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Application Note

# Application of LipidQuan to the Study of Prostate Cancer and the Response to Various Treatment Therapies

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## Abstract

Robust and reproducible targeted quantification methods for lipids that enable differentiation between various stages of prostate cancer (PCa) and treatment therapies are a key area of translational research. This application note demonstrates how the easy to deploy LipididQuan Platform can be implemented to develop a targeted assay, whilst method development and training costs are reduced when using the Quanpedia Library. The library contains over 2000 lipids with improved identification and specificity (***e.g.*** the use two fatty acyl chain fragments for phospholipid related MRMs increases specificity). Fast data processing and visualization using TargetLynx Software and third-party informatics (***i.e.***, Skyline) offers maximum flexibility to researchers. Parameters such as linear dynamic range, accuracy, and precision are assessed to show method reproducibility and robustness. Quantification of significant lipids provides differentiation between the PCa pilot study groups investigated using this methodology.

## Benefits

- Targeted quantification method for lipids that enable differentiation between various stages of prostate cancer and treatment therapies
- A robust and easy to deploy platform reducing method development and training costs using Quanpedia
- Improved identification and specificity of phospholipids using MRM transitions from the two fatty acyl chain fragments
- Fast data processing and visualization using TargetLynx Software and third-party informatics (*i.e.*, Skyline) for maximum flexibility

## Introduction

The diagnosis and prognostication for prostate cancer (PCa) remains challenging in this relatively common cancer (40% of all cancers worldwide).[1] Prostate-specific antigen (PSA) levels is the most common of the few blood-based protein biomarkers currently available in clinical practice, however, PSA by itself is not accurate especially as there is no reliable PSA range that is an explicit signifier for the presence of PCa.[2] Appropriate assay accuracy, precision, and sensitivity still need to be attained. Studies on potential biomarkers and measurable signatures for the disease thus remain a key area of translational research.

Across several studies, researchers have identified lysophosphatidylcholines (LPC), phosphatidylcholines (PC), lysophosphatidylethanolamines (LPE), phosphatidylethanolamines, and sphingomyelins (SM) as diagnostic signatures to characterize PCa.[4,5] More recently, a multi 'omics study combining lipid and protein measurements respectively has also identified biomarkers with improved utility in determining disease status of men likely to have prostate cancer.[6]

Here, we describe the utilization of the LipidQuan Workflow < https://www.waters.com/webassets/cms/library/docs/targeted_omics_method_library/LipidQuan%20Method%20Guide_22D > [7] to develop a polarity switching method for the quantification of the key lipid species involved in the biomolecular processes associated with prostate cancer and variations observed based on treatment therapies.

## Experimental

### Sample

Serum-based samples from 42 individuals were pooled to form phenotypic groups. These pools comprised of controls (individuals diagnosed with PCa) (n=6), active surveillance (AS) (n=6), Brachytherapy (n=6), hormone therapy only (n=6), healthy controls (n=6), combined radiotherapy and hormone therapy (n=6), and prostatectomy (n=6).

Avanti Odd-Chained LIPIDOMIX was used to prepare three spiking solutions at 10x, 20x, and 50x dilution in IPA. Neat standard mix and spiking solutions were spiked directly into pooled "control plasma" (anticoagulant, K2 EDTA) at less than 5% v/v (minimizing the impact of spiking on the matrix) to generate a 10-point calibration curve and 3 QC level (LQC, MQC, and HQC) samples at concentrations ranging from 5 ng/mL to 423,750 ng/mL in glass vials for all available lipid standards. A study reference (SR) pool of all 7 pool samples and NIST SRM 1950–Metabolites in frozen human plasma sourced through Sigma Aldrich (Gillingham, Dorset, UK) was also used for QC purposes.

## Sample Preparation

Firstly, 25 µL aliquots of calibrants, QCs, and samples were transferred to LoBind microtubes (Eppendorf, Hamburg, Germany). To this, 125 µL IPA/ACN (1:2, v/v) containing a 500-fold dilution of neat deuterated ceramide LIPIDOMIX and SPLASH LIPIDOMIX as internal standards were added to each well plate, except those of the double banks. The internal standard mix covers multiple lipids classes and is comprised of heavy (d7-d9) isotopes. Double blanks were prepared using IPA/ACN (1:2, v/v) with no internal standards. Samples were vortex mixed for 30 seconds before incubating with shaking at 5 °C for 2 hours to ensure complete protein precipitation. The extracted samples were centrifuged at 3000 g for 20 minutes at 10 °C before transferring the supernatant to Total Recovery UPLC Vials (Waters, p/n 186005669CV <https://www.waters.com/nextgen/us/en/shop/vials-containers--collection-plates/186005669cv-truview-lcms-certified-clear-glass-12-x-32-mm-screw-neck-total-r.html> ) for LC-MS analysis.

## LC Conditions

| | |
|---|---|
| LC system: | ACQUITY Premier I-Class Flow Through Needle (FTN) |
| Column(s): | ACQUITY Premier UPLC BEH Amide 2.1 x 100 mm, 1.7 µm, p/n: 186009505 |
| Column temperature: | 45 °C |

| | |
|---|---|
| Flow rate: | 0.6 mL/min |
| Mobile phase: | 95:5 Acetonitrile/water + 10 mM Ammonium acetate (A) and 50:50 Acetonitrile/water + 10 mM Ammonium acetate (B) |
| Gradient: | 0.1% to 20.0% B for 2 minutes, then 20% to 80% B for 3 minutes followed by 3 minutes re-equilibration |
| Run time: | 8 minutes |
| Injection volume: | 1 µL |

## MS Conditions

| | |
|---|---|
| MS systems: | Xevo TQ-XS |
| Ionization mode: | ESI (+/-) – Polarity switching |
| Capillary voltage: | 2.8 kV (+) 1.9 kV (-) |
| Acquisition mode: | MRM |
| Source temperature: | 120 °C |
| Desolvation temperature: | 500 °C |
| Cone gas flow: | 150 L/hr |
| Desolvation flow: | 1000 L/hr |
| Nebulizer gas: | 7 bar |

| | |
|---|---|
| Ion guide offset 1: | 3 V |
| Ion guide offset 2: | 0.3 V |

## Informatics

The Waters Targeted Omics Method Library (TOML) containing the LC conditions, MS method and associated TargetLynx processing method (including retention times and MRM transitions) was used to generate these methods. The resulting data were processed using either TargetLynx or Skyline < https://skyline.ms/project/home/begin.view?> (MacCoss Lab, University of Washington).

# Results and Discussion

Targeted lipid analysis of serum samples from individuals diagnosed with prostate cancer, patients undergoing various treatments and healthy subjects were conducted using the LipidQuan platform. The samples were prepared for LC-MS analysis using the sample preparation method described in the previous section. The LipidQuan method package was downloaded from the Waters Targeted Omics Method Library (TOML; www.waters.com/targetedomics <http://www.waters.com/targetedomics> ) and imported into MassLynx.

The Quanpedia method file included in the method package contained chromatographic settings and MRM transitions for over 2000 lipids. Using the Quanpedia file to import pre-populated method files eliminated manual input of LC-MS/MS settings and any potential transcription errors. For this study, a targeted polarity switch LC-MS method was generated to monitor 39 endogenous lipids across Cer, LPE, LPI, PG, PI, and SM classes (Figure 1) using the Quanpedia Library. The method measures and quantifies 4 LPE species in both polarity modes.

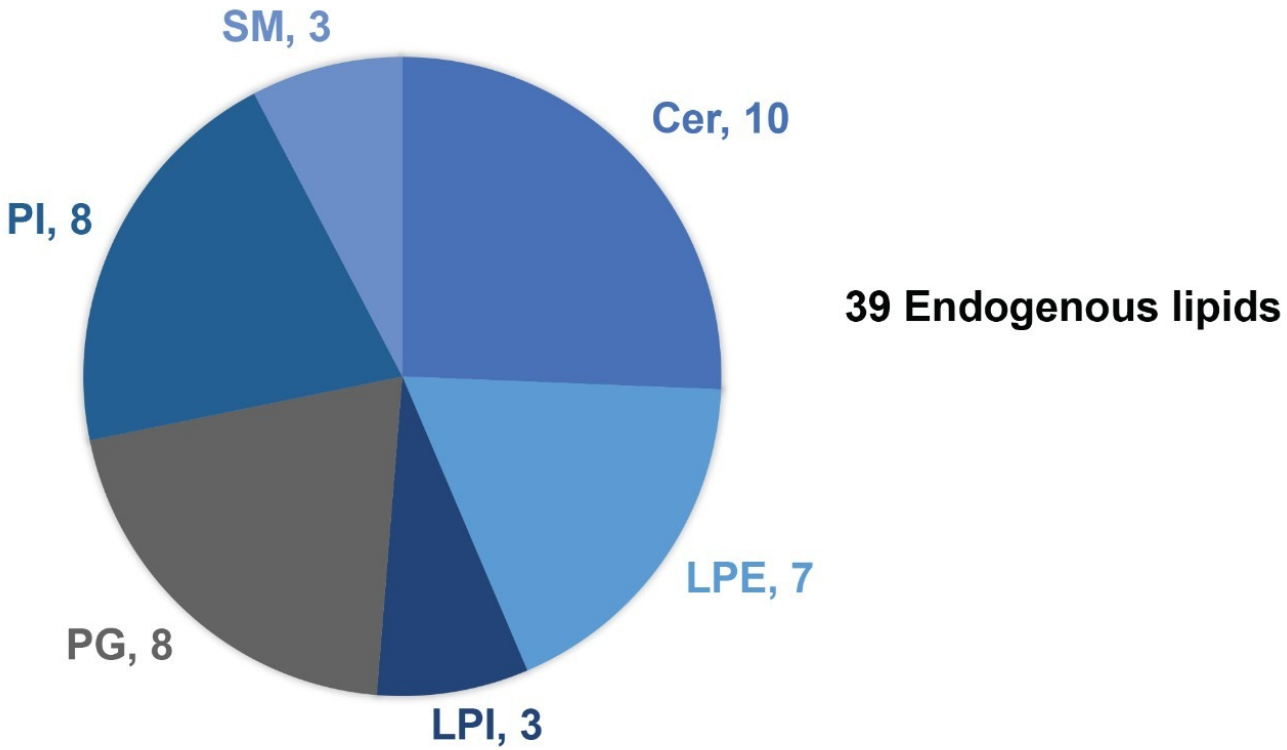


Figure 1. 39 lipids quantified using a polarity switching method. Four of the LPE species are measured in both positive and negative polarity modes.

A solution of 32 standards covering the key analytes where available and others from the classes of interest were used to confirm lipid identifications, retention times, and MRM transitions during method development. An example chromatogram is shown in Figure 2A. Replicates of pooled samples were injected in a randomized order, followed by injections of the QC sample, spiked with odd chain standards at 3 concentration levels, SR and NIST samples. Calibration curve samples were analyzed at the start of the run and at the end of the run. A representative chromatogram of study samples is displayed in Figure 2B.

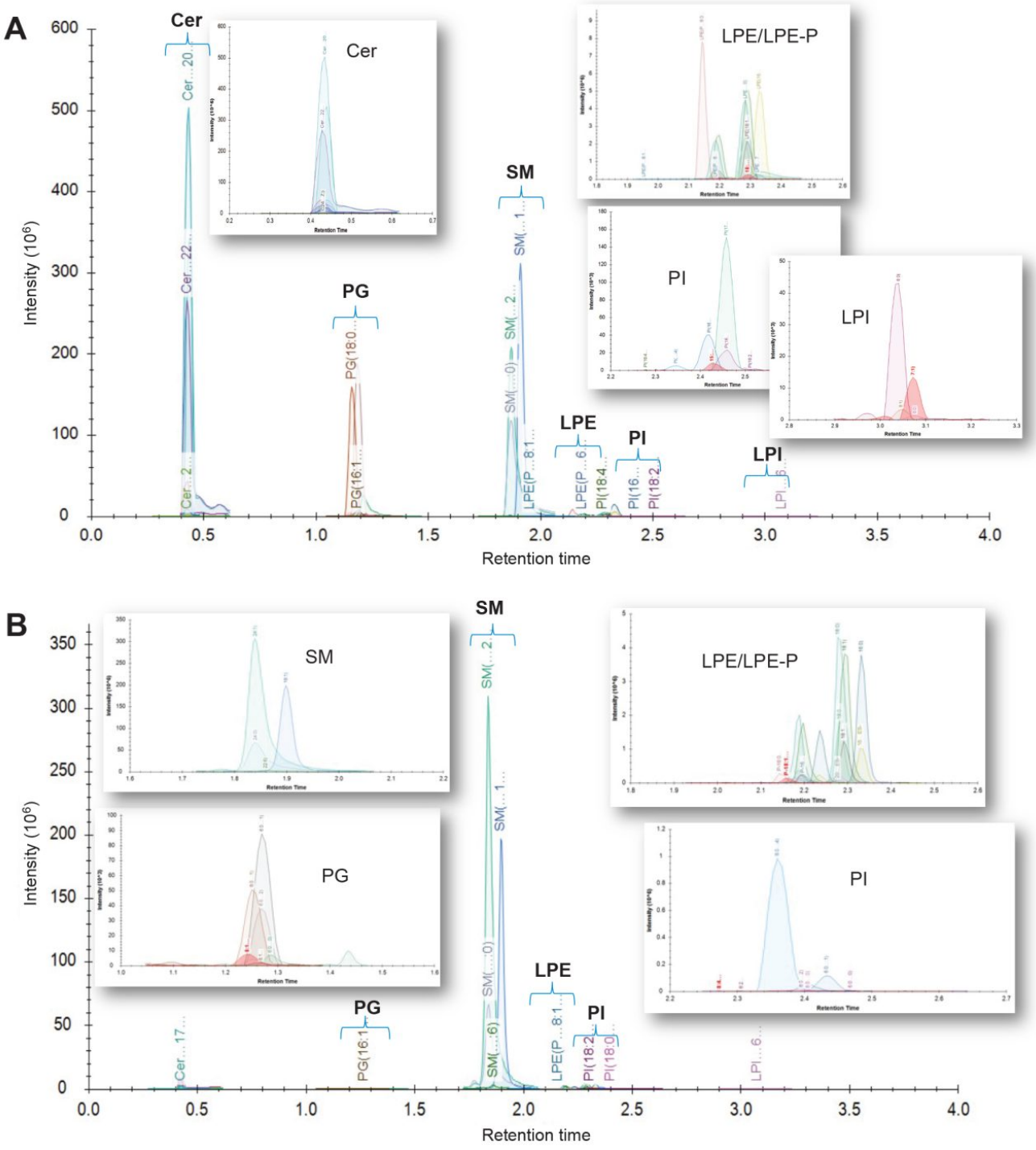


Figure 2. (A) Chromatograms representing the lipid standards used to confirm retention times and MRM transitions to be monitored in serum; (B) Chromatograms representing the various endogenous lipids in serum to be quantified.

Quantification was achieved using calibration curves of plasma spiked with known concentrations of standards prior to extraction. The area responses of calibrants and endogenous lipids were normalized to the deuterated SIL responses which are used as internal standards. Odd Chain lipid standards were used to assess linear

response to concentration, for the various lipid classes of interest across the ranges described in Table 1. Typical $R^2$ values ranged from 0.985–0.996, with example calibration curves shown in Figure 3. Odd Chain lipid standards spiked QC samples at 3 concentrations were used to monitor method precision. Box and whisker plots of these QC samples are represented in Figure 4. The results in Table 2 show that the coefficients of variance (CV) were all below 15% across the acquisition. These findings provide confidence in the data quality for the accurate and precise measurement of endogenous lipids of interest over the course of the analysis.

| Standards | Retention time (min) | Acquisition mode | Calibration range (ng/mL) | Correlation coefficient |
|---|---|---|---|---|
| LPE (17:1) | 2.33 | +ve | 4.8–600 | 0.987 |
| LPE (17:1) ES- | 2.33 | -ve | 4.8–600 | 0.990 |
| LPI (17:1) | 3.07 | -ve | 5.2–650 | 0.985 |
| PG (17:0/14:1) | 1.25 | -ve | 36–4500 | 0.996 |
| PI (17:0/14:1) | 2.46 | -ve | 80–10000 | 0.995 |
| SM (d18:1/12:0) | 1.99 | +ve | 260–32500 | 0.996 |

Table 1. Calibration parameters of standards spiked in plasma.

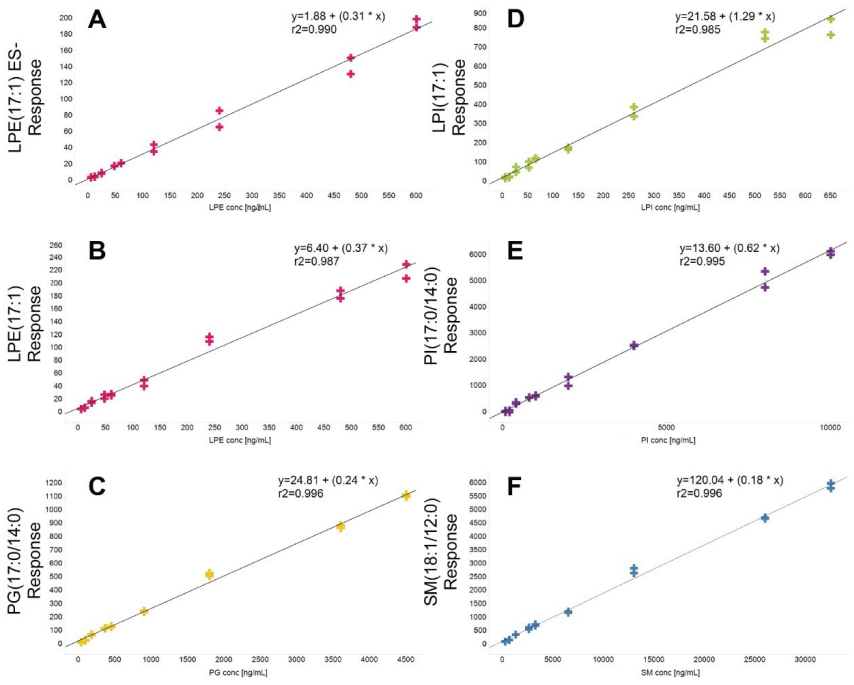


Figure 3. Example standard calibration curves representing LPE (ES-) R2= 0.99 (A), LPE (ES+) R2= 0.99 (B), PG R2= 0.99(C), LPI R2= 0.99 (D), PI R2= 0.99 (E), and SM R2= 0.99(F).

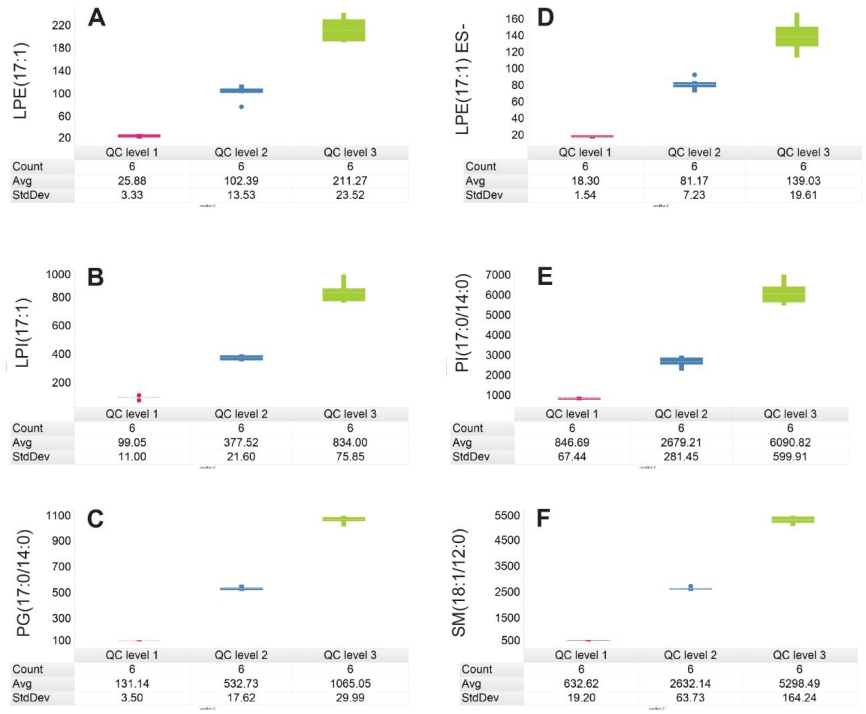


Figure 4. LQC, MQC, and HQC sample responses across the acquisition. QCs with Avanti Odd chain Lipidomix spiked at 3 concentration levels were injected following each set of sample replicates. The QC responses for LPE (ES-) (A), LPE (ES+) (B), PG (C), LPI (D), PI (E) and SM (F) spiked standards are displayed.

| Standards | n=6 | QC level 1 | QC level 2 | QC level 2 |
|---|---|---|---|---|
| | | LQC | MQC | HQC |
| LPE (17:1) | %CV | 12.85 | 13.22 | 11.13 |
| LPE (17:1) ES- | %CV | 8.41 | 8.91 | 14.11 |
| LPI (17:1) | %CV | 11.10 | 5.72 | 9.09 |
| PG (17:0/14:1) | %CV | 7.96 | 10.50 | 9.85 |
| PI (17:0/14:1) | %CV | 2.67 | 3.31 | 2.82 |
| SM (d18:1/12:0) | %CV | 3.03 | 2.42 | 3.10 |

Table 2. QC precision across the run determined using CVs of spiked standards. The table shows the CVs were <15%.

The lipidomic analysis highlighted a number of significant species, including SM, Cer, and LPE species. For example, LPE(20:3), LPE(P-16:0), and LPE(P-18:1) were key lipids for differentiating between patients categorized as controls (*i.e.* individuals diagnosed with PCa) and those undergoing hormone therapy. Box and whisker plots comparing the calculated concentration levels of these LPE species are displayed in Figure 5. The concentration of LPE(20:3) was determined in both positive and negative ion mode (Figure 5A and 5B) with the concentation

relating to control (PCa) individuals quantified as 79.7 ng/mL and 73.3 ng/mL in positive and negative mode respectively. Comparatively, the concentation of LPE(20:3) in hormone therapy patient pool samples was reported as 146.5 ng/mL in positive mode and 136.3 ng/mL for negative mode. LPE(P-16:0) and LPE(P-18:1) were only measured in negative mode but are also shown to be over expressed for the hormone treated group.

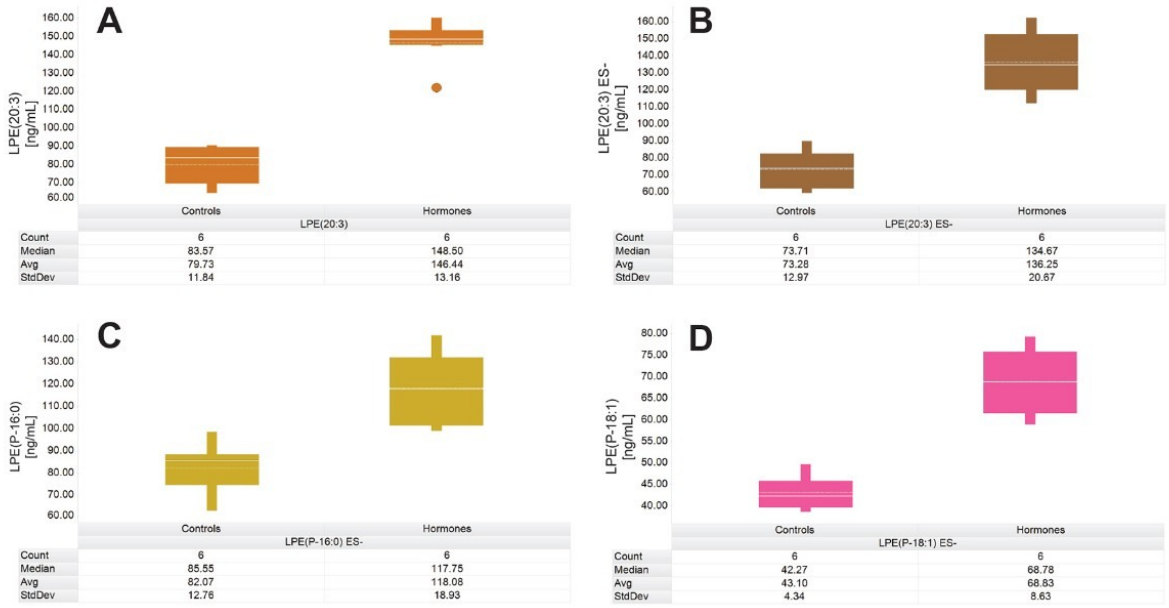


Figure 5. Box plots representing key LPE lipids, which show differential expression between controls (PCa diagnosed) and hormone therapy treated individuals. LPE(20:3) was analysed in both positive (A) and negative mode (B). LPE(P-16:0) (C) and LPE(P-18:1) (D) were analyzed in negative mode only. Quantification was performed using LPE(17:1) calibration curves.

## Conclusion

- A rapid, quantitative lipidomic method (LipidQuan) was developed and deployed for the analysis of serum in a prostate cancer related study.
- The method was shown to be linear over 3-orders of magnitude and had sufficient sensitivity to allow for the

analysis of lipids at endogenous levels in human serum.

- Employing HILIC-based chromatography allowed lipids to elute according to class thereby reducing the potential for isomeric/isobaric interferences and the number of calibrant and internal standards required for quantification (*i.e.*, cost reductions).
- The methodology allowed for the quantification of key lipid species identified from the integrated lipidomics and proteomics study.
- LPEs, Cer, and SM were found to be over expressed in prostate cancer samples compared to control samples.

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