Lipid analysis of eight human breast cancer cell lines with ToF-SIMS

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In this work, four triple negative (TN) cell lines, three ER+ and PR+ receptor positive (RP) cell lines, and one ER+, PR+, and HER2+ cell line were chemically distinguished from one another using time-of-flight secondary ion mass spectrometry (ToF-SIMS) and principal component analysis (PCA). PCA scores separation was observed between the individual cell lines within a given classification (TN and RP) and there were distinctly different trends found in the fatty acid and lipid compositions of the two different classifications. These trends indicated that the RP cell lines separated out based on the carbon chain length of the lipids while the TN cell lines showed separation based on cholesterol-related peaks (in the positive ion data). Both cell types separated out by trends in fatty acid chain length and saturation in the negative ions. These chemical differences may be manifestations of unique metabolic processes within each of the different cell lines. Additionally, the HER2+ cell line was distinguished from three other RP cell types as having a unique distribution of fatty acids including anticorrelation to 18-carbon chain fatty acids. As these cell lines could not be grown in the same growth media, a combination of chemical fixation, rinsing, C₆₀⁺ presputtering, and selection of cellular regions-of-interest is also presented as a successful method to acquire ToF-SIMS data from cell lines grown in different media. © 2015 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4929633>]

I. INTRODUCTION

According to statistics reported by Siegel *et al.*¹ breast cancer is expected to account for 29% of new cancers in women in 2015. Fortunately, breast cancer deaths have continued to fall since ~1990 (Ref. 2), in part, due to improvements in targeted therapies³ and personalized treatments,⁴ although the mortality rate still remains unacceptably high. The immunohistochemical classification of breast tumors based on the presence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) has resulted in development of targeted therapeutics for treating these receptor-positive breast tumors.⁵ Tumors lacking the presence of these three receptors are classified as triple negative (TN) and continue to have a higher mortality rate, as well as an absence of adequate tailored therapies.^{6,7} It is hoped that with a better understanding of tumor biology (such as tumor lipid metabolism), and improved subclassification of TN tumors, the number of deaths will fall at an increasing pace.

The contribution of metabolism to tumor growth and resistance to therapy is still not fully understood and continues

to be an intense area of research.⁸ In particular, lipid metabolism has increasingly been viewed as an important component of breast cancer biology.^{9,10} Studies have looked at lipogenesis¹¹ and fatty acid synthesis¹² in relation to tumor progression, and pathways involving lipid metabolism are being investigated for development of new therapeutic targets.^{13,14} One study has shown that inhibiting fatty acid synthase was selectively cytotoxic to breast cancer cells.¹⁵ The aggressiveness of several breast cancer cell lines have been related to levels of specific phospholipid species such as phosphatidylcholine and lysophosphatidylcholine.¹⁶ However, it is clear that further understanding of the lipid metabolic process is required to create rational approaches to new therapies.⁹

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a tool that is well suited for studying lipid metabolism in cancer cells because it can simultaneously detect a wide range of lipids, as has recently summarized,^{17,18} and has been used to specifically analyze breast cancer cells.¹⁹ Additionally, ToF-SIMS can map the distribution of different chemical species with very high spatial resolution (<1 μm),²⁰ enabling analysis of multiple cells in one region,²¹ allowing statistical averaging over 10–100 s of cells in a sample type, or analysis of single cells.^{22–24}

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ToF-SIMS spectra acquired from cells and tissues can be very complex and often contain hundreds of peaks. This has led to the use of multivariate analysis (MVA) methods to aid in the interpretation of ToF-SIMS data.²⁵ The combination of ToF-SIMS and MVA has been used successfully to study several cellular systems. In one study by Kulp *et al.*, freeze-fractured single cells from three human breast cancer cell lines were compared using principal component analysis (PCA) of the amino acid related peaks.²⁶ In a series of studies by Baker *et al.*, cells from three prostate cell lines (originating from a bone metastasis, lymph node metastasis, and a nonmalignant, normal adult prostatic cell line) were cryo-fixed, analyzed with ToF-SIMS, and distinguished from each other using discriminant function analysis of fatty acid and amino acid related peaks.^{21,27} Both of these studies demonstrated the utility of the ToF-SIMS and MVA combination to classify the cancerous cells.^{26,27} ToF-SIMS has also been used to discriminate individual cell types in a mixed population.²⁸ These studies show the potential of ToF-SIMS for comparison of chemical composition between cell types and even between individual cells. In other work, the distribution of choline metabolites in MCF-7 and MDA-MB-231 breast cancer xenograft models were visualized with SIMS, matrix-assisted laser desorption ionization MS, and MRI imaging,²⁹ showing the potential of ToF-SIMS for studying metabolic differences in cells and tissues.

In this work, ToF-SIMS was used in combination with MVA to investigate the complex lipid-related metabolite variations from eight breast cancer cell lines that have been grown in different media and chemically fixed. Mass spectra were collected from four triple negative classified cell lines (ER-, PR-, and HER-), three ER and PR receptor-positive lines (ER+ and PR+), and one line that was ER, PR, and also HER2 positive (ER+, PR+, and HER+) (identified in Table I). By using a short C₆₀ sputtering before analysis, we removed a portion of the outer membrane to enable collecting signal from the interior of the cell. ToF-SIMS imaging was used to generate regions of interest to help focus on only the cells for the chemical analysis. The methods used here demonstrate a process to handle analysis of the cellular interior of multiple (eight) cell lines grown in different media with a standard cell fixation preparation. PCA together with a comprehensive peak list was used to determine the chemical differences and similarities between these multiple types of breast cancer cells. The differences seen in the lipids and fatty acids may contribute toward an improved metabolic classification of breast cancers using tissue specimens.

II. EXPERIMENT

A. Cell line growth and preparation

The ER and PR receptor-positive cells lines that were used in this study were MCF-7, T47D, and HCC1428. The HER2 positive line was BT474. The triple-negative cell lines were MDA-MB-231, BT549, HS578t, and HCC1395. The cell lines type and receptor status are summarized in Table I.

TABLE I. Cell lines used in this study with ER, PR, and HER2 receptor status. The growth media is identified for each cell line along with the number of analysis spots for positive and negative ion modes.

	ER	PR	HER2	Media	No. of spots	
					(+)	(-)
MCF-7	+	+	—	DMEM	21	28
HCC-1428	+	+	—	RPMI	22	24
T47-D	+	+	—	RPMI	29	18
BT-474	+	+	+	DMEM	19	14
HS-578t	—	—	—	DMEM	27	19
MDA-231	—	—	—	DMEM	23	14
HCC-1395	—	—	—	RPMI	27	18
BT-549	—	—	—	RPMI	26	27

All cell lines were originally acquired from ATCC and independently authenticated by STR DNA typing (DNA Diagnostics Center Fairfield, OH). To reduce variation between culture conditions, the cell lines were paired in TN versus RP groups for Dulbecco's Modified Eagle Media (DMEM, Gibco, LifeSciences Technology, USA) (MCF7, HS578t, MDA-MB-231, BT474) or Roswell Park Memorial Institute (RPMI) media (HCC1428, HCC1395, BT549, T47D). All media were supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, USA) and 1% penicillin-streptomycin (Gibco, LifeSciences Technology, USA). Prior to plating, cells were cultured on 10 cm plates in their respective media and incubated at 37 °C with 5% CO₂. Cells were fed one day prior to plating on silicon chips. After plating, cells were then grown on silicon chips for 24 h to allow attachment. Cells were seeded onto 1 cm × 1 cm silicon chips that were previously cleaned with 2 × successive sonications in dichloromethane, acetone, and methanol. The cleaned chips were dried with nitrogen and stored in a laminar flow hood until cell seeding. Cells were grown on chips for 24 h. Prior to ToF-SIMS analysis, the silicon chips were removed from the cell growth media, briefly rinsed with 150 mM ammonium acetate,³⁰ fixed in 4% formaldehyde for 30 min,³¹ then rinsed with 150 mM ammonium acetate to remove intracellular salts, buffer salts introduced during fixation, and components from the growth media. The cells were dried overnight in air and analyzed the following day.

It is possible that some lipids are lost or rearranged during this chemical fixation combined with rinsing and drying process, but this method has proven to be an effective sample preparation process for the analysis of cells with ToF-SIMS.³¹ As shown in the paper by Robinson and Castner,³¹ different sample preparation methods (e.g., chemical fixation and frozen hydrated) will have different advantages.

B. TOF-SIMS

ToF-SIMS experiments were performed using an ION-TOF ToF-SIMS 5-100 (ION-TOF GmbH, Münster, Germany) equipped with two ion sources. A liquid metal ion gun (LMIG) was used to generate a pulsed 25 keV Bi₃⁺

beam, whereas an electron impact gun was used to generate a 20 keV C_{60}^{++} beam. Both beams hit the target at a 45° angle. Using a cycle time of 100 μ s, the mass range used for analysis was $m/z = 1$ –846. All organic peaks with sufficient intensity to be identified were picked within this mass range. With the ToF-SIMS tuned for the best mass resolution, the mass resolution ($m/\Delta m$) for the $C_2H_3^+$ ion was predominantly greater than 4500. Positive ion spectra were calibrated to CH_3^+ , $C_2H_3^+$, $C_3H_3O^+$, $C_4H_7^+$, $C_7H_7^+$, and $C_{27}H_{45}^+$. Negative ion spectra were calibrated to CH^- , OH^- , C_2H^- , $C_{16}H_{31}O_2^-$, and $C_{18}H_{33}O_2^-$. Target currents were measured before acquiring each data set using a Faraday cup. The Bi_3^+ current was typically 0.15 pA. In all cases, a Bi_3^+ dose of 5.0×10^{11} ions/cm² was used to acquire spectra from a 250 μ m $\times$ 250 μ m area. Each spectrum contained 128 $\times$ 128 pixels. Low energy electrons (several microamps at 21 eV) were flooded onto the surface of the samples to compensate for charge buildup during analysis.

A C_{60}^{++} etching sequence was used prior to data collection to sputter the surface of the cells, as this has been previously shown to improve cell sample separation²⁸ as well as expose a portion of the cell interior. The C_{60}^{++} etch covered a 500 μ m $\times$ 500 μ m area with primary ion currents between 0.5 and 1.0 nA and an etching dose at 1.6×10^{13} ions/cm². A dose of 1.25×10^{13} ions/cm² of 20 keV C_{60}^{++} was previously shown to remove ~ 10 nm of material from NIH/3T3 fibroblasts²³ (or $\sim 0.13 \times 10^{13}$ ions/cm² removed 1 nm of material). Thus, a dose of 1.6×10^{13} ions/cm² should be sufficient to remove the exposed plasma membrane during this sputter cycle, exposing intracellular material. With the removal of the outer cellular membrane, the data shown in this study contains information from the intracellular components. However, due to overlap of cells and the likely presence of the edges of the plasma membrane, it is expected that there will still be some signal from the membrane walls.

C. TOF-SIMS data analysis

Data were collected from three different cultures (days) containing multiple replicate samples for each cell line for the positive data, and at least two different sets (days) containing multiple duplicate samples for the negative ion data. For details on number of data points acquired, see Table I.

The data used in this study were preprocessed to put them in a form appropriate for PCA. The first step consisted of selecting spectra only from the cellular areas (regions of interest, ROI) for each data file by thresholding the total ion image in the high mass resolution mode. An example of this can be found in Fig. S-1 where a comparison of images shows the cells in a high spatial resolution mode as well as the high mass resolution mode and a typical ROI mask. With the ROI mask applied to the image, only spectra from the cell areas were used for the PCA. PCA was carried out using the SpectraGUI (Dan Graham, Ph.D., NESAC/BIO, University of Washington) in MATLAB (MathWorks, Natick, MA). Peak lists were created for both positive and negative ion spectra that included all peaks whose maximum

intensity was twice or more that of the average background intensity to the left and right of the peak, excluding known salt, salt adduct, inorganic and substrate peaks (as specified for each PCA figure in Sec. III). Sample negative ion spectra from BT549 cells can be found in supplementary material (Fig. S-2).³² Peak lists were created for all peaks meeting the specified criteria and for peaks with $m/z > 200$. For the $m/z > 200$ peak lists, this resulted in 284 peaks for the positive ion list and 207 peaks for negative ion list. In order to generate peak lists that included all peaks from all sample types, representative spectra from all eight cell lines were overlaid, and peaks were chosen manually and integration limits were set manually. The spectral data were normalized to the sum of the intensities of the peaks in the given peak list, square-root transformed, and mean centered before PCA was applied. This data preprocessing was chosen to minimize nonsample related differences, reduce the dynamic range of the data and give more influence to higher m/z values, more chemically specific peaks and to allow the data to be compared across a common mean of zero.

The normalized intensity plots were generated by dividing the intensity of the selected peak by the sum of the intensities of all of the peaks in the peak list. These values were then averaged over all of the data files for a given cell line and plotted as normalized intensities.

III. RESULTS AND DISCUSSION

A. Results

ToF-SIMS and PCA were used to compare chemical differences between the eight human breast cancer cell lines summarized in Table I. Sample preparation was designed to allow comparison between cells regardless of the growth media and to emphasize the lipid chemistry within the cell. Initial PCA using all the masses did not show any separation in the scores plots that correlated with cell growth media (example of positive ion PC1 vs PC2 shown in supplementary Fig. S-3). This suggests that the effects of the growth media have been minimized using our workflow. Scores plots using only peaks with $m/z > 200$ also did not show any separation that correlated with cell growth media. Secondary ion peaks that showed high PCA loadings from the $m/z > 200$ peak lists are detailed in Table II for positive and negative ions, including proposed chemical compositions and biomolecule identifications. In general, the prominent positive ion peaks were found to be from phosphocholine (Pchol), monoacylglycerols (MAG), diacylglycerols (DAG), and cholesterol, while the negative ions peaks provided more information about a variety of fatty acids (FA).

B. Comparison of cell lines using positive ions

Principal component scores and associated loadings plots generated using the positive ion $m/z > 200$ peak list for all eight breast cancer cell lines are shown in Fig. 1. The larger amount of variance that is observed in the BT474 and T47D scores is potentially due to different biological factors between the cells on different days of data acquisition

TABLE II. Key positive and negative ion *m/z* and fragment identification for peaks that show the largest variance in PCA.

Positive ion peaks					Negative ion peaks				
Mass (m/z)	Composition	Deviation (ppm)	Possible biomolecule	Reference	Mass (m/z)	Composition	Deviation (ppm)	Possible biomolecule	Reference
224.11	C ₈ H ₁₉ PNO ₄	-4.46	PChol. + C ₃ H ₄	32	227.20	C ₁₄ H ₂₇ O ₂	-4.40	Myristic acid, 14:0	33–35
237.22	C ₁₆ H ₂₉ O	29.51	FA, (16:0)	Possible ID	253.22	C ₁₆ H ₂₉ O ₂	-7.90	Palmitoleic acid, 16:1	33,34
263.24	C ₁₈ H ₃₁ O	7.60	FA, (18:1)	Possible ID	255.23	C ₁₆ H ₃₁ O ₂	7.84	Palmitic acid, 16:0	33,34
311.26	C ₁₉ H ₃₅ O ₃	9.64	MAG, (16:1)	33	265.25	C ₁₈ H ₃₃ O	11.31	18:0 fragment	Possible ID
313.27	C ₁₉ H ₃₇ O ₃	9.58	MAG, (16:0)	34	277.22	C ₁₈ H ₂₉ O ₂	-10.82	Linolenic acid; 18:3	34
337.27	C ₂₁ H ₃₇ O ₃	5.93	MAG, (18:2)	Possible ID	279.24	C ₁₈ H ₃₁ O ₂	-28.65	Linoleic acid; 18:2	33–35
339.29	C ₂₁ H ₃₉ O ₃	-2.95	MAG, (18:1)	33,34	281.25	C ₁₈ H ₃₃ O ₂	-7.11	Oleic acid; 18:1	33–35
341.30	C ₂₁ H ₄₁ O ₃	11.72	MAG, (18:0)	33	283.26	C ₁₈ H ₃₅ O ₂	14.12	Stearic acid; 18:0	33–35
369.35	C ₂₇ H ₄₅	16.24	Cholesterol	33,36	303.24	C ₂₀ H ₃₁ O ₂	-26.38	Arachidonic acid; 20:4	35,36
495.42	C ₃₁ H ₅₉ O ₄	42.39	DAG, (28:0)	Possible ID	305.25	C ₂₀ H ₃₃ O ₂	-6.55	Dihomo-γ-linolenic acid; 20:3	35
521.45	C ₃₃ H ₆₁ O ₄	13.42	DAG, (30:1)	33,34	307.26	C ₂₀ H ₃₅ O ₂	13.02	Eicosadienoic acid; 20:2	35
523.46	C ₃₃ H ₆₃ O ₄	24.83	DAG, (30:0)	33,34	309.28	C ₂₀ H ₃₇ O ₂	-3.23	Eicosenoic acid; 20:1	35
547.46	C ₃₅ H ₆₃ O ₄	23.75	DAG, (32:2)	33,34	311.29	C ₂₀ H ₃₉ O ₂	16.06	Arachidic acid, 20:0	Possible ID
549.49	C ₃₅ H ₆₅ O ₄	-3.64	DAG, (32:1)	33,34,36	329.25	C ₂₂ H ₃₃ O ₂	-6.07	Docosadienoic acid; 22:5	Possible ID
551.49	C ₃₅ H ₆₇ O ₄	25.39	DAG, (32:0)	33,34	331.26	C ₂₂ H ₃₅ O ₂	12.07	Adrenic acid; 22:4	35,37
575.49	C ₃₇ H ₆₇ O ₄	24.33	DAG, (34:2)	33,34	333.28	C ₂₂ H ₃₇ O ₂	-3.00	22:03	Possible ID
577.51	C ₃₇ H ₆₉ O ₄	17.32	DAG, (34:1)	33,36	385.35	C ₂₇ H ₄₅ O	-7.79	Cholesterol	35
579.53	C ₃₇ H ₇₁ O ₄	8.63	DAG, (34:0)	34	465.31	C ₂₇ H ₄₅ SO ₄ -	-12.89	Cholesterol sulfate	38
599.49	C ₃₉ H ₆₇ O ₄	23.35	DAG, (36:4)	33,34					
601.51	C ₃₉ H ₆₉ O ₄	16.62	DAG, (36:3)	33,34					
603.53	C ₃₉ H ₇₁ O ₄	8.28	DAG, (36:2)	33,34,36					
605.54	C ₃₉ H ₇₃ O ₄	18.17	DAG, (36:1)	34					
607.55	C ₃₉ H ₇₅ O ₄	26.33	DAG, (36:0)	34					

(passage number, serum batch to batch variation, etc.). It is interesting to note that in the PC1 vs. PC2 scores plot (top left), the four TN cell lines data (red symbols) separated from one another. This separation can be related to significant differences in phospholipids and cholesterol as well as MAG, and DAG-related peaks. In contrast, the RP cell lines (gray and blue symbols) did not show significant separation until PC4, where the separation corresponded to loadings that were dominated by MAGs and DAGs with very little contribution from phospholipids and cholesterol.

The TN cell lines separate across PC1 with the phosphocholine fragment (phosphocholine + C₃H₄),³³ referred to here as Pchol. (m/z 224⁺) and the cholesterol fragment (m/z 369⁺) predominantly loading with the MDA-MB-231 and MAG and DAG peaks (m/z 339⁺, 549⁺, 575⁺, 577⁺, 601⁺, 603⁺, and 605⁺) loading with the HS578t cells. In PC2, the cholesterol and Pchol showed the largest loadings (Fig. 1, bottom middle) with little contribution from other peaks, indicating that the composition of cholesterol and Pchol were significant initial differences found to separate the BT549 and HCC1395 cell lines.

PC4 scores (Fig. 1, top right as PC1 vs PC4) showed a better separation of the receptor positive cell lines (including the HER2 + line) than the TN cell lines. The PC4 loadings (Fig. 1, bottom right) were dominated by MAG, DAG and fatty acid fragments with very little contribution from Pchol and cholesterol. The only HER2 + cell line, BT474 (blue

points), stands out as having the highest positive scores across PC4. These positive scores correspond with MAG, DAG, and fatty acid fragments that can be attributed to having hydrocarbon tails of 16-carbon atoms or less (m/z 237⁺, 313⁺, 495⁺, 521⁺, 523⁺, 549⁺, 551⁺, and 577⁺). In contrast, the HCC1428 and MCF-7 lines have negative scores on PC4, where the major contribution to the negative loadings were from MAG, DAG, and fatty acid fragments containing unsaturated 18-carbon chains (m/z 263⁺, 337⁺, 599⁺, 601⁺, and 603⁺).

This analysis does identify different cell metabolomics profiles within the TN and RP cell lines based on lipid profiles. However, this approach does not separate cell types by receptor status classification (i.e., all triple negative lines do not separate from all receptor positive lines). The TN cell lines show separation based on the relative levels of Pchol and cholesterol related peaks (Fig. 1, PC1 vs PC2), but little to no separation was seen for the TN cell lines in PC4, indicating that the MAG and DAG tail chain length is not a predominant factor in the chemical difference between these cell lines. In contrast, the RP cell lines showed the most separation in PC4, indicating that fatty acid tail chain length and saturation are important differences between these cell lines.

The scores and loadings plots highlight the different chemistries of cell lines. The specific chemical contributions to these differences are identified by normalized intensities of peaks in the PCA loading plots. Key positive ion peaks

Fig. 1. PCA of eight breast cancer cell lines using the positive ion $m/z > 200$ peak list. (Top left) The PC1 vs. PC2 scores plot. The TN cell lines are colored with red, the HER2+ line in blue and the ER+, HER2- cell lines are gray. (Top right) The PC1 vs. PC4 scores plot. The TN cell lines are colored with red and the RP cell lines are gray. (Bottom left) The loading plot for PC1. (Bottom middle) The loading plot for PC2. (Bottom right) The loading plot for PC4. The cell lines are plotted with the following symbols: ■ MDA-MB-231, ▲ BT549, ● HCC1395, ◆ HS578t, ▲ BT474, ◆ MCF7, ● HCC1428, and ■ T47D. Ellipses show the 95% confidence limits.

identified by PCA are plotted in Fig. 2 (and supplementary Fig. S-4).

Consistent with PC1 scores and loadings from Fig. 1, the BT549 and MDA-MB-231 cells showed the highest relative intensity of cholesterol, while the other cells showed varying levels corresponding with their location on PC1. The normalized ToF-SIMS cholesterol intensities were compared with relative cholesterol values from the Metabolon data available for five of the cell lines [Fig. 2(f)] (acquired from the DTP Molecular Target program³⁴). Metabolomic Profiling was performed using the platform described in Lawton *et al.*³⁵ This data illustrate the trend toward higher cholesterol levels in the BT549 and MDA-MB-231 cells for both datasets. Note that relative intensities are different due to the different platforms used. The presence of a similar trend in the data suggests that comparison of ToF-SIMS normalized intensities might be useful for lipidomic comparison between cell lines. However, it should be remembered that the ToF-SIMS sampling depth is only ~ 20 angstrom and thus is sampling only a part of the cells (i.e., primarily the inside of the cell after the C_{60} sputtering) and it is difficult to directly compare the data to any analytical method that might be sampling the entire cell composition.

The normalized intensities of the MAG 16:0 (m/z 313⁺) and the DAG 32:0 (m/z 551⁺) are shown in Figs. 2(b) and

2(d). While the MAG 16:0 can only contain a 16 carbon chain, the DAG 32:0 may be comprised of two chains of the same length (16:0/16:0) or different lengths (e.g., 18:0/14:0). Due to the fragmentation process of ToF-SIMS, it is possible that a single chain ion peak (such as MAG 16:0) can originate from a larger molecule (such as DAG 32:0) that contains chains of the same length. Here, we see that the intensity pattern of MAG 16:0 is similar to that from DAG 32:0, suggesting that ions attributed to this MAG species very likely originated from a parent DAG molecule, which helps support an identification of the larger mass peak. The normalized intensities of other MAGs and DAGs with different chain lengths and saturations are shown in supplementary Fig. S-4 for comparison. It has been hypothesized previously in ToF-SIMS studies that both MAG and DAG peaks may actually be fragments from larger (TAG) molecules.^{36,37} It is important to remember that the peaks attributed here to MAG and DAG species may actually be fragments from larger TAG molecules.

The intensity patterns of many of these lipid peaks show that there is a different distribution of the saturated and unsaturated species, as well as from different chain lengths, for different cell types. This can be seen with a comparison between the intensity patterns of saturated DAG 36:0 (m/z 607⁺) and unsaturated DAG 36:3 (m/z 601⁺) in Fig. 2 as

FIG. 2. Normalized intensities of selected positively charged ions. The species shown are (a) MAG 16:0, m/z 313⁺, (b) DAG 36:0, m/z 607⁺, (c) cholesterol, m/z 369⁺, (d) DAG 32:0, m/z 551⁺, and (e) DAG 36:3, m/z 601⁺. In (f), cholesterol relative values mined from metabolic data Metabolon dataset (Ref. 33). The TN cell lines are colored with red, the HER2⁺ line in blue, and the ER⁺, HER2⁻ cell lines are gray. All intensities were normalized by the sum of the intensities of all of the peaks in the $m/z > 200$ peak list.

well as other saturated and unsaturated species shown in Fig. S-4. These results indicate that the degree of saturation and chain length of these tails may play an important factor in understanding the chemical differences between these cell lines. This theory is supported by a recent clinical study by Hilvo *et al.* where fatty acid alkyl tail saturation for TAGs was shown to be related to patient pathologic complete response.³⁸ These normalized intensities and ratios of different DAGs and MAGs demonstrate the unique lipid compositions of each of the cell lines.

C. Comparison of cell lines using negative ions

The negative ion spectra from cells and tissues are often dominated by individual fatty acids peaks for $m/z > 200$. In our data, PCA of negative ion peaks with $m/z > 200$ showed a large variance in PC1, resulting in overlap of most of the cell lines (supplementary Fig. S-5). The exception was MDA-MB-231, and this line was separated from the other TN cell lines by the cholesterol peaks. However, scores plots for the cell lines were better separated in PC2 and PC3 (Fig. 3). PC2 largely separates the receptor positive lines from one another and PC3 predominantly separates out the TN cell lines from each other.

The HER2⁺ BT474 cell line showed large positive scores along PC2 and was well-separated from the ER⁺ and TN cell lines, which are all negative for the HER2 receptor. It is significant that the HER2⁺ cell line is so well separated from the others since HER2⁺ tumors have been shown to have unique metabolic signatures.³⁹ The fatty acids that correlate with the BT474 (HER2⁺) cell line are myristic (14:0, m/z 227⁻), and palmitic (16:0, m/z 255⁻), acids which have high positive loadings in PC2 (Fig. 3, bottom left) along with fatty acids 16:1 (m/z 253⁻: attributed to palmitoleic acid), 20:4 (m/z 303⁻: attributed to arachidonic acid)⁴⁰ and 20:3 (m/z 305⁻: attributed to dihomo-gamma-linolenic acid).⁴¹ In contrast, peaks attributed to linoleic (18:2, m/z 279⁻) and oleic acid (18:1, m/z 281⁻) had the largest negative loadings. Linoleic acid is a precursor fatty acid (FA) species for synthesis of arachidonic acid. Increased arachidonic acid has been found in breast cancer tissue and is associated with cancer cell proliferation.^{42,43} The high arachidonic acid (AA) and dihomo-gamma-linolenic acid (DHA) in HER2⁺ cells concomitant with lower linoleic and oleic acid suggests depletion of these lipids to provide for AA and DHA synthesis.

Overall, the RP cell lines have decreased correlation with arachidonic acid but increased correlation with the linoleic

FIG. 3. PCA of eight breast cancer cell lines using the $m/z > 200$ peak list and negative ions. The TN cell lines are colored with red, the HER2+ line is blue, and the ER+ cell lines are gray. (Top right) The PC2 vs. PC3 scores plot. The TN cell lines are colored red, and the RP cell lines are gray. (Bottom left) The loading plot for PC2. (Bottom right) The loading plot for PC3. The cell lines are plotted with the following symbols: ■ MDA-MB-231, ▲ BT549, ● HCC1395, ◆ HS578t, ▲ BT474, ◆ MCF7, ● HCC1428, and ■ T47D. Ellipses show the 95% confidence limits.

acid precursor that provide substrates for arachidonic acid synthesis. Since it is the receptor positive cell lines which separate most clearly on PC2, it is possible that altered linoleic acid metabolism is more important for receptor positive cell lines than the TN lines. These data are interesting because of the association of linoleic acid metabolism with more aggressive breast cancers. For example, the ratio of the linoleic acid (LA) metabolites (C18:3 n-6, C20:3 n-6, and AA) to LA, a proxy measure of delta-6 desaturase enzyme activity, was increased in cancerous tissue, suggesting greater conversion of LA to AA and was higher in ER- than in ER+ patients, indicating genotype-related trends.⁴²

The TN lines separated from one another on PC3. The MDA-MB-231 and BT-549 lines had the highest positive scores correlating with peaks attributed to stearic acid (18:0, m/z 283⁻), dihomo-gamma-linolenic acid (20:3), and arachidonic acid (20:4), which had the highest positive loadings (Fig. 3, bottom right). The data points from the TN HCC1395 cell line had negative scores, where peaks

attributed to oleic acid (18:1) had the highest negative loadings. While again many of the fatty acids in the arachidonic acid synthesis pathway appear as high loading peaks, both the linoleic precursor and arachidonic acid having positive loadings indicate that this metabolic pathway may not play a prominent role in separating out these TN cells.

The normalized intensities of key negatively charged ions that were identified from PCA are shown in Fig. 4 (and supplementary Fig. S-6).

While mathematically it is not expected that the normalized intensities will show similar trends to PC scores higher than PC1, there is a good correlation to the trends seen for the FA16:0 (palmitic acid) with the PC2 score trends and as well as a trend for FA 18:0 (stearic acid) with PC3 likely because they showed strong loadings with only one of the PC scores and did not load as strongly with the other. However, the 18:1 (oleic acid) normalized peak, with strong loading for both PC2 and PC3, did not show a trend that could be easily related to the PCA scores plots. This is likely

FIG. 4. Normalized intensities of selected negatively charged ions. The species shown are (a) palmitic acid, m/z 255⁻, (b) oleic acid, m/z 281⁻, (c) stearic acid, m/z 283⁻, and (d) dihomogamma-linolenic acid, m/z 305⁻, (e) arachidonic acid, m/z 303⁻, and (f) linoleic acid, m/z 253⁻. The TN cell lines are colored with red, the HER2+ line in blue, and the RP (HER2-) cell lines are gray. All intensities were normalized by the sum of the intensities of all of the peaks in the $m/z > \times 200$ peak list.

due to the fact that each PC is calculated from the covariance matrix after subtracting previous PCs.

The arachidonic acid 20:4 species [Fig. 4(e)] is interesting not only because it was identified as a peak with a high loading in both PC2 and PC3, but also, as mentioned above, arachidonic acid has been found to be associated with breast cancer cell proliferation. Unsurprisingly, the normalized intensity for the 20:4 species is high for the BT474 HER2+ cell line, consistent with the PCA results, and a similar trend is noted for the 20:3 species (dihomogamma-linolenic acid). However, the triple negative lines BT549 and MDA231 also show significantly higher normalized intensity of the 20:4 species. The PC2 results indicated that there was an anticorrelation between linoleic and arachidonic acid (Fig. 3). Comparison of the normalized intensities of these species [Figs. 4(e) and 4(f)] indicates that this is most significant for the HER2+ cell line BT474 and the RP line HCC1428, and these were also the two cell types with the highest positive and negative scores on the PC2 scores plot.

IV. SUMMARY AND CONCLUSIONS

The ToF-SIMS data acquired from eight human breast cancer cell lines were compared, and chemical differences

between the cell lines were found using PCA. A robust method to acquire ToF-SIMS data from multiple cell lines grown in different media is presented that includes chemical fixation, C₆₀ presputtering, and selecting cellular ROIs. Using this cell preparation method, there was no separation seen in PCA scores that could be related to the effect of the growth media. When the peak lists were truncated to m/z 200 – 852, for both positive and negative secondary ions, the lipid related chemistry could be emphasized and trends were found in the PCA scores for the different breast cancer receptor classifications (i.e., triple negative and receptor positive).

For the positive ion data, PCA identified phosphocholine and cholesterol as the largest sources of variance for triple negative classification of cell lines while MAG and DAG tail chain length separated out the receptor positive cell lines. In a study by Lehmann *et al.*,⁴⁴ triple negative breast tumors were classified into six different subtypes using gene expression analysis. The MDA-MB-231, HS578t, and BT549 cell lines were identified within the “mesenchymal-like” subgroup of TN lines, while the HCC1395 line was unclassified.⁴⁵ In our data, a similar grouping of MDA-MB-231, HS578t, and BT549 cell lines separated from HCC1395 is seen across PC2 positive ion data, where all of the data

points of the HCC1395 line have negative scores possibly due to a different ratio of PChol to cholesterol from the other cell lines.

In the negative ion data, both the length and saturation index of fatty acids were important sources of variance. The HER2 + line (BT474) was found to have a unique chemical signature from three other receptor positive cell lines that was specifically related to the composition of key fatty acids (anticorrelated with 18-carbon chain fatty acids). The data suggest that fatty acid metabolism, particularly elongation and saturations pathways,⁴⁵ can be used to distinguish the cell lines from one another using ToF-SIMS.

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