow-up free 16:4(n-3) by treatment response in non-small cell lung cancer patients.

Supplementary Files

- Fatty Acid Validation (Summary in MS Word)
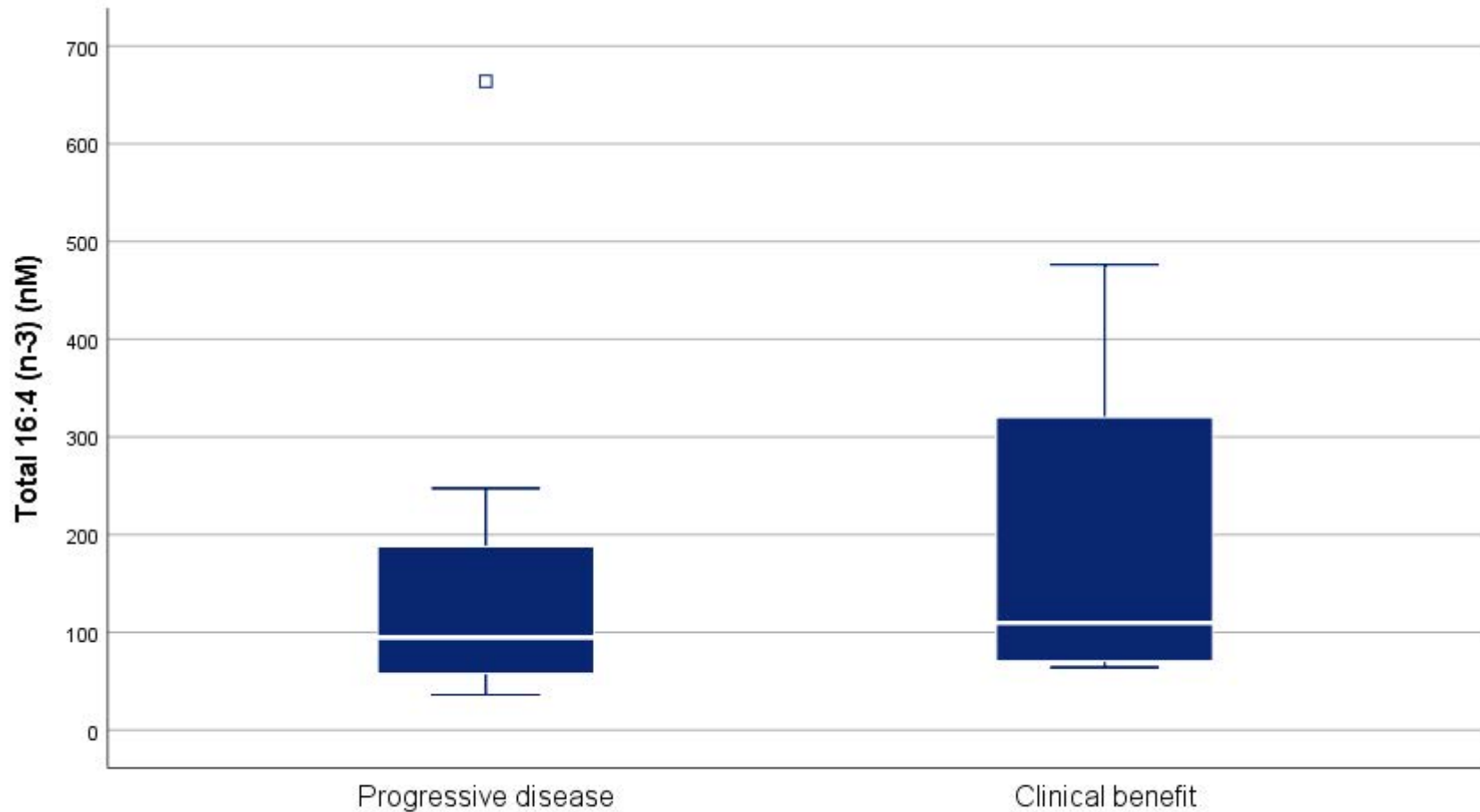- Stability Tests (Data in MS Excel)

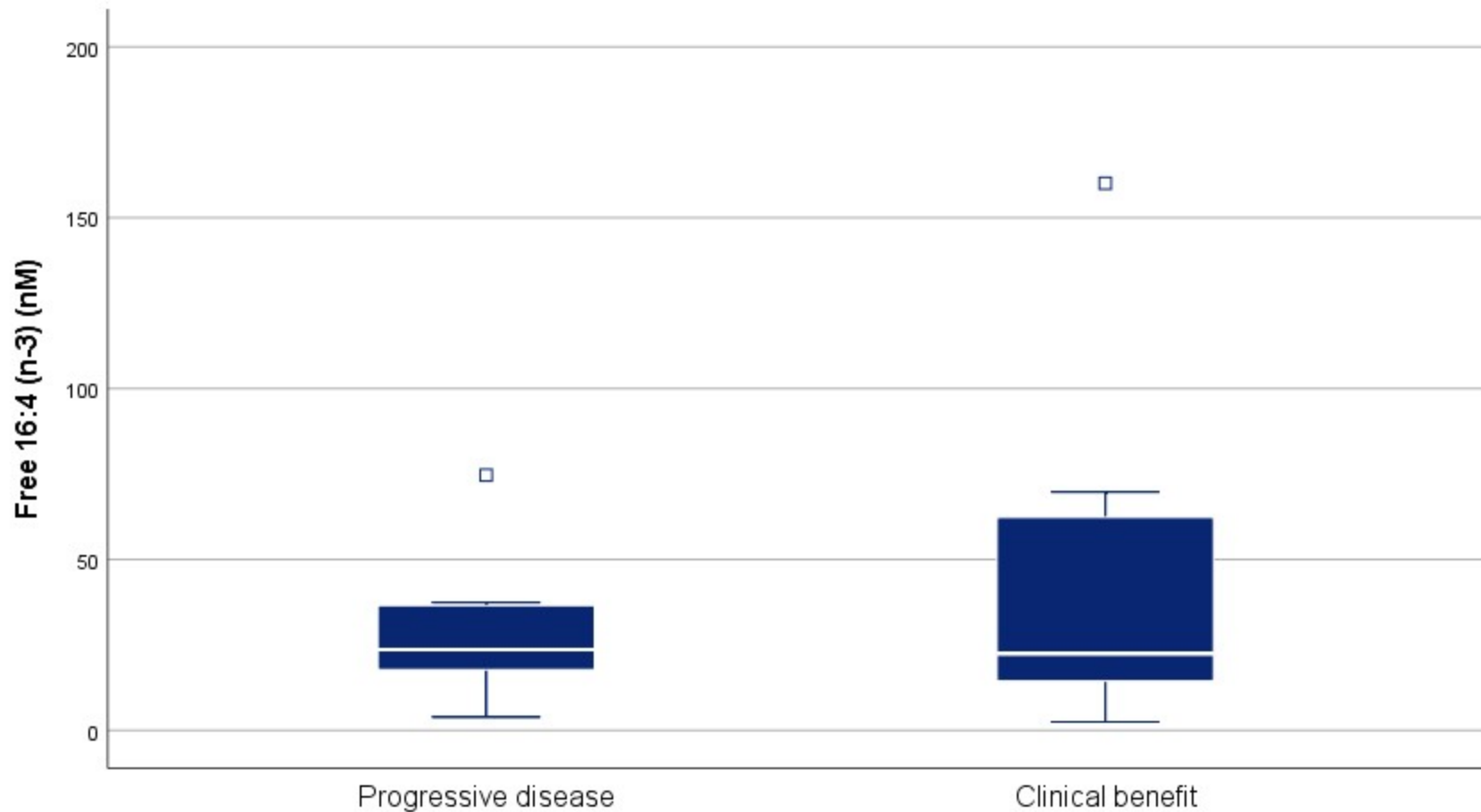**Figure 1. Follow-up total 16:4 (n-3) by treatment response in non-small cell lung cancer patients**

Figure 2. Follow-up free 16:4 (n-3) by treatment response in non-small cell lung cancer patients

6 hr on a benchtop at room temperature

Sample Name	Actual Conc. (nM)	Value #1	Value #2	Value #3	Mean	Std. Dev.	Percent CV	Accuracy (%)
Plasma Blank	16.76	17.26	17.14	16.55	16.98	0.38	2.23	**101.35**
QC Low	24.75	25.71	24.19	24.65	24.85	0.78	3.14	**100.38**
QC Mid	36.75	35.27	34.95	36.45	35.56	0.79	2.22	**96.76**
QC High	96.73	104.73	98.33	97.46	100.17	3.97	3.96	**103.56**

30 days stored at -30 ℃ (Freezer)

Sample Name	Actual Conc. (nM)	Value #1	Value #2	Value #3	Mean	Std. Dev.	Percent CV	Accuracy
Plasma Blank	16.76	15.11	15.63	16.32	15.69	0.61	3.88	**93.61**
QC Low	24.75	25.13	24.30	25.16	24.86	0.49	1.97	**100.44**
QC Mid	36.75	35.10	35.31	34.50	34.97	0.42	1.20	**95.16**
QC High	96.73	98.24	99.75	100.34	99.44	1.08	1.09	**102.80**

Supplementary information

Plasma levels of Platinum-Induced Fatty Acid [16:4n-3] do not affect response to platinum-based chemotherapy: a pilot study in non-small cell lung cancer patients
BS van der Meij, L Teleni, AE Stanislaus, RA Murphy, L Robinson, VL Damaraju, Q Chu, MB Sawyer, VC Mazurak

Fatty Acid Validation

Methods

The analytical method used in the quantification of fatty acid in human plasma was validated for selectivity, carryover, extraction recovery, matrix effect, linearity, accuracy and precision, and stability.

Selectivity

Specificity was evaluated according to the following: 80 µL water (done in triplicate) was saponified, extracted and derivatized according to the methods outlined and were analyzed for interferences at the retention times of the analyte and internal standard.

Accuracy and Precision

Accuracy and precision were determined by extracting plasma spiked at three concentrations on three separate days. QC samples spiked at 8, 20 and 80 nM were extracted and analyzed in triplicate. Precision was assessed by calculating the coefficient of variation and accuracy was calculated using the following equation below. Precision should be less than 15% and accuracy should be in the range of 85-115%.

$$\text{Accuracy (\%)} = \frac{\text{Final concentration calculated}}{(\text{Endogenous concentration} + \text{Spiked concentration})} \times 100$$

Stability

Stability was investigated by extracting and analyzing triplates of QC samples (spiked concentrations 8, 20, 80 mM) at the following conditions: 1) autosampler condition (10 °C) for 6 and 12 hours; 2) benchtop

for 6 hours; 3) four freeze/thaw cycles; and 4) storage at -30 °C for 30 days. The results were calculated in the same manner as accuracy and reported.

Results

Selectivity

There were no interferences observed at the retention times of FA($16:4_{n-3}$) and its internal standard. The retention time of the fatty acid and internal standard is approximately 5.22 min and 5.20 min, respectively.

Isooctane Method Blank

Plasma Blank
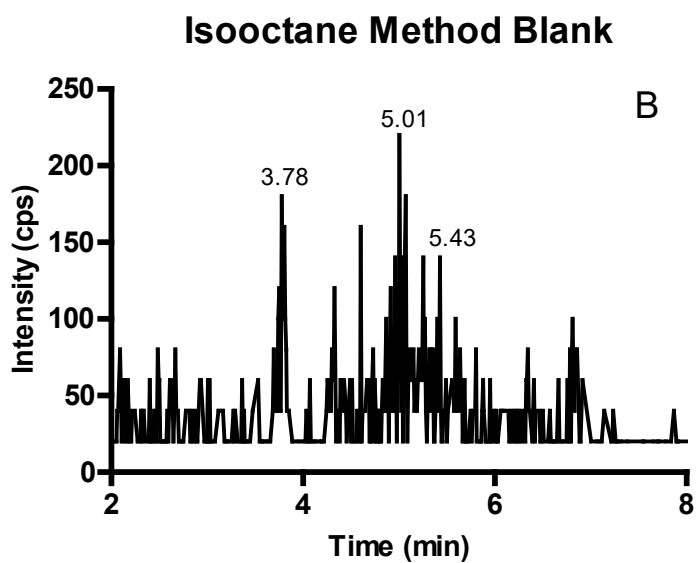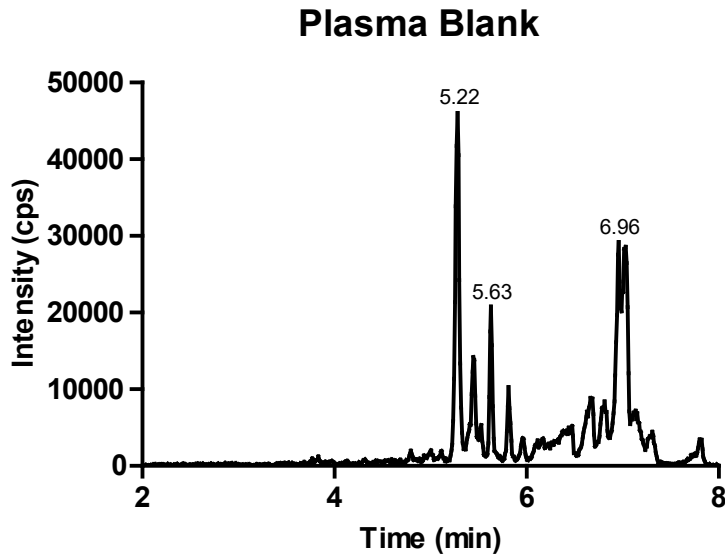
Accuracy and Precision

The following tables summarize the accuracy and precision for the QC samples (n=3). Each day is listed individually and the replicates, mean, standard deviation, precision (%CV) and accuracy are shown. The results show acceptable accuracy and precision, illustrating that the analytical method used was precise and accurate.

Stability

The following tables summarize the results of the stability assessments. The accuracy of the QC samples were all in the acceptable range (85-115%), which is an indication that there was no significant degradation of the analyte under the conditions tested.