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Identification of Lipidomic Profiles Associated with Drug-Resistant Prostate Cancer Cells

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Abstract

Background: The association of circulating lipids with clinical outcomes of drug-resistant castration-resistant prostate cancer (CRPC) is not fully understood. While it is known that increases in select lipids correlates to decreased survival, neither the mechanisms mediating these alterations nor the correlation of resistance to drug treatments are well characterized.

Methods: We addressed this gap-in-knowledge using *in vitro* models of non-cancerous, hormone-sensitive, CRPC and drug-resistant cell lines combined with quantitative LC-ESI-Orbitrap-MS lipidomic analysis and subsequent analysis such as Metaboanalyst and Lipid Pathway Enrichment (LIPEA).

Results: This approach identified several lipid regulatory pathways associated with Docetaxel resistance in PCa. These included those controlling glycerophospholipid metabolism, sphingolipid signaling pathways and ferroptosis. In total, 7,460 features were identified as being dysregulated between the cell lines studied, and 21 lipid species were significantly altered in drug-resistant cell lines as compared to nonresistant cell lines. Docetaxel resistance cells (PC3-Rx and DU145-DR) and had higher levels of phosphatidylcholine (PC), oxidized lipid species, phosphatidylethanolamine (PE), and sphingomyelin (SM) as compared to parent control cells (PC-3 and DU-145). These cells also had higher levels of ceramides release into the media.

Conclusion: These data identify lipids whose levels may correlate to Docetaxel sensitivity and progression of prostate cancer.

Introduction

Drug resistance is a major obstacle for development of PCa treatments. Although reports show that taxane-mediated microtubule stabilization differentially affects the androgen receptor, Docetaxel, a first-line chemotherapy for metastatic CRPC, has a known associated mechanism of resistance. The actual mechanisms linking the changes in lipids levels to the generation of drug resistance in prostate cancer cells are unclear. This is despite several studies correlating unbalanced cellular lipid composition and quantity to altered cellular functions that contribute drug resistance [1].

Cancer cells regulate their cellular lipids in a multifaceted process. Extensive studies provide strong evidence for reprogramming of lipid metabolism in cancer [2-4]. Many of these studies are fueled by advances in mass spectrometry allowing for enhanced analysis of changes in the cellular or blood lipidome. While these studies have identified specific lipids, they have been hampered by the fact that changes in these lipids have not been put into context with changes in the regulation of overall lipid metabolism pathways. Further, changes in these lipids have not been correlated to changes in androgen or drug resistance. One reason for this gap-in-knowledge is that, unlike genomic and proteomics, analysis tools allowing for assessment of lipid regulation pathways in tandem with lipidomic outcomes are not common. Recent advances have resulted in the development of such tools, such as the Lipid Pathway Enrichment Analysis, or LIPEA [5]. However, this approach has only seen limited application and has not been applied to studies in cancer cells.

PCa cells, in part, obtain and employ lipids to meet increasing energy demands for cell proliferation in a nutrient-deprived tumor microenvironment [6-12]. Many of the

mechanisms mediating changes in lipid profiles during this process are not well understood. The present study used LIPEA, in conjunction with data derived from Metaboanalyst to identify the underlying regulatory lipid pathways associated with Docetaxel resistance in PCa. Further, we focused on lipidomic changes in multiple models, including non-cancerous, hormone-sensitive and CRPC and Docetaxel resistant cell lines. We then validated this pathway analysis using LC-ESI-MS/MS. To our knowledge, these studies represent one of the most comprehensive identification of differences in lipid profiles in drug resistant prostate cancer cell lines, castration-resistant and hormone-sensitive cells and corresponding media.

Materials and Methods

Cell Culture

PC-3, LNCaP, 22RV-1, DU-145, RWPE1 and PNT2 cell lines were purchased from ATCC (Manassas, VA). The Docetaxel resistant human DU145-DR cell line was acquired from Dr. Begona Mellado's laboratory in the Medical Oncology Department, Hospital Clinic de Barcelona, Spain. The Docetaxel resistant human PC3-Rx cell line was acquired from Prof. Lisa G Horvath's laboratory in the Garvan Institute of Medical Research in Darlinghurst, Australia. Cell supplements, including antibiotics and primary cell culture media, were purchased from ATCC (Manassas, VA). Standard cell culture media were purchased from Corning Inc (Corning, NY). Human prostate cancer cells were cultured in 10% FBS (Seradigm, Radnor, PA) and 1% penicillin/streptomycin supplemented RPMI-1640, respectively. All cells were incubated in 95% humidity and 5% CO₂ at 37°C. Docetaxel resistant cell lines maintained resistance by receiving a range of nM concentrations of Docetaxel at every 2nd and 4th passage. Docetaxel dose response curves were consistently generated to check resistant levels using MTT assays.

Bligh-Dyer Lipid Extraction

Media from cells were collected followed by centrifugation. Cells were washed twice and harvested in 1x phosphate buffered saline (PBS), followed by centrifugation. Phospholipids from both cells and media were immediately extracted using chloroform and methanol according to the method of Bligh and Dyer [13]. Briefly, cell lines and media were suspended in 3 ml of methanol and 3 ml of chloroform. Tubes were vortexed for 30 s, allowed to sit for 10 min on ice, centrifuged (300 x g; 5 min), and the bottom chloroform

layer was transferred to a new test tube and spiked with a commercial mix of SPLASH Lipidomix internal standards (Avanti Polar Lipids, Inc.). SPLASH Lipidomix Mass Spec standards includes all major lipid classes at ratios similar to that found in human plasma. The extraction procedure was repeated three times and the chloroform layers combined. The collected chloroform layers were dried under nitrogen, reconstituted with 50 μ l of methanol: chloroform (3:1 v/v), and stored at 80°C until analysis.

Liquid Phosphorus Assay

Lipid content was quantified by determining the level of inorganic phosphorus using the Bartlett Assay [14]. Sulfuric acid 400 μ l (5M) was added to lipid extracts (10 μ L) in a glass test tube, and heated at 180-200°C for 1 h. H₂O₂ (100 μ l of 30 % v/v) was then added while vortexing, and heated at 180-200°C for 1.5 h. Reagent (4.6 ml of 1.1 g ammonium molybdate tetrahydrate in 12.5 ml sulfuric acid in 500 ml ddH₂O) was added followed by vortexing, which was followed by addition of 100 μ l of 15% ascorbic acid (v/v), and further vortexing. The solution was heated for 7-10 min at 100°C, and a 150 μ l aliquot was used to measure the absorbance at 830 nm.

ESI-MS/MS Analysis of Cells and Media

Lipid extracts (500 pmol/ μ l) were prepared by reconstitution in chloroform: methanol (2:1, v/v). ESI-MS was performed as previously described [15-17] using a LCQ Deca ion-mass spectrometer (LCQ Finnigan mass spectrometer (Thermo Fisher-Fenning Institute, CA)) with a nitrogen drying gas flow-rate of 81/min at 350 °C and a nebulizer pressure of 30 psi. The scanning range was from 200 to 1000 m/z on 5 μ l of the samples scanned in

positive and negative mode for 2.5 min with a mobile phase of acetonitrile; methanol; water (2:3:1) in 0.1% ammonium formate. Samples were run in triplicate (n=3) and overlap of confident identifications or most abundant species was defined as the core lipid pool [18].

NanoHRLC-LTQ-Orbitrap MS

NanoHRLC-LTQ-Orbitrap MS (NanoLC High Resolution Mass Spectrometry analyses) Lipid extracts were also analyzed by using a Thermo-Fisher LTQ Orbitrap Elite Mass Spectrometer coupled with a Proxeon Easy NanoLC system (Waltham, MA) located at Proteomics and Mass Spectrometry Facility, University of Georgia.

Individuals running samples were blinded to sample conditions. Mass spectra were acquired in the positive ion mode. Mass spectrometric parameters for lipid extracts were as follows: spray voltage: 1.7-1.8 kV, ion transfer tube (or capillary); temperatures: 200°C, respectfully. Full scan, data-dependent MS/MS (top8-ddMS2), were collected at m/z 150–2000 (350-1800), corresponding to the mass range of most expected cellular lipids. External calibration was applied before each run to allow for LC-HRMS analysis at 120,000 resolution (at m/z 400) and MS/MS at 15,000-30,000.

Lipids were separated on a nanoC18 column (length, 130 mm; i.d., 100 µm; particle size, 5 µm; pore size, 150 Å; max flow rate, 500 nL/min; packing material, Bruker Micron Magic 18). Mobile phase A was 0.1% formic acid/water; mobile phase B was 0.1% formic acid/acetonitrile. 1.5 µL of each sample was injected for analysis. A constant flow rate of 450-500 nL/min was applied to perform a gradient profiling with the following proportional change of solvent A (v/v): 0-2 min at 98% A, in 40 min from 100% A to 5%

A, kept at 5 % A for 10 min, then lowered to 50% A in 10 min. Between runs, a wash run with high-organic gradient and an equilibrium run were inserted to minimize the carryover. The autosampler temperature was maintained at 7 °C for all experiments. Solvent extraction blanks and samples were jointly analyzed over the course of a batch (10–15 samples).

Data Processing

Full scan raw data files were acquired from Xcalibur™ (Thermo Fisher Scientific), centroided and converted to a useable format (mzXML) using MSConvert. Data processing and peak area integration were performed using MZmine [21], and XCMS [22], resulting in a feature intensity table. Feature tables and MS/MS data were placed into a directory for each substrate analyzed. Each folder contained each sample type, feature tables end in “pos.csv” for positive mode. LipidMatch developed by Koelmel et al [23] was used to identify features. Peak areas were normalized to a mixture of deuterium labeled internal standards for each sample (SPLASH® LIPIDOMIX® Mass Spec Standard).

Multivariate Statistical Analysis of Cells and Media

Multivariate principal component analysis (PCA) was performed using MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/>). Automatic peak detection and spectrum deconvolution was performed using a peak width set to 0.5. Analysis parameters consisted of interquartile range filtering and sum normalization with no removal of outliers from the dataset. Features were selected based on volcano plot analysis and were further

identified using MS/MS analysis. Significance for volcano plot analysis was determined based on a fold-change threshold of 2.00 and $p \leq 0.05$. Following identification, total ion count was used to normalize each parent lipid level, and the change in the relative abundance of that phospholipid species as compared to its control was determined. This method is standard for lipidomic analysis as reported in our previous studies [17, 24]. Schematic diagram of analytical strategy and sequence of analysis using the different LC/MS instruments and software (see **Supplemental Figure 1**).

Pathway Enrichment Analysis

Pathway enrichment analysis of metabolites was performed using LIPEA software [5]. LIPEA is a web tool for over-representation analysis of lipid signatures detection and enriched in biological pathways [5]. Total lipid compounds from all the pathways are extracted and the over-representation analysis (ORA) starts in parallel for each pathway. When all the ORA analysis are completed, the server computes the Benjamini and Bonferroni p -values corrections. Once this process is finished, the server returns a list of enriched pathways sorted by p -value. Finally, the results are shown in an interactive table. Significance of the pathway fit is calculated with comparison to Fisher's exact test performed on numerous permutations of random features within the total feature list. Hierarchical clustering of these data identified differentially expressed lipid pathways from the set of lipids identified in our study. The module predicted biological activity directly from the mass spectrometric peak list data, and implements the *mummichog* algorithm [25], which was cross referenced with the KEGG database. Biochemical pathways were derived from transformed KEGG IDs, using the internal mapping process

(connected to Swiss Lipids, Lipid Maps, HMDB and KEGG databases) [5]. Columns represent individual sample type; rows refer to distinct metabolites, lipids and genes. Shades of green represent low levels and shades of red represent high levels ($p < 0.05$).

Statistical Analyses

All statistical analyses were compiled using GraphPad Prism for windows version 8.2.1 (GraphPad Software, Inc., La Jolla, CA). For all analysis, the experimental unit was individual samples obtained from a minimum of 4 ($n = 4$) groups were assessed. One passage of cells was equivalent to one group of sample (n). We controlled for the effect of multiple testing by measuring the statistical significance of each association using both the p value and the q value. Using a FDR of $q < 0.05$, the q value quantifies significance in terms of the false discovery rate (FDR) rather than the false positive rate and forms a measure of how likely a particular p value is to represent a genuine association. For all analyses, significance was set at $p \leq 0.05$ where data are expressed as mean $\pm$ SEM based on t-test for pairwise analysis and/or ANOVA analysis (with Kruskal-Wallis *post hoc* test).

Results

Comprehensive LC-ESI-MS/MS Analysis between PCa Cancer Cells

While its well known that the development and progression of prostate cancer is associated with abnormal changes in lipids, the association of these lipids with specific signaling pathways has not received as much attention. Further, even less attention has been given to identifying these lipids, or the signaling pathways involved, with the development of drug resistance. We address this gap-in-knowledge by constructing a heat map using MS Peak to Pathway-Metaboanalyst [26] comparing changes in lipid levels, as determined by LC-ESI-MS/MS, between non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media (**Figure 1**). Pathway analysis was also conducted using the MS Peaks to Pathway Activities module from Metaboanalyst, which generated a heat map-specific pathway visualization [27, 28]. This analysis resulted in several common dysregulated metabolic pathways including glycerosphingolipid metabolism. We confirmed these data with a LIPEA analysis, which showed that glycerophospholipid and sphingolipid metabolism were highly ranked and significantly associated to the set of lipids identified in our study. Other pathways identified included ferroptosis (20%) and choline metabolism in cancer (20%) (**Figure 2**, **Supplemental Table 1**) [5].

To validate these data, and to further analyze lipid changes, an additional LC-ESI-MS/MS analysis was conducted comparing changes in lipid levels between hormone-sensitive, castration-resistant and drug resistant human prostate cancer cell lines in comparison to non-cancer cell lines (**Table 1**). This resulted in the identification of 7,460

dysregulated ion features, encompassing 21 different lipid species (**Figure 3A**). This was supported by a heat map analysis (**Figure 3B**). Furthermore, OPLS-DA comparing hormone-sensitive cell-lines to control cells in the positive mode (ESI+) showed distinct separation of each prostate cell line (**Supplemental Figure 2A**). This is further analyzed in (**Supplemental Figure 2B**), which presents a cloud plot demonstrating directional fold changes, significance, retention times and m/z values. This analysis identified 84 altered lipidomic features between hormone-sensitive cells as compared to normal cells. These lipids were also identified via heat map analysis (**Supplemental Figure 2C**). OPLS-DA showed clear separation between castration-resistant and non-cancerous prostate cancer cell lines (**Supplemental Figure 3A**), suggesting differential lipidomic profiles with the cell types. This was supported by a cloud plot analysis (**Supplemental Figure 3B**) that identified 45 lipids whose levels differed between castration-resistant cell-lines and non-cancer control cells. The relative abundance of each lipid species levels varied significantly across all samples identified in CRPC cell lines in comparison to normal cells (**Supplemental Figure 3C**).

Alterations in PC Lipids

In agreement with the LIPEA analysis, PC lipids were augmented in all eight PCa cell lines analyzed, when compared to non-cancerous RWPE1 and PNT2 cells (**Figure 4A**). Amongst the PC lipids, 36:1 PC was significantly increased in both Docetaxel resistant cells types analyzed (**Figures 4B**). 12:0-24:1 PC was also identified as a dominant PC species (**Figure 4C**). Interestingly, 38:4 PC (**Figure 5A**) was significantly enriched in PC3-Rx cells, as compared to its content level in PC-3 parent and non-cancer control

cells. 18:0-22:6 PC (**Figure 5B**) was another dominant PC species that was increased in select cells. Most interestingly, LPC were enriched specifically in PC-3 parent cells, with 16:0 and 20:4 LPC being particularly prominent in this cell line (**Figure 6**). These data support the LIPEA, which suggested increased glycerophospholipid metabolism in prostate cancer cells.

LIPEA also suggested increase pathways correlating to ferroptosis. This may correlate to an increase in oxidative stress and metabolism, among other events, in these cells. In support of this hypothesis, the levels of oxidized PC (OxPC) were enriched in prostate cancer cells as opposed to non-cancer cells (**Figure 7A**). While a specific lipid species was not identified, the data did demonstrate an increase in oxidized LPC (OxLPC) in some cancer cells, especially PC-3 cells from both the parent and Docetaxel resistant strain. This correlates very well with data suggesting that LPC-species are specifically increased in PC-3 cells (**Figure 6**).

Alteration in PE Lipids

PC are not the only glycerophospholipids. As such we investigated changes in PE, PG, PI and PS lipids as well. For the most part, there was not any observable significant enrichments of PA, PG, PS or PI lipid species between non-cancer and cancerous prostate cancer cells, with the exception of increased PA and PG in PC-3 cells when compared to PNT2 and RWPE1 cells (**Supplemental Figure 4A and 4B**). This trend was only observed when comparing androgen-insensitive cells to non-cancer cells, and was not observed between parent PC-3 and DU-145 cells and their Docetaxel resistant counter parts (data not shown). A general enrichment of plasmalogens was also

observed in most prostate cancer cells, when compared to non-cancer cells (**Supplemental Figure 4C**); however, no observable trend was identified between the cancer cell lines.

In contrast to other glycerophospholipids, PE was significantly enriched in 22RV1, DU-145 and PC3-Rx cells as compared to non-cancer cells (**Figure 8A**). Further, significant enrichment of PE species were observed in PC3-Rx cells as compared to its parent cell lines, or even to all other cells. 38:4 PE identified as 18:0-20:4 PE, was the most dominant of the PE lipid species in PC3-Rx cells, as compared to PC-3 parent control (**Figures 8B and C**). Similar to PC lipids, OxPE was also significantly enriched in PC-3 and PC3-Rx cells, as compared to non-cancerous cells (**Figure 8D**). A similar trend was seen for LPE, which similar to LPC, was enriched in PC-3 and PC3-Rx cells (**Supplemental Figure 5**).

Alterations in Sphingolipids

LIPEA also suggested an increase in sphingolipids metabolism and in the sphingolipid signaling pathways. In support of this, SM was significantly enriched in PC3-Rx cells as compared to PC-3 parent control and non-cancerous cells (**Figure 9A**). 34:1+H was the dominant SM species identified in PC3-Rx cells (**Figure 9B**). We assessed ceramide enrichment as a de-facto marker of sphingolipid metabolism (**Figure 10**). While we did not identify substantial differences in the enrichment of ceramides between different cancer cells types, there was a significant enrichment of ceramide species in the media collected from PC-3 cells. This trend was amplified in PC3-Rx cells and media, and was observed in DU145-DR cells as well. A dominant ceramide species was not identified.

313 Nonetheless, these data support LIPEA suggesting that sphingolipid metabolism and
314 signaling is enriched in prostate cancer cells.

315

Discussion

Drug resistance in prostate cancer remains an un-solved challenge and is one of the primary drivers of low survivability among prostate cancer patients. Owing to the high complexity and diversity among lipid molecules, while studies have identified lipid species as biomarkers in various cancers, fewer attempts have been made for drug resistance prostate cancer. Recent evidence suggests elevated plasma levels of phospholipids are associated with an increased risk of PCa [29]. Unfortunately, these studies did not link individual lipids to the generation of drug resistance. Our initial MS Peak to Pathway-Metaboanalyst approach identified multiple pathways altered in the panel of prostate cancer cells analyzed. Hierarchical clustering found several nodes that correlated to glycerophospholipid and sphingolipid metabolism. This is not surprising as the extraction methods used may be biased in this analysis towards glycerophospholipids. Further, Metaboanalyst is not perfected for lipids species. We addressed the later limitation using the LIPEA pathway analysis, which was recently identified to mine pathways significantly associated to a set of lipids. LIPEA, which works with compound IDs for lipids found in KEGG, also identified glycerophospholipid metabolism as being significantly enriched in prostate cancer cells compared to non-cancer cells.

The enrichment of glycerophospholipid metabolism, as identified using multiple analysis, aligned with the fact that lipids generated from glycerophospholipid metabolism are also increased in prostate cancer cells, including LPC and LPE. Metabolites from these molecules regulate many signaling pathways, as well as cell growth [30, 31].

LIPEA also identified sphingolipid metabolism as being enriched in prostate cancer cells, which agrees with the analysis generated by Metaboanalyst and hierarchical

clustering. Several studies have shown that sphingolipid-mediated gene expression plays a critical role in cancer by several mechanisms [32-34]. This includes the regulation of lipid-lipid interaction, membrane structure and/or regulation of the interaction of membrane proteins with the membrane bilayer [35].

The identification of ferroptosis as being enriched in prostate cancer cells was unexpected. Ferroptosis is a regulated form of iron-dependent, non-apoptotic cell death [36]. Ferroptosis can be driven by extensive lipid peroxidation that alters the physical properties of the membrane or degrade reactive compounds that cross-link DNA or proteins [37-39]. Ferroptosis is linked to numerous diseases of the kidney heart, liver and brain [36, 40]. To date, few studies have linked changes in ferroptosis specifically to prostate cancer; however, excitingly, one recent study did identify that certain cancer cells were vulnerable to ferroptotic cell death induced by inhibition of a lipid peroxidase pathway as a feature of therapy-resistant cancer cells [41, 42]. As such, our data support this study by demonstrating that an enrichment of lipids corresponding to ferroptosis aligns with drug-resistance prostate cancer cells. Further, these data suggest that targeting ferroptosis may alter the sensitivity of prostate cancer cells to Docetaxel.

Data demonstrating the significant enrichment of four lipid classes (namely, PC, PE SM, Cer) in hormone-sensitive cells in comparison to normal control cells is consistent with previous reports in plasma from prostate cancer patients [29, 43, 44]. Elevated concentrations of SM in plasma were previously reported in patients with PCa in comparison to the control group [45]. Other reports have found elevated levels of Cer [34, 46, 47]. These data further support the previously stated hypothesis that sphingolipids have a potential role in regulating prostate cancer cells response to

chemotherapy [48]. Further, these data support that these lipids may serve to identify drug-resistance prostate cancer. Finally, these data show that at least part of the lipidomic profile seen in prostate cancer patients can be recapitulated in vitro (see below). This allows for a model to begin to investigate the molecular mechanism involved in changes in lipids during prostate cancer progression and development of drug resistance.

Data from this study also showed elevated levels of plasmalogens in prostate cancer cells. To the best of our knowledge, an increase in plasmalogens in these cell lines has not been previously reported. However, a previous study reported increased levels of plasmalogen phospholipids in neoplastic human breast tissue as compared to benign tissue, and correlated this with metastatic properties of human cancer [49]. The significance of these data to prostate cancer progression or drug resistance is not known.

The increase in specific PC lipids, such as 36:1 and 12:0-24:1 in PC-3 cells, but not in DU-145 cells mirrored data seen for LPC, OxPC and OxLPC. Elevated PC in plasma has been associated with prostate cancer progression. For example, 20:4 LPC was suggested as top biomarker for prostate cancer [50]. LPC has also been shown to be elevated in tissues exposed to radio/chemotherapy treatments [51-53]; however, it's not known if chemotherapy increased LPC levels, or if LPC was elevated prior to treatment [54]. Other reports have demonstrated that the LPC is increased in ovarian cancer patients and the fatty acid composition of this LPC is changed [55].

The elevation of PE in prostate cancer cells agrees with the findings of increased in PE in patient plasma samples [43, 56], as well as in prostate cancer cells (LNCaP, 22RV1 and DU-145), as compared to PNT2 cells [44]. PE has also been detected in high abundance in exosomes derived from PC-3 cells [57]. Similar to LPC levels in PC-3 cells,

enrichment in LPE was primarily seen in PC-3 cells from both parent and Docetaxel resistant line. Previous studies also compared PE plasmalogens in normal, benign and neoplastic samples from human prostate, breast, and lung tissues, and suggested that these are lipid tumor markers for distinguishing between benign vs. neoplastic tissue, and identifying *in-vivo* metastatic progression.

Ceramide is another sphingolipid reported to accumulate in patient plasma after chemotherapy treatment [48]. While our data did not demonstrate great difference in Ceramide between the prostate cancer cell studies, it did demonstrate increased ceramides in media isolated from several prostate cancer cells. This was especially true for media isolated from Docetaxel resistant cells. These data agree with the above mentioned studies showing that chemotherapeutics resistance may involve sphingolipid defects in apoptotic regulation. For example, etoposide and doxorubicin chemotherapeutics elevated ceramide during indication of cell death in leukemia cells [51]. Further, the combination of paclitaxel and tamoxifen increases ceramide and overcomes drug resistance in ovarian cancer [58]. Inhibition of *de novo* generation of ceramide also decreases the cellular response to cytotoxic agents [48].

While this study represents one of the most comprehensive analysis of lipid composition in prostate cells to date, it is limited by the facts that the actual concentrations for lipid species were not provided. This was in part intentional and these data are meant to springboard further studies focusing on the how specific lipids identified are being altered in these cells. Further, it important to point out that standards for many of the specific lipid species, as opposed to general classes, are not commercially available.

Future studies will focus on quantifying these specific lipids in these cells as well as validating their existence in human plasma.

These data, as well as previous studies demonstrates that there are fundamental differences between the lipidome of non-cancer and cancer cells [44, 59-61]. Non-cancer cells typically exhibit neutral total membrane charge due to the presence of zwitterionic phospholipids (PC and SM) on the outer leaflet of the membrane and PS and PE located in the inner leaflet of the membrane [62-64]. Unlike normal cells, cancer cells typically lose their capacity to maintain asymmetrical distribution leading to abnormal exposure of (PS and PE) to the cells outer membrane and/or PC and SM to cytosolic leaflet causing changes in cell signaling and down-stream gene expression. Our data further identify the novel finding that the lipidomic profile of drug-resistant prostate cancer cells also differs, even from their parent cells. As such, these lipids may be useful for identifying drug-resistant prostate cancer *in vivo*. These findings are important as understanding lipidomics, including the underlying molecular machinery of lipid metabolism, would assist in the discovery of novel and potential targets and develop new predictors for personalized cancer treatments. Finally, these data support the conclusion that changes in these lipidomic profiles mirror those reported in patient samples.

424 **List of Abbreviations**
425 Cer = Ceramide
426 CRCP = Castration-resistant prostate cancer
427 DU145-DR = DU145 based docetaxel resistant cells
428 KEGG = Kyoto encyclopedia of genes and genomes
429 LC-ESI-MS/MS = Liquid Chromatography-Electrospray Ionization-Tandem Mass
430 Spectrometry/Mass Spectrometry
431 LPC = Lysophosphatidylcholine
432 LIPEA = Lipid Pathway Enrichment
433 LPE = Lysophosphatidylethanolamine
434 MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
435 ORA = Over representation analysis
436 OxLPC = Oxidized lysophosphatidylcholine
437 OxPC = oxidized phosphatidylcholine
438 OxPE = Oxidized phosphatidylethanolamine
439 PA = Phosphatidic acid
440 PC = Phosphatidylcholine
441 PC3Rx = PC-3 based docetaxel resistant cell lines
442 PCa = Prostate cancer
443 PCA = Principle component analysis
444 PE = Phosphatidylethanolamine
445 PG = Phosphatidylglycerol
446 PI = Phosphatidylinositol
447 PS = Phosphatidylserine
448 SM = Sphingomyelin (SM)
449

Declarations

Ethical Approval and Consent to participate

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Authors provide formal consent to publish work

Availability of data and material

Authors will make data available on National Metabolomics Data Repository (NMDR)

Competing Interest

The authors declare no conflict of interest.

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Authors' contributions

L.M.I. and B.S.C. conceived and designed the experiments; L.M.I. and M.M. performed the experiments; C-W.C performed the mass spectrometry in a blind fasion; L.M.I. and B.S.C. analyzed the data; B.S.C. contributed reagents/materials/analysis tools; L.M.I. and B.S.C. wrote the paper.

485

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Table 1: Prostate Cell Line Characteristics

Cell Line	Cell Type	Tissue	Androgen Receptor Expression	Disease	Grade	Ethnicity	Age
PNT2	Epithelial	Prostate	+	Benign	N/A	Caucasian	33-year-old male
RWPE-1	Epithelial	Prostate	+	Benign	N/A	Caucasian	54-year-old male
22Rv1	Epithelial	Prostate	+	Adenocarcinoma	N/A	N/A	N/A
LNCaP	Epithelial-like	Prostate	+	Adenocarcinoma	N/A	Caucasian	50-year-old male
DU145	Epithelial	Prostate	-	Adenocarcinoma	II	Caucasian	69-year-old male
PC3	Epithelial	Prostate	-	Adenocarcinoma	IV	Caucasian	62-year-old male
DU145-DR	Epithelial	Prostate	-	(treated with 2nM of Docetaxel to maintain DR)	II	Caucasian	69-year-old male
PC3-Rx	Epithelial	Prostate	-	(treated with 2nM of Docetaxel to maintain DR)	IV	Caucasian	62-year-old male

Figure Legends

Figure 1. Heat map of differentially altered metabolites associated with non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media as determined by LC-ESI-MS/MS. Data were derived from a minimum of 3 extractions from 3 different passages per cell line.

Figure 2. LIPEA pathway analysis. Identification of lipids pathways enriched in prostate cancer cells (LNCaP, 22RV1, PC-3, DU-145, PC3-Rx and DU145-DR) as compared to non-cancerous cells (PNT2 and RWPE1). Data were derived from a minimum of 3 extractions from 3 different passages, where increases in lipids were mapped to genes identified in the KEGG database.

Figure 3. A) Differential cloud plot demonstrating dysregulated features between hormone-sensitive, castration-resistant, Docetaxel resistant cells and non-cancerous cells (PNT2 and RWPE1) as determined by LC-ESI-MS/MS (p -value < 0.05 threshold, fold change > 1.5 threshold). **B)** Differential expression of lipid features in non-cancerous prostate cells (B) as compared to hormone-sensitive (HS), castration-resistant (CR) and Docetaxel resistant (DR) prostate cancer cells. Only those features who levels that vary significantly (p < 0.05) are projected on the heat map. Each row represents a metabolite feature and each column represents a sample.

Figure 4. Comparison of phosphatidylcholine (PC) in non-cancerous (PNT2 and RWPE1) and hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* q < 0.05 ** q < 0.01 *** q < 0.001). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for **A)** phosphatidylcholine (PC) **B)** 36:1 PC and **C)** 12:0-24:1 PC

Figure 5. Comparison of **A)** 38:4 and **B)** 18:0-22:6 PC levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* q < 0.05 ** q < 0.01 *** q < 0.001). Each symbol represents an individual lipid feature as identified by MS/MS.

Figure 6. Comparison of **A)** lysophosphocholine (LPC), **B)** LPC and **C)** 20:4 LPC levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* q < 0.05 ** q < 0.01 *** q < 0.001). Each symbol represents an individual lipid feature as identified by MS/MS.

Figure 7. Comparison of oxidized phosphatidylcholines (OxPC) levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3

and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for **A**) oxidized phosphatidylcholine (OxPC), **B**) oxidized lysophosphatidylcholine (OxLPC).

Figure 8. Comparison of phosphatidylethanolamine (PE) levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for phosphatidylethanolamine (PE), **B**) 38:4 PE and **C**) 18:0-20:4 PE and **D**) OxPE.

Figure 9. Comparison of sphingomyelin (SM) levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for **A**) sphingomyelin (SM) and **B**) 34:1 +H SM.

Figure 10. Comparison of ceramide levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1) cells, and castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS.

Figures

Figure 1

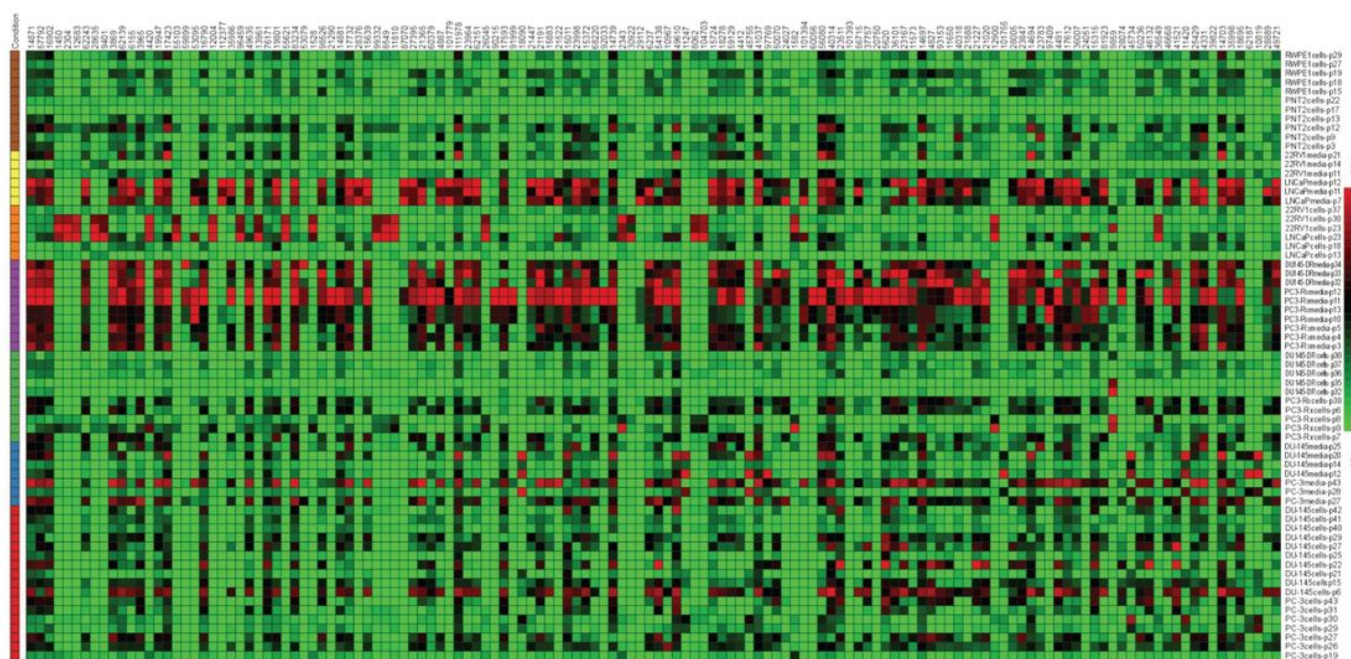


Figure 1

Heat map of differentially altered metabolites associated with non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media as determined by LC-ESI-MS/MS. Data were derived from a minimum of 3 extractions from 3 different passages per cell line.

Figure 2

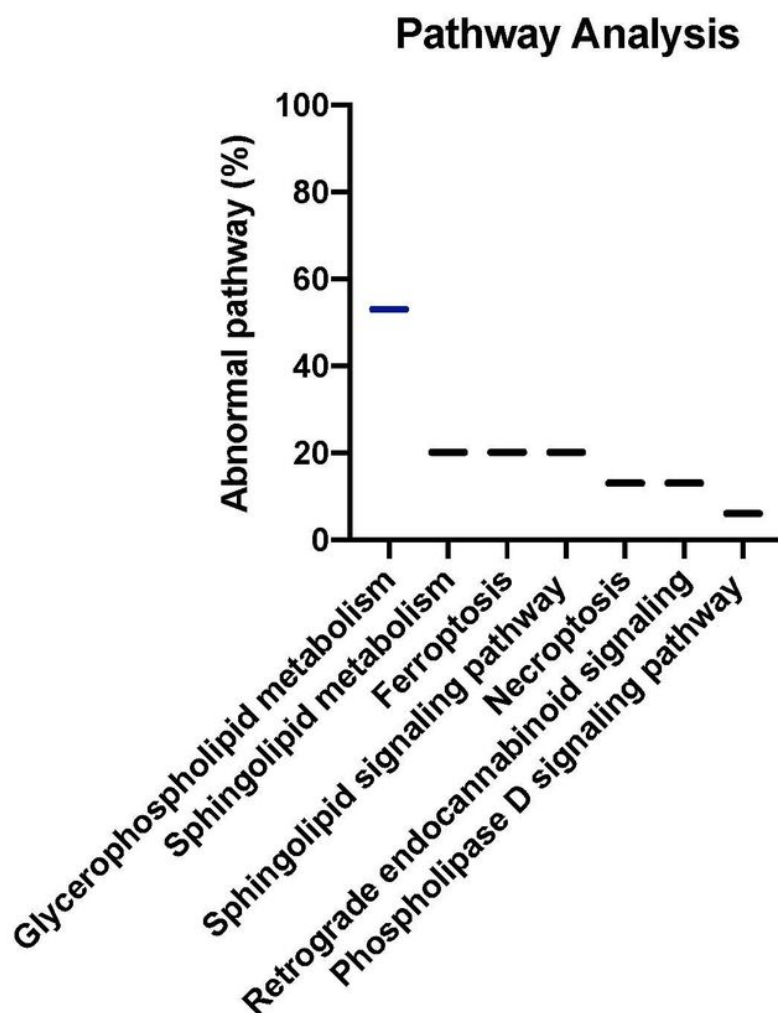


Figure 2

LIPEA pathway analysis. Identification of lipids pathways enriched in prostate cancer cells (LNCaP, 22RV1, PC-3, DU-145, PC3-Rx and DU145-DR) as compared to non-cancerous cells (PNT2 and RWPE1). Data were derived from a minimum of 3 extractions from 3 different passages, where increases in lipids were mapped to genes identified in the KEGG database.

Figure 3

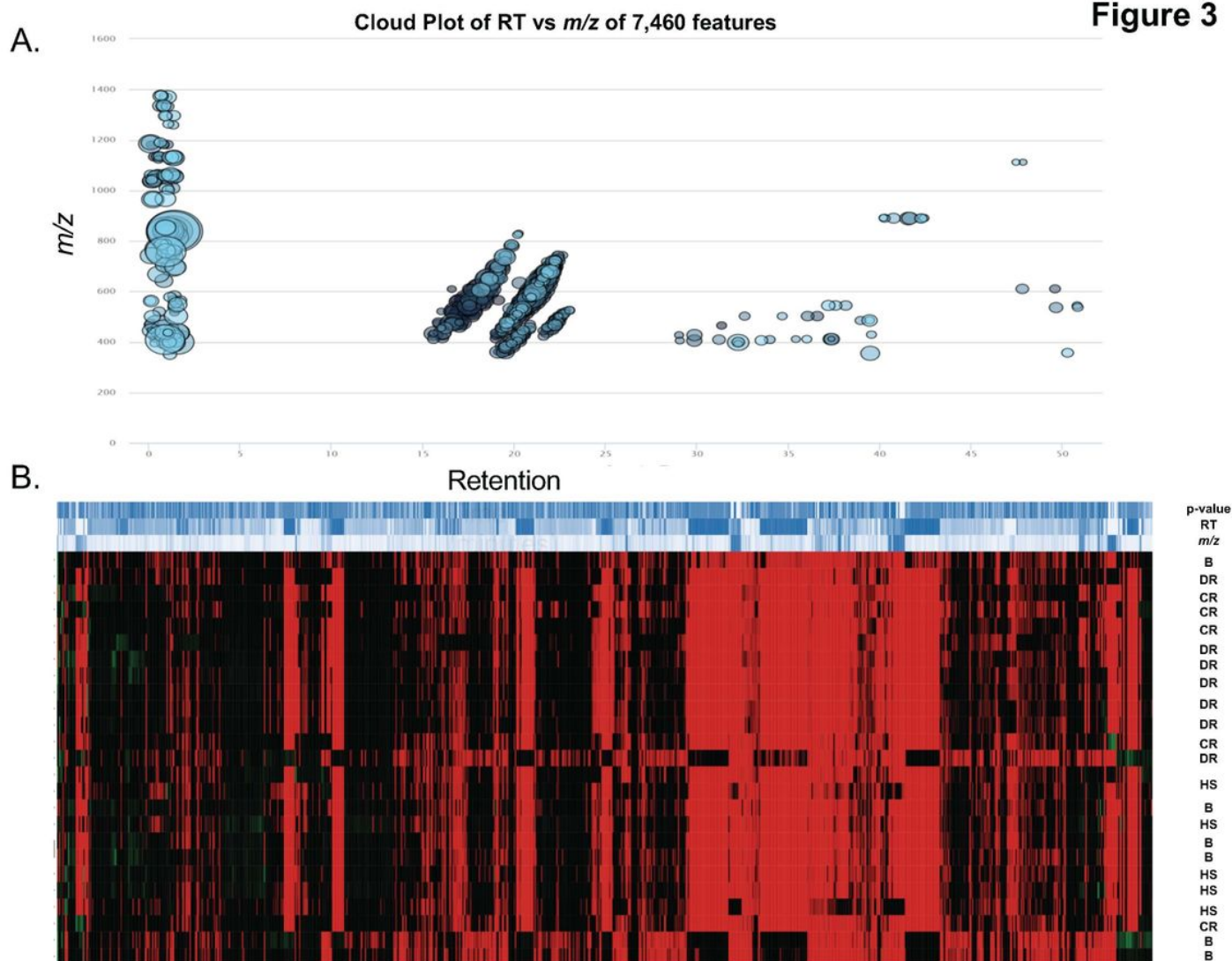
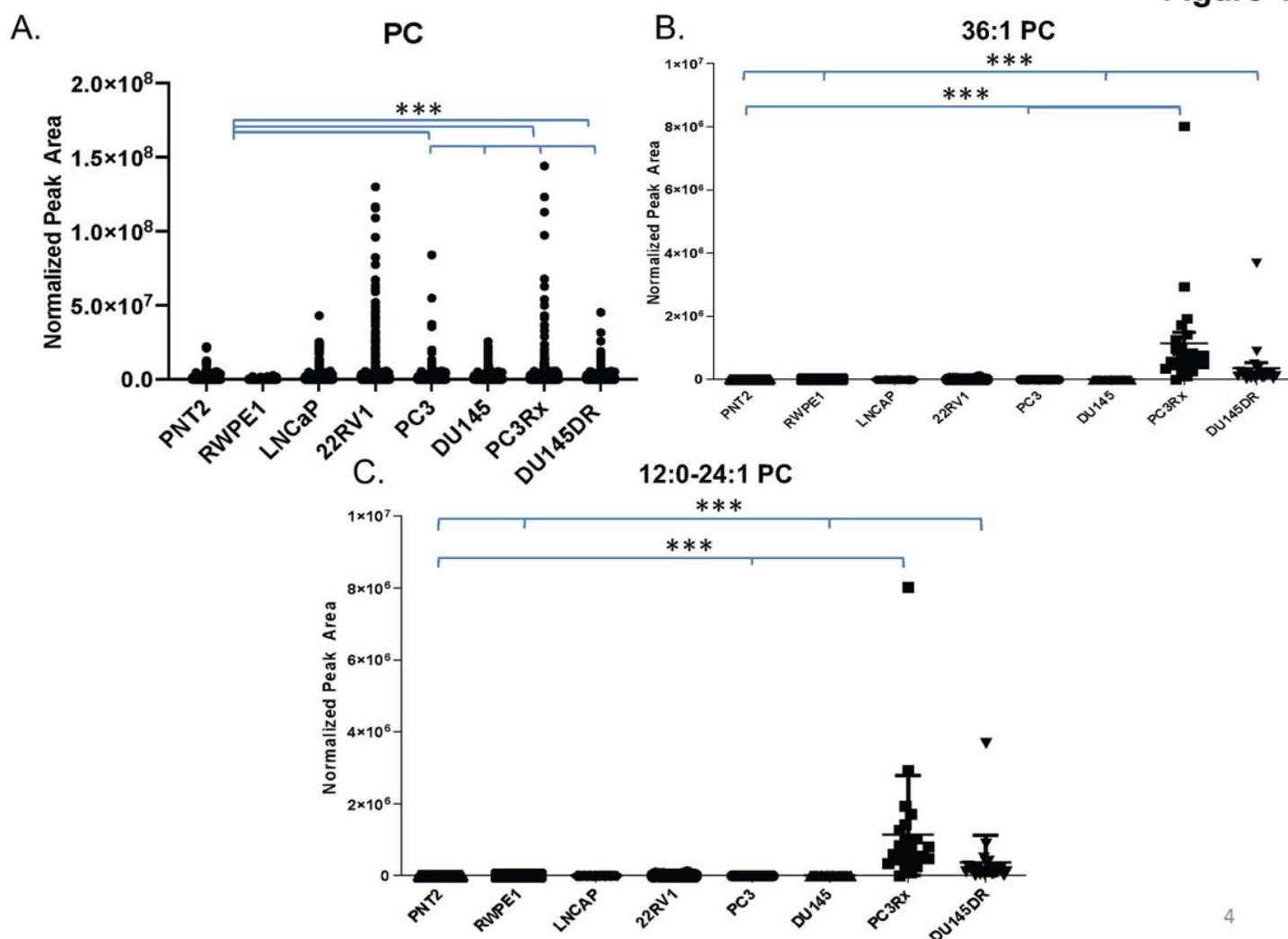


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Figure 4

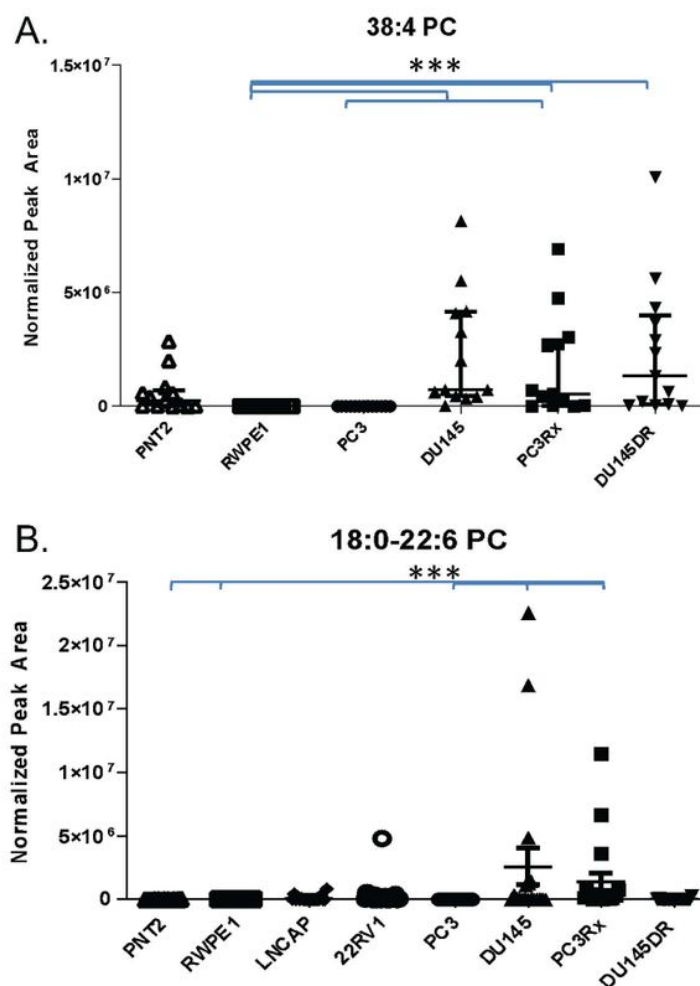


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Figure 4

Comparison of phosphatidylcholine (PC) in non-cancerous (PNT2 and RWPE1) and hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for A) phosphatidylcholine (PC) B) 36:1 PC and C) 12:0-24:1 PC

Figure 5

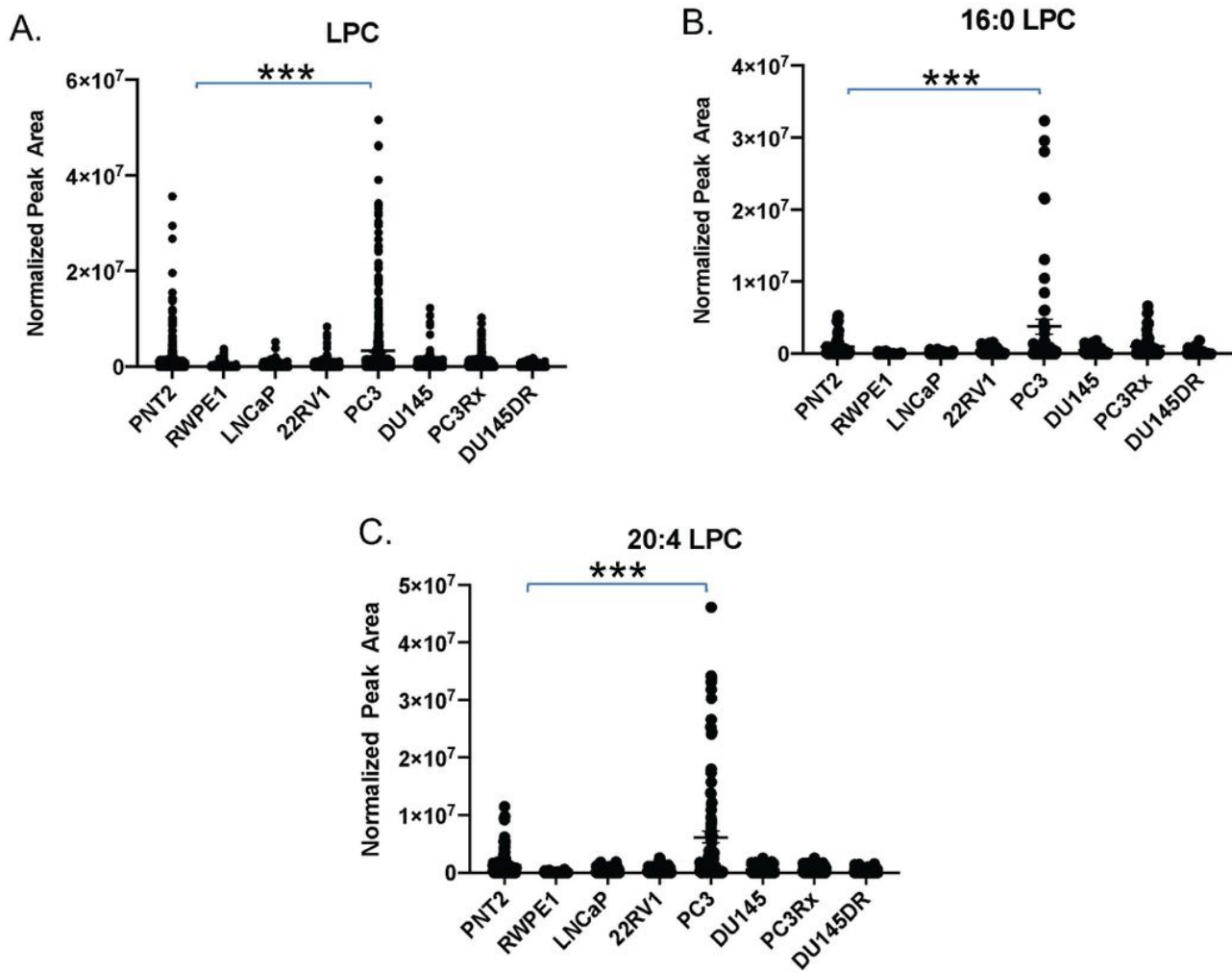


5

Figure 5

Comparison of A) 38:4 and B) 18:0-22:6 PC levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS.

Figure 6



6

Figure 6

Comparison of A) lysophosphocholine (LPC), B) LPC and C) 20:4 LPC levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS.

Figure 7

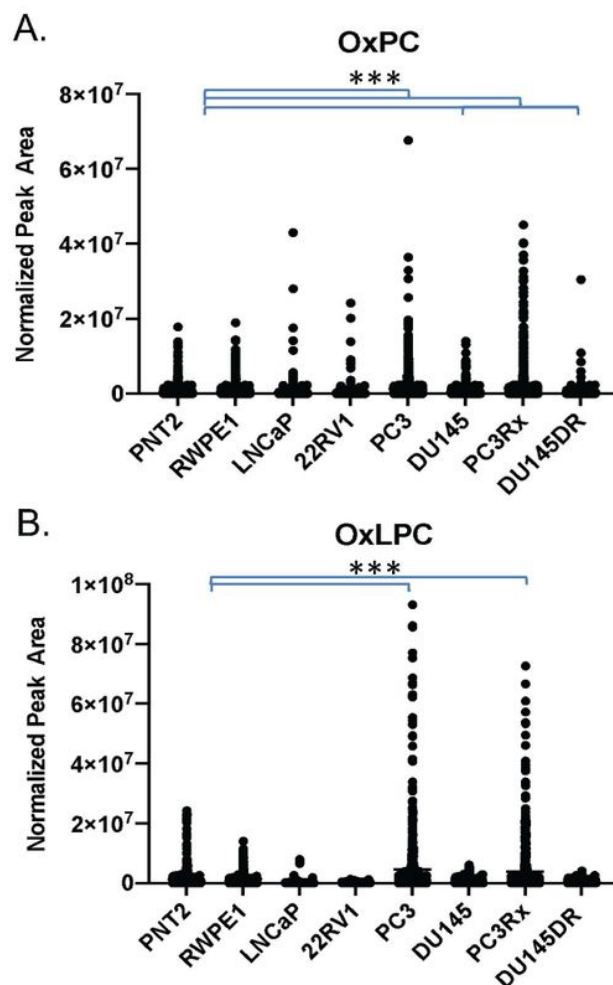


Figure 7

Comparison of oxidized phosphatidylcholines (OxPC) levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 31 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for A) oxidized phosphatidylcholine (OxPC), B) oxidized lysophosphatidylcholine (OxLPC).

Figure 8

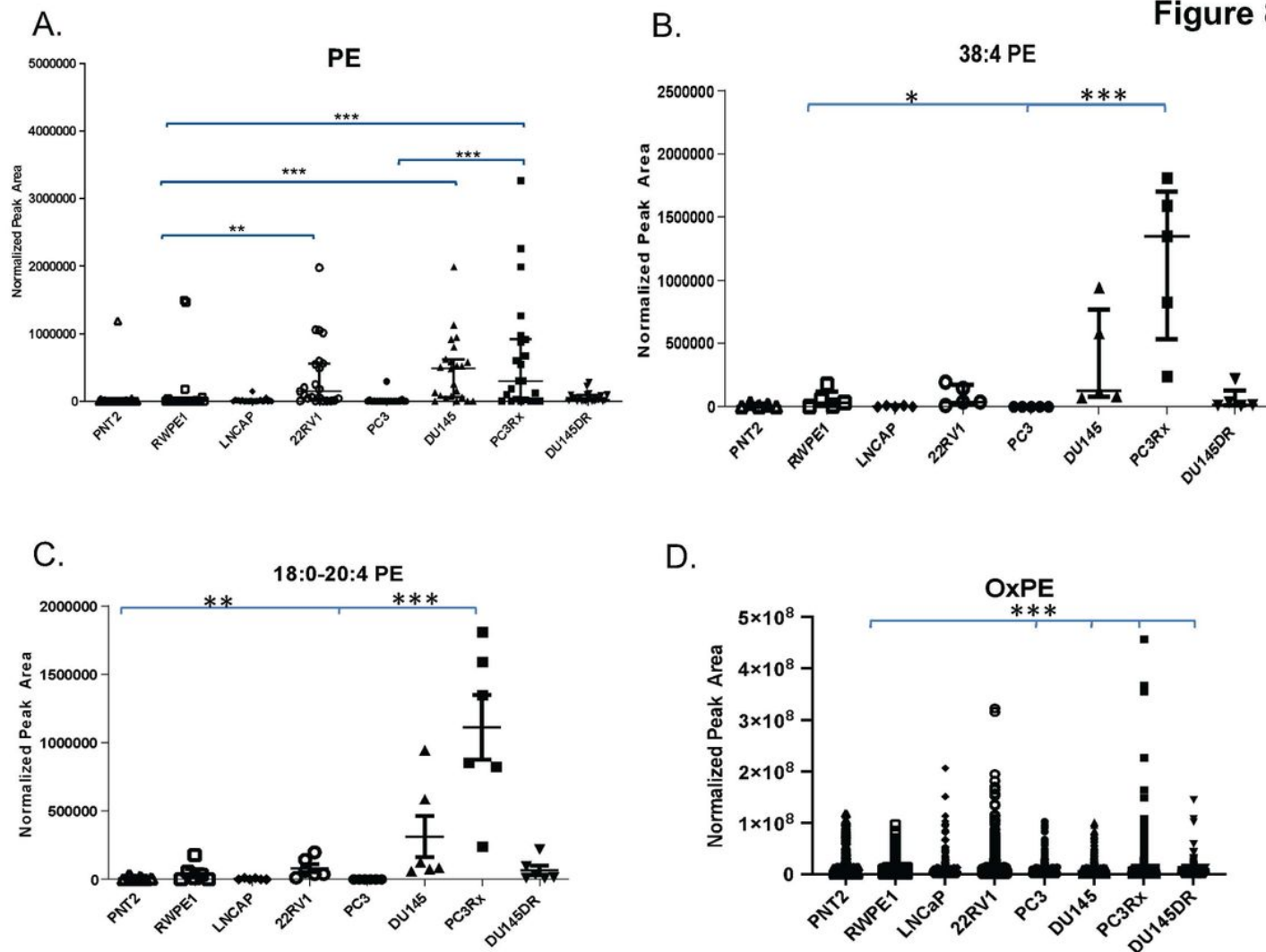


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Figure 9

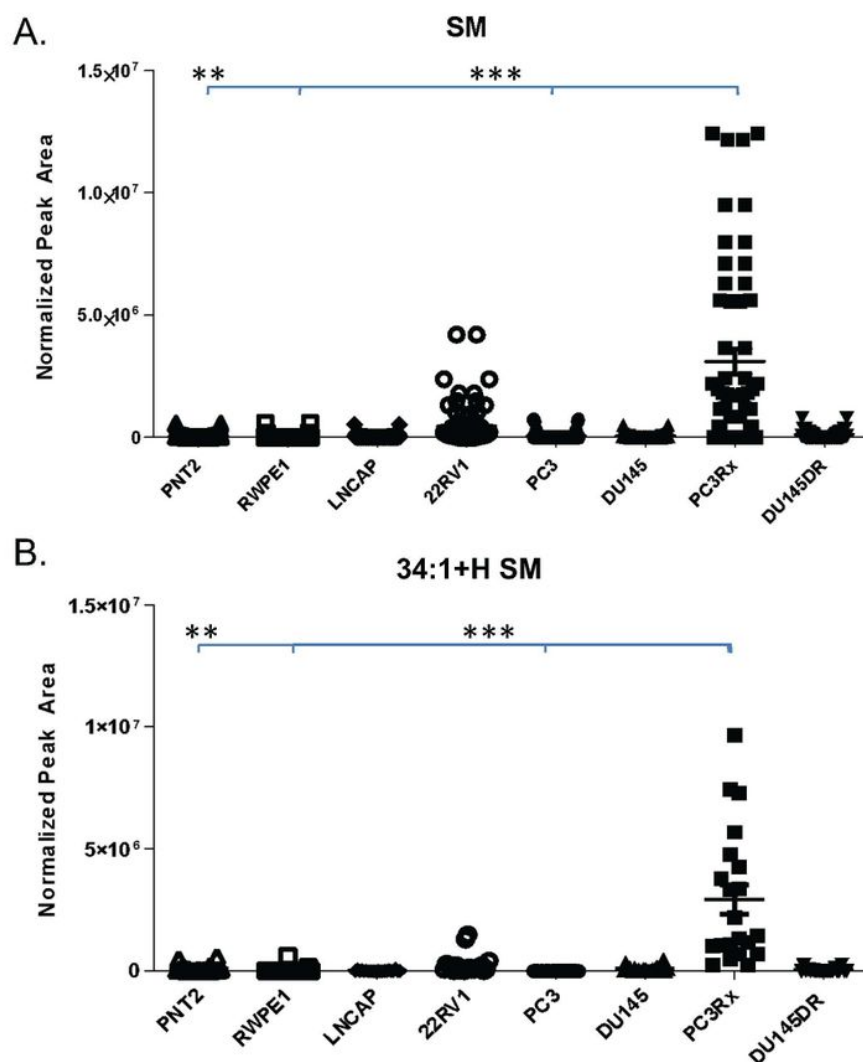


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Comparison of sphingomyelin (SM) levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1), castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS. Normalized peak areas between all cells are shown for A) sphingomyelin (SM) and B) 34:1 +H SM.

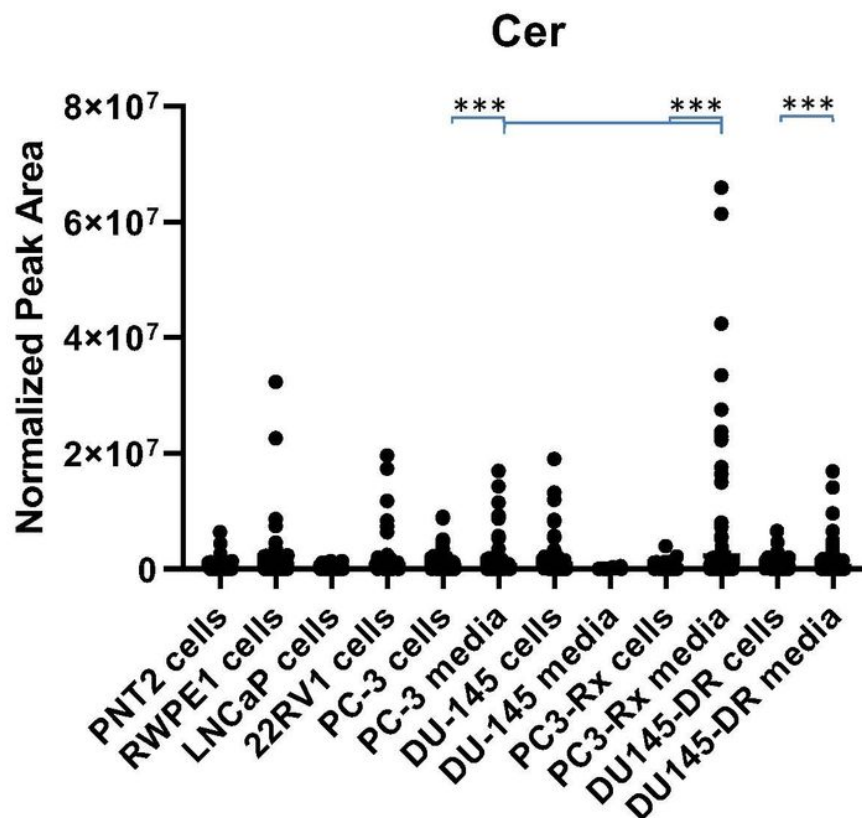


Figure 10

Comparison of ceramide levels in non-cancerous (PNT2 and RWPE1), hormone-sensitive (LNCaP and 22RV1) cells, and castration-resistant (PC-3 and DU-145) and Docetaxel resistant (PC3-Rx and DU145-DR) prostate cell lines and media. Data are indicative of 6 samples (6 distinct passages) per group and are expressed as mean $\pm$ the SEM (* $q < 0.05$ ** $q < 0.01$ *** $q < 0.001$). Each symbol represents an individual lipid feature as identified by MS/MS.

Supplementary Files

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