LETTER

Superpixel Raman spectroscopy for rapid skin cancer margin assessment

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Funding information
Cancer Prevention and Research Institute of Texas, Grant/Award Number: RP130702

Abstract
Spontaneous Raman micro-spectroscopy has been demonstrated great potential in delineating tumor margins; however, it is limited by slow acquisition speed. We describe a superpixel acquisition approach that can expedite acquisition between \( \times 100 \) and \( \times 10,000 \), as compared to point-by-point scanning by trading off spatial resolution. We present the first demonstration of superpixel acquisition on rapid discrimination of basal cell carcinoma tumor from eight patients undergoing Mohs micrographic surgery. Results have been demonstrated high discriminant power for tumor vs normal skin based on the biochemical differences between nucleus, collagen, keratin and ceramide. We further perform raster-scanned superpixel Raman imaging on positive and negative margin samples. Our results indicate superpixel acquisition can facilitate the use of Raman microspectroscopy as a rapid and specific tool for tumor margin assessment.

KEYWORDS
basal cell carcinoma, Raman imaging, Raman spectroscopy, rapid acquisition, skin cancer, tumor margin

1 INTRODUCTION

Raman spectroscopy is a sensitive in detecting molecular differences between tumor and healthy tissue without requiring tissue sectioning or staining. Raman microspectroscopy offers the potential for accurate tumor margin detection for many cancer types, including skin [1], oral cavity [2], breast [3] and brain [4]. The detection is usually implemented in an imaging mode with a spatial resolution of \( \sim 1 \mu m \). However, point-by-point scanning is extremely time-consuming: a single point typically requires \( \sim 1 \) second in tissue; thus, to scan the complete surface of a multicentimeter tissue would take hours or even days.

A few approaches have been proposed to speed up Raman acquisition. Stimulated Raman techniques such as CARS [5] and SRS [6] produce images akin to hematoxylin and eosin (H&E) stained histology slides without staining, but under most implementations trade imaging speed for...
spectral content. Several studies have accelerated spontaneous Raman measurements using sparse sampling techniques (sampling at 10-20 μm increments [1, 7–9]) collecting a full spectrum at each pixel at the expense of limited coverage of the tissue surface area (2% or less). Other methods that have been developed include line-scanning confocal [10], 2D multifocal arrays [11] and Wiener estimation [12], with speed up factors reported between ×10 and ×100. One attractive approach is superpixel acquisition whereby a spectrum is averaged over a larger pixel (aka a superpixel of ~25 × 25 μm²-100 × 100 μm²) on the sample surface while integrating only once on the detector. Thus, the acceleration mainly comes from the reduction of total detector reading time. This approach was recently used for small superpixel sizes from 1 × 1 μm² to 30 × 30 μm² for human bone characterization [13].

In this letter, we aim at the proof of concept that superpixel acquisition performs statistically the same as our previous point-by-point scanning approach in classifying basal cell carcinoma (BCC) from normal skin structures while substantially speeding up acquisition. The speed up factor is proportional to the area of the superpixel. For a superpixel size between 25 × 25 μm² and 100 × 100 μm², the maximum speed up factor varies between ×625 and ×10 000, as compared to point-by-point scanning with 1 μm² point when sampling the complete surface area. We demonstrate the equivalence of superpixel acquisition in tissue simulating phantoms and human skin cancer specimens. Furthermore, we demonstrate raster-scanned superpixel Raman classification images of both positive and negative margin samples, and emphasize the need to determine the optimum superpixel size for the application of skin cancer diagnosis.

2 | EXPERIMENTAL

2.1 | System description

The system is based on a custom-built confocal Raman microspectroscopy integrated with a reflectance confocal microscope as described previously [14]. Superpixel acquisition still utilizes a confocal setup. The 1 μm laser spot is rapidly scanned across a user-defined region by using galvanometer scanners, while the CCD camera collects one average confocal Raman spectrum. As shown in Figure 1A, the speed up factor is proportional to the superpixel area and results in a trade-off in resolution. In this way, one can trade-off spatial resolution for speed and acquire an image that covers the complete surface. For this study, we mainly focus on a superpixel size of 100 × 100 μm², as that closely matches the resolution of a dermatopathologist analyzing frozen section histopathology.

2.2 | Comparison between point-by-point scanning and superpixel acquisition

The spectral difference and signal-to-noise ratio (SNR) were compared between point-by-point scanning and superpixel acquisition using phantom experiments [13]. All raw spectra underwent wavenumber calibration, dark noise subtraction, cosmic ray removal, spectral response calibration, smoothing and fluorescence background removal.

For the spectral difference test, mixed phantoms of synthetic collagen type I and elastin were measured using either point-by-point scanning (2 μm step size, 1 second per step) or superpixel acquisition (1 second per spectrum, repeated 10 times).

For the SNR test, SNR was calculated as follows [16]:

$$SNR(\nu) = \frac{I_{mean}(\nu)}{SD(\nu)}$$

where $I_{mean}(\nu)$ is the mean intensity and $SD(\nu)$ is the SD at a given wavenumber (ν). Because $SD(\nu)$ is not only influenced by the signal variations from the experimental setup but also the inhomogeneity of the sample [13], we used a pure collagen type I phantom. We measured the SNR of point-by-point scanning by taking 20 spectra only at the central position, and the SNR of superpixel acquisition by taking 20 spectra across the entire region. Integration time is 1 second per spectrum.

2.3 | Sample preparation

Ten skin tissue samples were obtained from eight patients undergoing Mohs micrographic surgery. Seven samples were found to have both BCC and normal tissue, and three contained only normal tissue. For each sample, a skin section of 20 μm thickness was cut at −22°C with a microtome, and then transferred onto an MgF₂ substate for the experiment. The serial section went through H&E staining for histopathological diagnosis by a board-certified dermatologist. This study was approved by the Institutional Review Board at The University of Texas at Austin and the Seton Healthcare Family.

2.4 | Superpixel acquisition experiment and model establishment

Multiple locations were sampled on each skin section as shown in Figure 2A. By visual comparison of reflectance, bright-field and histopathology images, each superpixel was annotated as either BCC or one of the seven primary normal skin structures, including epidermis, dermis,
inflamed dermis, hair follicle, hair shaft, sebaceous gland and fat. The corresponding average spectra were also saved in the database.

A previously established biophysical inverse model [14] was fitted to the average tissue spectra by nonnegative least squares fitting. The model has been recently applied to in vivo skin cancer diagnosis [17], and ex vivo skin tumor margin assessment [15]. The model describes the tissue spectra as a linear combination of the basis spectra of collagen, elastin, triolein, nucleus, keratin, ceramide and water. The fit coefficients were then used as the input variables of a logistic regression classifier to discriminate BCC from normal skin structures. Receiver operator characteristic (ROC) curve was built on leave-one-out cross validation. The optimal combination of input model components was determined by maximizing the area under the ROC curve.

2.5 | Raster-scanned superpixel imaging

Two samples from two new patients were used to test the raster-scanned superpixel imaging. One sample contains both BCC and normal dermis (positive margin), while
the other sample contains only normal tissue, mainly dermis and fat (negative margin). Superpixel imaging was performed by translating the sample in two dimensions using a linear motorized stage. Two superpixel sizes were compared: $100 \times 100 \mu m^2$ and $50 \times 50 \mu m^2$. The classification model established in Section 2.4 was applied to each superpixel, labeling it as positive or negative. A binary tumor heat map was generated by prioritizing high specificity.

3 | RESULTS AND DISCUSSION

We observed that point-by-point scanning and superpixel acquisition exhibit visually similar spectra (Figure 1B). Signal intensity is similar for both the point scanning setup and superpixel setup, and the SNRs are also similar (Figure 1C). The major reason that SNRs are similar is because when the shot noise is dominant, SNR should be proportional to the square root of the signal intensity [16]. Although the signal intensity of individual point fluctuates under the superpixel setup, the overall intensity retains the same after integrating over the entire region.

Figure 2A shows a typical example of a superpixel acquisition experiment. In total, we collected 154 annotated tissue spectra, including 28 spectra from BCC and 126 spectra from normal skin structures (epidermis [N = 17], dermis [N = 29], inflamed dermis [N = 14], hair follicle [N = 33], hair shaft [N = 13], sebaceous gland [20]).
The model fitting results are shown in Figure 2B.

Figure 3A shows the scatter plots of the fit coefficients of primary model components. We found that nucleus is the most important component to discriminate BCC from normal tissues. BCC has a significantly larger amount of nucleus compared to normal structures. BCC also has a significantly higher amount of keratin compared to dermis/inflamed dermis, and a lower amount of keratin compared to epidermis and hair follicle. In addition, BCC has significantly lower collagen and higher ceramide than dermis/inflamed dermis.

The optimum classification result was achieved by combining the fit coefficients of nucleus, collagen, keratin and ceramide as the input parameters, leading to an area under the ROC curve of 0.95, as shown in Figure 3B. By selecting a balanced tumor score threshold, the specificity and sensitivity reached 94% and 82%, respectively. This ROC was then statistically compared to our recent study in 30 patients using R software to determine if the superpixel acquisition provides equivalent potential for BCC classification compared with the point-by-point scanning.

Our results show that the areas under the two ROC curves are not statistically different ($P$ value = .34). Because the $P$ value does not convey the statistical power of the comparison, we also estimated the sample size required to demonstrate a difference in the area under the ROC curves at the level observed in our study. Since the required sample size is very large ($N > 1500$), we

[$N=15$] and fat [$N=5$]).
conclude that the effect size is small, that is, the observed difference in the area under the ROC curves is not meaningful. Therefore, the discriminant capabilities of superpixel acquisition and point-by-point scanning were not statistically distinguishable for BCC classification.

Figure 4 demonstrates an example of raster-scanned superpixel imaging of partial tissue samples. For these two samples, the negative and positive margins are correctly classified. In Figure 4A, BCCs are identified in both the $100 \times 100 \ \mu m^2$ and $50 \times 50 \ \mu m^2$ superpixel Raman classification images. Although prioritizing high specificity would lead to discontinuous positive pixels, it guarantees that only true positive pixels are classified as positive (high positive predictive value). In Figure 4B, the whole image is classified as normal tissue.

4 | CONCLUSION

In this study, we demonstrate a significant speed advantage of superpixel acquisition Raman spectroscopy for BCC tumor margin assessment. When compared to point-by-point scanning, the speed up factor is dictated by the ratio of the superpixel area to the laser spot size. Our superpixel approach provides an alternative speed up method to sparse sampling approach in applications where such high resolutions are not needed, enabling more complete sampling of the tissue surface area. It is worth mentioning that sparse sampling techniques [1, 7–9] have also demonstrated capability in detecting BCC margin. The speed up factor would be between $\times 25$ and $\times 100$ compared with sparse sampling using a step size between 10 and 20 $\mu m$. Further experiments are needed to compare between the performance of superpixel acquisition and point-by-point scanning with a matching step size (eg, $100 \ \mu m$) for the same tissue surface area.

The total estimated scan time for tissue samples with areas of $1 \times 1 \ \text{cm}^2$ would be around 2.7 hours ($100 \times 100 \ \mu m^2$ superpixel, 1 second per step). Future developments could combine this approach with other speed up approaches (eg, line-scanning confocal [10], multifocal [11] and Wiener estimation [12]) aimed to further reduce the acquisition time for intraoperative use (eg, $<1 \ \text{hour}$). When compared to gold standard diagnostics such as histopathology, the sensitivity of 82% is still not acceptable for cancer detection. The goal of this study was demonstrating equivalence between the superpixel approach and traditional point scanning approach. It is our hope that combining this technique with other imaging modalities (such as autofluorescence imaging [8, 9] and reflectance confocal microscopy imaging [20]) the sensitivity can be improved upon without sacrificing speed.

**FIGURE 4**  Raster-scanned superpixel Raman classification images of, A, positive and, B, negative margin samples. H&E, reflectance confocal microscope and Raman classification images of the same region are compared. The positive region is marked on the hematoxylin and eosin (H&E) image. Two superpixel sizes were compared: $100 \times 100 \ \mu m^2$ and $50 \times 50 \ \mu m^2$. Scale bar: 200 $\mu m$. 
Our next step involves fully characterizing the trade-offs of the superpixel approach by expanding to imaging of samples and more diversity of samples. We are currently collecting larger superpixel data sets containing diverse tissue structures (eg, hair follicle, inflamed dermis and epidermis), and including more tissue types per image to help refine our diagnostic algorithm and improve sensitivity further. We will also compare between different superpixel sizes to determine the optimum size for the application of skin cancer diagnosis that is larger than a single cell but smaller than the point when mixed microanatomies begin to obscure the identification of tumor cells.

ACKNOWLEDGMENTS
This work was funded by Cancer Prevention & Research Institute of Texas (CPRIT) (RP130702). We thank the Seton Healthcare Family and the Austin Dermatological Surgical Center for collaboration. We thank Sandra Esparza and Leandra Turner for their help in collecting fresh tissue specimens, Prof. Aaron Baker for providing the histology facility, and Greg Lyness and Esther Maier for H&E staining. We also thank UT Statistical consulting service and Erika Hale for providing statistical guidance.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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How to cite this article: Feng X, Fox MC, Reichenberg JS, et al. Superpixel Raman spectroscopy for rapid skin cancer margin assessment. J. Biophotonics. 2020;13:e201960109. https://doi.org/10.1002/jbio.201960109
In Situ DESI-MSI Lipidomic Profiles of Breast Cancer Molecular Subtypes and Precursor Lesions
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ABSTRACT

Clinically meaningful molecular subtypes for classification of breast cancers have been established, however, initial and progression of these subtypes remain poorly understood. The recent development of desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) facilitates the convergence of analytical chemistry and traditional pathology, allowing chemical profiling with minimal tissue pretreatment in frozen samples. Here, we characterized the chemical composition of molecular subtypes of breast cancer with DESI-MSI. Regions of interest were identified, including invasive breast cancer (IBC), ductal carcinoma in situ (DCIS), and adjacent benign tissue (ABT), and metabolomic profiles at 200 μm elaborated using Biomap software and the Lasso method. Top ions identified in IBC regions included polyunsaturated fatty acids, deprotonated glycerophospholipids, and sphingolipids. Highly saturated lipids, as well as antioxidant molecules [taurine (m/z 124.0068), uric acid (m/z 167.0210), ascorbic acid (m/z 175.0241), and glutathione (m/z 306.0765)] were able to distinguish IBC from ABT. Moreover, luminal B and triple-negative subtypes showed more complex lipid profiles compared with luminal A and HER2 subtypes. DCIS and IBC were distinguished on the basis of cell signaling and apoptosis-related ions [fatty acids (341.2100 and 382.3736 m/z) and glycerophospholipids (PE (P16:0/22:6, m/z 746.5099, and PS (38:3, m/z 812.5440)]. In summary, DESI-MSI identified distinct lipid composition between DCIS and IBC and across molecular subtypes of breast cancer, with potential implications for breast cancer pathogenesis.

Significance: These findings present the first in situ lipidomic findings of the four molecular subtypes of breast cancer, DCIS, and normal tissue, and add to the understanding of their pathogenesis.

Introduction

Breast cancer is the most common cancer affecting women and represents a complex group of diseases that exhibit great variability at clinical presentation and biologic aggressiveness. Numerous clinical studies of breast cancer coupled with advances in genomic profiling provided data that support the existence of clinically useful molecular subtypes (1). In particular, assessments of hormonal receptor [estrogen receptor (ER) and progesterone receptor (PR)] and HER2 status have contributed to translation of this molecular subtype into clinics (2). Despite all genomic characterization, our understanding of initiation and progression of breast cancer remains incomplete. Cell processes driving invasiveness from ductal carcinoma in situ (DCIS) stage and differentiation into each molecular subtype of breast cancer are complex, and analyses beyond genomics are needed to better understand them.

Metabolic reprogramming in cancer cells, referred to as the Warburg effect, was described nearly a century ago and the interest on this effect has been recently renewed as it is considered one of the hallmarks of cancer cells (3, 4). Lipids play a key role in cell membranes’ structure and trafficking and are substrates for energy production (5), and de novo lipogenesis has roles in many other cellular processes, such as oxidative stress–induced cell death, regulation of chemotherapeutic agent uptake (6), and the generation of signaling molecules (5). Genomic changes in breast cancer, such as deletion of chromosome 8p, lead to de novo lipogenesis and that loss has been recognized as a hallmark of aggressive cancers (7–10).

The convergence of two powerful disciplines, pathology and analytical chemistry, has facilitated studies of discrete molecular changes that occur in cancer cells. In situ mass spectrometry has helped to elucidate the complex connections between metabolism, genomics, and cellular functions (11). Desorption electrospray ionization (DESI) represents a recent and robust advance among ambient ionization techniques that allows scientists to study cancer cell metabolism coupled to mass spectrometry imaging (MSI) with minimal sample pretreatment (12–15). Global levels of metabolites and their spatial distributions with a 200 μm resolution can be determined from frozen tissue samples and comparisons across normal, precancerous, or invasive regions of interest (ROI) stained with hematoxylin and eosin (H&E) bring new insights to cancer pathogenesis. To date, desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) has been used to study the metabolite profiles of various types of cancer tissues, including breast (16, 17), brain (18), gastric (14), prostate (11), and has been considered a promising tool diagnosis and tumor margin evaluation (19).

The aim of this study was to integrate lipidomic and metabolomic data to distinguish molecular subtypes of breast cancer using in situ DESI-MSI. Metabolomic profiles of frozen slides containing paired tumor and normal samples were created in two-dimensional (2D) maps. Small molecules 2D maps were overlaid with digital H&E images of the same samples, harboring invasive breast cancer (IBC), in situ lesions, and normal tissue. These data were examined considering the ROI to better understand the lipidomic differences across subtypes of breast cancer.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Cancer Res 2020;80:1246–57
doi: 10.1158/0008-5472.CAN-18-3574
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Materials and Methods

Ethics statements

This study was approved by the Institutional Research Board of the AC Camargo Cancer Center (São Paulo, Brazil; No. 1830/13) and adheres to the tenets of the Declaration of Helsinki. Written informed consent for collection of tissues for research was obtained from all patients.

Patient samples

Frozen tumor samples were collected from 68 patients. These samples included 122 tissues (67 breast tumor tissues and 55 paired normal tissues) representing different molecular subtypes of breast cancer: luminal A (n = 13), luminal B (n = 17), triple-negative (n = 12), HER2+ (n = 10), pure DCIS (n = 5, of which, 2 are ER+/HER2-, 2 are ER-/HER2+, and 1 is ER-/HER2+), and DCIS adjacent to an invasive tumor (n = 10, of which, 6 are ER+/HER2+, 3 are ER-/HER2+, and 1 is ER-/HER2+; Supplementary Table S1). The samples were resected with sterile surgical blades and were snap-frozen in liquid nitrogen within 30 minutes of collection. The samples were stored at −140°C until they were sectioned. At least three sequential sections (each 10 μm thick) were prepared from each sample with a Leica Multicut 2045 Cryotome. The cryotome chamber was chilled to −29°C and the specimen holder was chilled to −20°C. The samples were kept on dry ice before and after the sections were cut and they were subsequently stored at −80°C. For DESI-MSI samples were transported on dry ice. Prior to DESI-MSI, the glass slides were dried in a desicator for approximately 15 minutes. After DESI-MSI, the slides were stained with H&E and scanned with the Aperio XT Scanscope (Leica Biosystems). Digital H&E images were overlaid with their corresponding 2D metabolic images.

Mass spectrometry imaging

A 2D DESI Source (Prosidia, Inc.) coupled to a QExactive HF hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) was operated at 70,000 resolving power for tissue imaging. Data were acquired in the negative-ion mode (−5 kV) over a mass-to-charge (m/z) ratio range of m/z 100–1,200 for 42 samples (batch 1 from sample A1 to A42) and over a range of m/z 100–1,000 for 26 samples (batch 2 from sample A43 to A68). Analyses were performed in a matrix design, with 200 μm spatial resolution. A histologically compatible solvent, 100% methanol (HPLC grade; Merck), was used for analyses at a flow rate of 1.5 μL/min. Nitrogen was used as the sheath gas for electrosprey nebulization at a pressure of 175 psi. DESI-MSI data were collected from entire tissue sections. These data were used to generate a m/z spectra every 200 μm, and these spectra were used to build 2D metabolic maps. XCalibur 2.2 Software (Thermo Fisher Scientific) was used to acquire data and Firefly was used to convert processed mass spectral data into image files (.img). These files were read by the biomedical image analysis software, Biomap (freeware, https://ims-imaging.org/wp/). All imaging experiments were performed under identical experimental conditions, including identical geometrical parameters. Data were preprocessed by background subtraction and normalization based on total ion current. DESI-MSI intersection and intrasample reproducibility were assessed by analyzing different tissue sections of mouse brain tissue on different days under the same experimental conditions. Very similar mass spectra profiles were observed confirming that DESI-MSI is reproducible between serial sections of tissue and from scan to scan within one experiment. Full spectra profile reproducibility was also investigated, and %RSD values of approximately 1% were obtained, showing that similar mass spectra profiles are obtained from similar samples.

Histopathologic and molecular classifications

After DESI-MSI, the same tissue sections were stained with H&E for histopathologic evaluation by an expert pathologist. Careful histologic evaluations were performed by a breast pathologist (V.P. de Andrade) who used digital images to assign the following ROI: adjacent benign tissue or normal lobules (ABT), DCIS, and IBC. Molecular classification of each sample was recorded from the original diagnostic IHC and ISH assays. These classifications included the following antibody reagents as appropriate: a rabbit mAb recognizing ER (SP1, Roche), an anti-PR rabbit monoclonal primary antibody (1E2, Roche), a rabbit monoclonal primary antivirus antibody for HER2 [IHC, Anti-HER-2/neu (4BS), Roche] or a dual color-dual hapten ISH antibody (as needed), and a rabbit primary anti-Ki-67 antibody (30-9, Roche). The luminal A subtype was defined as ER+ and/or PR+, HER2+, and Ki-67 < 20%. The luminal B subtype was defined as ER+ and/or PR+, as well as PR < 20%, HER2+, or Ki67 >20%. The HER2 subtype was defined as ER-/PR+ and HER2+. The triple-negative subtype was defined as ER, PR, and HER2+. IHC was performed on automated protocols recommended for the Roche/Ventana Benchmark XT Autostainer (Roche/Ventana).

Imaging evaluation

ROI were overlaid on 2D molecular images with Biomap software to correlate m/z spectra with ABT, DCIS, and IBC tissues (Fig. 1). A corresponding spectrum was generated for each selected ROI and was exported as a .txt file for statistical analysis.

Statistical analysis

The large amount of molecular data obtained for each sample hindered a direct interpretation of the data. Mass spectra .txt files obtained from ROIs were imported into the R environment for statistical analysis using the Lasso method (least absolute shrinkage and selection operator; refs. 20, 21). Forty-two samples analyzed by DESI-MSI (from sample A1 to A42) were analyzed by Lasso, the other 26 samples (from sample A43 to A68) were not included in the Lasso analyses because their mass spectra were obtained over a different m/z range. Lasso is a method of regression analysis with a regularization term, which leads to variable selection. Briefly, Lasso minimizes the usual sum of squared errors (as other regression methods for supervised learning); however, while performing this, it restrains the sum of the absolute values of the regression coefficients to be less than a fixed value. This constraint shrinks the coefficient estimates toward zero and forces some of them to be exactly equal to zero. Models generated from the Lasso are therefore simpler, sparse, and generally more accurate and much easier to interpret. Lasso regularization also avoids overfitting to data and selects variables that are most important to the model. Particularly, when applied to a classification problem, Lasso will select a subset of the variables that most discriminate the sample groups. In this study, Lasso was initially applied to m/z values of both invasive and benign tissues that were obtained from ROI. The resulting models contained sets of variables that were able to discriminate IBC from ABTs. Next, Lasso was applied to ratios of m/z values for invasive and ABTs to generate models that could discriminate the following groups: (i) IBC × DCIS and (ii) subtypes of breast tumors (e.g., luminal A, luminal B, triple-negative, and HER2+). Briefly, a mathematical weight for each statistically informative m/z value was calculated according to Lasso depending on the importance of the mass-spectral feature in characterizing a certain group. Features that were not statistically
informative received a weight of zero and were disregarded. An anion whose peak height (abundance) was important to characterize a certain group received a positive weight value. In contrast, important ions with low abundances, or those that were absent, received a negative weight. Because the features selected according to Lasso can occur at a valley or a shoulder of an actual mass spectra peak, m/z ion peaks were selected by characterizing the nearest mass spectra peak to the statistically selected feature. The generated models were cross-validated according to the leave one out method, with each sample in the set being ignored during data modeling at least once.

**Database searches**

Each ion was selected on the basis of visual inspection of 2D molecular images and the Lasso method. Each ion was subsequently compared with available literature and identified on the basis of searches of lipid maps, metabolomics, human metabolome database data available at www.lipidmaps.org, www.metabolomicworkbench.org, and www.hmdb.ca respectively, based on high mass accuracy and isotopic distribution considering a mass error of 5 ppm. When the isomerism of the double bonds in the fatty-acid chains of complex lipids, and isobaric species, resulted in more than one proposed structure, the information about the category to which they belonged was considered.

**Metabolites identification**

Selected ions were submitted, after ultra-high-performance liquid chromatography (UHPLC) separation, to higher energy collision-induced dissociation and compared the corresponding fragmentation profile with that of the standard from the human metabolome database (HMDB) to confirm the identity of this species. To each tissue sample,
400 µL of methanol (HPLC grade; Merck), were added and samples were vortexed for 3 minutes, followed by centrifugation at 13,000 rpm for 5 minutes. Samples were analyzed on a Q Exactive Orbitrap System (Thermo Fisher Scientific) connected to an Ultimate 3000 RS UHPLC chromatographic system (Dionex). Mobile phases were water + 0.1% formic acid (A) and acetonitrile (B; HPLC grade; Merck), and the column was an Acquity HSS C18 2.1 × 100 mm, 1.8 µm (Waters). Separation was performed on a 6-minute gradient from 2% to 100% B, followed by cleaning and conditioning steps. Column (40°C) and samples (10°C) were kept at a constant temperature. The Orbitrap was run on MS Scans on both polarities, using the following parameters: capillary voltage +3.5/−2.5 kV; capillary temperature 275°C; sheath gas 55; auxiliary gas 15; spare gas 3; probe heater temperature 450°C; and S-Lens RF level 50. MS scans were acquired from m/z 50 to 750, with 70,000 resolution, automatic gain control target of 5e6 and maximum IT time of 200 ms. Whenever possible, collision energy was tuned for optimized fragmentation.

Results
Comparison between areas of normal tissue versus tumor tissue
An inspection of the 2D molecular images that were collected from the 122 samples (67 tumors and 55 ABTs) resulted in the observation of more than 100 ions that delimited ROI. Figure 2 and Supplementary Fig. S1 (SI Appendix) show representative mass spectra that were extracted from ABT and IBC regions of two samples for each analyzed subtype. Figure 1E and F provides an example of the ROI that were identified with H&E staining and that corresponded with 2D maps of lipid and metabolite distribution for a sample of luminal B subtype (sample A2), and Supplementary Fig. S2 (SI Appendix) shows examples for each subtype analyzed.

The 2D ion images obtained from the DESI-MSI analyses performed exhibited high heterogeneity in their molecular distribution (Fig. 2; Supplementary Fig. S1, SI Appendix). The anions identified mainly included metabolites, xenobiotics, and deprotonated lipids (Supplementary Tables S2 and S3, SI Appendix). Most of the metabolites, such as glutamine (m/z 145.0612) and glutamate (m/z 146.0453), were present at higher relative abundances in the IBC regions than in the ABT regions. (Fig. 3; Supplementary Fig. S3, SI Appendix). Similarly, anions from antioxidant molecules, such as uric acid (m/z 124.0068), uric acid (m/z 167.0210), ascorbic acid (m/z 175.0241), and glutathione (m/z 306.0765), were present at higher relative abundances in the IBC tissues (Fig. 3; Supplementary Fig. S3, SI Appendix). Meanwhile, in the ABT regions, the most abundant anions included caprylic acid (m/z 143.1072), palmitoleic acid (m/z 253.2173), oleic acid (m/z 281.2487), and some organosulfur compounds such as benzyl sulfate (m/z 187.0065) docetylbenzensulfonic acid (m/z 325.1841), and sulfated steroids (m/z 367.1585 and 395.1897). The anion of m/z 187.0065 was the one that presented the best capacity to delimit the ABT (Fig. 4; Supplementary Fig. S4, SI Appendix). Some anions from xenobiotics, including picric acid (m/z 281.2487) and pantoprazole (m/z 382.067; Fig. 5), were also observed at higher relative abundances in ABT. In the m/z 400–950 range, the spectra for the IBC regions exhibited greater complexity and higher abundances than the ABT regions (Fig. 2; Supplementary Fig. S1, SI Appendix). HER2 tumors presented a different profile, with much lower relative abundances for the IBC and ABT regions when compared with other subtypes (Fig. 2; Supplementary Fig. S1, SI Appendix). Polysaturated fatty acids (FA) were among the top abundant anions in the IBC tissues. These polysaturated FAs included deprotonated eicosanoids (e.g., arachidonic acid, m/z 303.2331), glycerophospholipids (GP, e.g., glycerophosphoinositol (PI); m/z 885.5479 and glycerophosphoserine (PS; m/z 788.5438), and sphingolipids (SP; e.g., Cer(d42:2), m/z 682.5906).

It was further observed that unsaturated GPs, including PS, glycerophosphoethanolamine (PE), phosphatidic acid (PC), PI, and glycerophosphoglycerol (PG), presented different degrees of unsaturation in different ROI. The amount of saturated FAs was relatively higher in the IBC regions than in the other ROI (Fig. 6; Supplementary Fig. S5, SI Appendix). Supplementary Table S4 (SI Appendix) lists some of the molecules that were less unsaturated and in which subtypes were observed.

The Lasso method identified 18 ions with different m/z values in the m/z 100–800 m/z range as being important to the morphologic characteristics of the ROI identified and for the differentiation of IBC from ABT regions (Fig. 3; Supplementary Fig. S3; Supplementary Table SS A, SI Appendix). The metabolites selected included glutamic acid (m/z 146.0453) and ascorbic acid (m/z 175.0241). The xenobiotics selected included 2-(4’-chlorophenyl)-3,3-dichloropropenoate (m/z 248.9286). The former two metabolites were more concentrated in IBC regions, whereas the latter was present in greater abundance in ABT regions. In the range of m/z 300–800, Lasso selected anions from unsaturated FAs including eicosanoid such as arachidonic acid (m/z 303.2331), sphingolipids such as Cer(d36:1) (m/z 600.5140) and glycerophospholipids such as PS(36:1) (m/z 788.5438). Figure 3 and Supplementary Fig. S3 present comparisons of ion images obtained with results obtained by Lasso.

Comparison between IBC and DCIS tissues
The results obtained from the DESI-MSI analyses of DCIS samples (68% ER+, 37% HER2+), and 6% triple-negative) showed lower abundances and complexity compared with the luminal B and triple-negative IBC subtypes and higher abundances and complexity compared with the IBC HER2 subtype. Lasso subsequently selected four ions of different m/z that were present at higher abundances in DCIS. These ions included FAs (m/z 341.2100 and 382.3736) and GPs (PE(P-160)/22:6, m/z 746.5099 and PS(38:3), m/z 812.5440) (Supplementary Table SSB, SI Appendix). Figure 7 and Supplementary Fig. S6, SI Appendix, show the 2D molecular images for PE(P-160)/22:6 (m/z 746.5099 and PS(38:3) m/z 812.5440 in four DCIS samples.

Molecular subtypes
The spectra of each molecular subtype exhibited distinct metabolic profiles, with differences evident within the m/z 400–950 range (Fig. 2; Supplementary Fig. S1, SI Appendix). For example, the HER2 subtypes showed a much lower abundance of anions compared with the other subtypes (Fig. 2; Supplementary Fig. S1, SI Appendix). In contrast, the triple-negative and luminal B subtypes presented the highest relative abundance of anions, and this was mostly attributed to the presence of glycerolipids, GPs, and SPs (Supplementary Fig. S1, SI Appendix).

Lasso also selected 13 anions for the differentiation of molecular subtypes (Supplementary Table S6, SI Appendix). Five anions were selected as important for assigning a luminal A subtype. These included two anions with positive weights [linoleic acid, m/z 279.2330, and Cer(d40:1), m/z 656.5760] and three anions with negative weights (Unknown-3, m/z 107.9900 and GPs, m/z 765.5190 and m/z 772.5200). Four lipids with positive weights were selected as important for assigning the HER2 subtype. The anions were those of m/z 359.2720 (C27H44D2O2, assigned as steroid (ST) or FA); the PE, C16H29NO5P (m/z 751.5530); the PG, PG(36:3) (m/z 892.5906).
For classification of the luminal B subtype, two GPs [PG(32:3), m/z 751.4860 and PI(36:2), m/z 861.5330] were selected, whereas classification of the triple-negative samples included ascorbic acid (m/z 175.0241) with a positive weight and the GP (m/z 751.4860) with a negative weight.

Metabolite identification

Supplementary Figure S7A (SI Appendix) shows the extracted ion chromatogram (m/z 384) for sample A2. Supplementary Fig. S7B shows the exact mass spectrum (electrospray in the positive-ion mode), from which the molecular formula was calculated as C16H16F2N3O4S, compatible with the protonated molecule [M+H]+ of pantoprazole. To confirm this hypothesis, this cation was fragmented to generate its MS/MS spectrum (Supplementary Fig. S7C). Comparison of this spectrum with the ones at the HMDB database confirmed the identity of the proposed structure of pantoprazole. Supplementary Fig. S8A shows the extracted ion chromatogram (m/z 187) for sample A2, whereas Supplementary Fig. S8B shows the MS spectrum of m/z 187 (electrospray in the negative-ion mode). As Supplementary Fig. S8B clearly shows, this ion coelutes with several interfering species, some of them being isobaric, which in that case did not allow the acquisition of a proper fragmentation spectrum. Nevertheless, data processing resulted in the molecular formula of C7H7O4S, corresponding to the deprotonated molecule of benzyl sulfate. Closer inspection in the isotopic pattern of this ion is also compatible with the presence of a sulfur atom in this species, whose identity should be confirmed by further experiments.

Figure 2.

A, Representative DESI-MSI data (m/z 100-1200) in the negative-ion mode obtained from the region delimited by the black square on H&E staining and overlaid on the DESI-MSI image of ABT (benign) from a HER2 subtype sample (sample A43); the 2D molecular image presented was obtained for the anion of m/z 187.0065. B, IBC regions from a HER2 subtype sample (sample A43); the 2D molecular image presented was obtained for the anion of m/z 303.2331.
DESI-MSI is a fast and robust tissue-based imaging method that does not require fixation, staining, or any complex preparation protocol; however, it allows detection of numerous metabolites and their relative abundances. DESI-MSI has been able to distinguish benign from malignant metabolomic profiles (11) and was recently proven to be a reproducible technique for rapid breast cancer diagnosis (22). It has been applied to many types of tumors. In Supplementary Table S7 (SI Appendix), DESI-MSI data from thyroid (23), brain (18), gastric (14), pancreas (24), and prostate tumors are compared with our data, including, in bold and underlined, the lipids that presented different degrees of saturation in each type of tumor.

Herein we demonstrate the capacity of DESI-MSI to differentiate molecular subtypes of breast cancer, and to provide a better understanding of the metabolism inherent to particular ROI in breast tissue. Two-dimensional molecular images (with a spatial resolution of 200 \( \mu \)m) were correlated with histology to provide valuable information regarding the metabolism of specific regions of IBC, DCIS, and distinct molecular subtypes of breast cancer. Differences in molecular profiles between DCIS and IBC were observed before morphologic changes become apparent in malignant cells. Because very few frozen DCIS samples are available from biobanks due to challenges in macroscopic identification, our understanding of their biology and behavior has been therefore incomplete (25). In 2008, Castro and colleagues showed that gene subsets are differentially expressed between pure DCIS and the in situ component of lesions that coexist with invasive ductal carcinoma (26). We examined 15 DCIS, with 5 pure DCIS and 10 DCIS with invasive component, the larger cohort using DESI-MSI to date.

Lipidomic profiling was able to distinguish DCIS from IBC. When Lasso was applied, anions from four molecules were selected for identification of DCIS, two FAs and two GPs. Among these FAs anions, the most important one selected by Lasso was from a docosanoid molecule whose signaling activity contributes to an inflammatory mechanism (27). Docosanoids are produced from the oxidation of polyunsaturated fatty acids by more than one pathway including the oxygenation via cyclooxygenases (COX; ref. 28), lipoxygenases (29), and cytochrome P450 monooxygenases. COX enzymes control a wide spectrum of processes (30) and many studies, comparison of this spectrum with the ones at the HMDB database confirmed the identity of the proposed structure of pantoprazole. Supplementary Fig. S8A shows the extracted ion chromatogram (m/z 187) for sample A2, whereas Supplementary Fig. S8B shows the MS spectrum of m/z 187 (electrospray in the negative-ion mode). As Supplementary Fig. S8B clearly shows, this ion coelutes with several interfering species, some of them being isobaric, which in that case did not allow the acquisition of a proper fragmentation spectrum. Nevertheless, data processing resulted in the molecular formula of \( C_22H_{25}O_S \), corresponding to the deprotonated molecule of benzyl sulfate. Closer inspection in the isotopic pattern of this ion is also compatible with the presence of a sulfur atom in this species, whose identity should be confirmed by further experiments.
from 1977 to the present, show the involvement of cyclooxygenase, including COX-2 and COX-1, in breast cancer (31–33). The two GPs are intercorrelated in mammalian cells and include a PE and a PS (34). This PS is primarily localized to the internal leaflet of the plasma membrane. In apoptotic cells, this PS translocates to the external leaflet, and this translocation is considered a crucial step in the recognition of apoptotic cells by macrophages (35). Taken together, these results suggest that mechanisms mediating signaling and apoptosis are more active in DCIS compared with IBC.

Reprogrammed activities for supporting cell survival under stressful conditions or allowing cells to grow and proliferate at abnormal levels included altered bioenergetics, enhanced biosynthesis, and redox balance (36). Changes in levels of some metabolites involved in these processes were revealed by DESI-MSI. The higher relative abundance of glutamine in IBC regions of luminal subtypes could be one example of enhanced biosynthesis and altered bioenergetics. Glutaminolysis is an important anaplerotic flux in cancer, which generates TCA cycle intermediates (36). The relative abundance of glutamine in the two more aggressive subtypes examined (e.g., HER2 and triple-negative) was lower than in the luminal subtypes, suggesting that more aggressive tumors are characterized by higher glutamine consumption, and this trend is consistent with a previous report that described low

![Discussion](https://bloodcancerdiscov.aacrjournals.org/content/80/6/1253/F5.large.jpg)

**Discussion**

DESI-MSI is a fast and robust tissue-based imaging method that does not require fixation, staining, or any complex preparation protocol; however, it allows detection of numerous metabolites and their relative abundances. DESI-MSI has been able to distinguish benign from malignant metabolomic profiles (11) and was recently proven to be a reproducible technique for rapid breast cancer diagnosis (22). It has been applied to many types of tumors. In Supplementary Table S7 (SI Appendix), DESI-MSI data from thyroid (23), brain (18), gastric (14), pancreas (24), and prostate tumors are compared with our data, including, in bold and underlined, the lipids that presented different degrees of saturation in each type of tumor. Herein we demonstrate the capacity of DESI-MSI to differentiate molecular subtypes of breast cancer, and to provide a better understanding of the metabolism inherent to particular ROI in breast tissue. Two-dimensional molecular images (with a spatial resolution of 200 μm) were correlated with histology to provide valuable information regarding the metabolism of specific regions of IBC, DCIS, and distinct molecular subtypes of breast cancer. Differences in molecular profiles between DCIS and IBC were observed before morphologic changes become apparent in malignant cells. Because very few frozen DCIS samples are available from biobanks due to challenges in macroscopic identification, our understanding of their biology and behavior has been therefore incomplete (25). In 2008, Castro and colleagues showed that gene subsets are differentially expressed between pure DCIS and the in situ component of lesions that coexist with invasive ductal carcinoma (26). We examined 15 DCIS, with 5 pure DCIS and 10 DCIS with invasive component, the larger cohort using DESI-MSI to date.

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Glutamine availability in aggressive tumor tissues (36). This data also may suggest that (i) these tumors could have other ways to enhance biosynthesis and obtain energy as described previously by Choi and colleagues; and (ii) protein expression of glycolysis markers such as Glut-1, CAIX, and MCT-4 was highest in HER2 and lower in luminal A and B (37). HER2 subtype is characterized by enhanced glycolytic metabolism and HER2-mediated expression of glycolysis-related genes (38), whereas less aggressive luminal subtypes appear to rely on a balance between de novo fatty acid synthesis and oxidation as sources for both biomass and energy requirements (39).

The HER2 tumors presented a singular lipid profile, which is consistent with data from previous transcriptomic and genomic studies, whereas HER2+ breast cancers have a molecular signature that distinguishes these cancers from other types of breast cancer (2). This trend is also consistent with the observation that amplification of HER2 does not have a dramatic effect on lipid metabolism in breast cancer (40).

A higher relative abundance of antioxidant molecules was detected in the IBC when compared with ABT. These results are consistent with the model in which the elevated activity of tumor cells neutralizes the...
large amount of reactive oxygen species (ROS) that is generated in tumors (41). Cancer cells present higher level of ROS-scavenging enzymes than normal cells, such as glutathione peroxidase, preventing ROS-mediated activation of death-inducing pathway (36). Another possible strategy for the neutralization of ROS in cancer cells, which is also consistent with our data, is an increase of GP saturation degree. A higher concentration of saturated and monounsaturated GPs in membranes has shown the potential to confer extra protection from oxidative and chemotherapeutic damage induced by reducing lipid peroxidation (6). A higher concentration of lipids with higher degrees of saturation in the membrane can also facilitate the formation of relatively ordered regions (e.g., lipid rafts), which can recruit other specific lipids and proteins, and regulate many cellular processes, including immune signaling and host–pathogen interactions (42).

A higher degree of saturation is also indicative of de novo lipogenesis. Indeed, higher levels of lipids in cancer and their precursors lesions have been found to unexpectedly undergo exacerbated endogenous fatty acid biosynthesis (43). The terminal catalytic step in FA synthesis is done by fatty acid synthase (FASN); its gene overexpression and increased activity are among the most frequent alterations in cancer cells (43).

Higher levels of lipids in cancer tissues have previously been described in DESI-MSI studies conducted with various types of cancer, including prostate (11), gastric (14), and breast cancers (16, 17). The higher level of lipids in cancer is also consistent with a usual catabolic reaction involving the Krebs cycle in tumor cells whereby the cycle is altered from producing citrate to contributing to lipogenesis (43). The various classes of lipids that have been identified have been associated with specific functions, including proinflammatory responses (eicosanoids; ref. 27), biological membrane composition (phospholipids), apoptotic mechanisms (PSs; ref. 44), and secondary messengers (PIs; ref. 45).

Some of the most abundant ions detected in benign breast tissues derive from organosulfur compounds, including benzyl sulfate (m/z 187.0063) and steroids sulfated (m/z 395.1897 and 465.3040; Fig. 4). And indeed, sulfation is an important process in the metabolism and inactivation of estrogen, and thus modulates the concentrations of active estrogens (46). The sulfation of estrogens is catalyzed by several members of a superfamily of cytosolic sulfotransferase (SULT) enzymes (47). Estrogen SULT activity has been demonstrated in a variety of steroid target tissues in human, including breast, and may well be important in affecting the biologic activity of estrogens within those tissues (48). Xenobiotics in ABT was also observed, including pantoprazole (m/z 382.0670). Higher levels of xenobiotic molecules are consistent with the contemporary view that adipose tissue is widely contaminated with various lipophilic xenobiotics, such as organochlorines, and these are agents that can act on hormones. Deposits of lipophilic xenobiotics in fat tissue have been observed in breast tissue, and they may contribute to the development of breast tumors (49).

Overall, DESI-MSI is an easy-to-use technique, comparison with pathologic slides is feasible and straightforward, but the analysis of the huge amount of data it generates can be rather complex. On the basis of our expertise with genomic analyses, we extrapolated metabolomic and lipidomic data analyses by using Lasso. However, improvements in the computational methods currently available for data acquisition and processing are needed to successfully translate this new technology into clinical practice.

In conclusion, we have demonstrated that data from both DESI-MSI and morphologic inspection can be combined, and that this approach can be used to obtain metabolomic profiles able to glutamine availability in aggressive tumor tissues (36). This data also may suggest that (i) these tumors could have other way to enhanced biosynthesis and obtain energy as described previously by Choi and colleagues; and (ii) protein expression of glycolysis markers such as Glut-1, CAIX, and MCT-4 was highest in HER2 and lower in luminal A and B (37). HER2 subtype is characterized by enhanced glycolytic metabolism and HER2mediated expression of glycolysisrelated genes (38), whereas less aggressive luminal subtypes appear to rely on a balance between de novo fatty acid synthesis and oxidation as sources for both biomass and energy requirements (39).

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In conclusion, we have demonstrated that data from both DESI-MSI and morphologic inspection can be combined, and that this approach can be used to obtain metabolomic profiles able to...
characterize normal parenchyma, DCIS, and various molecular subtypes of breast cancer. These findings also reveal a potential role for lipids in the development of selected molecular subtypes. For example, lipids that mediate signaling involved in inflammation and apoptotic mechanisms were found to have a key role in differentiating DCIS from IBC, whereas lipids with an overall higher degree of saturation and antioxidant molecules appeared to have essential roles in IBC. The results obtained for xenobiotic molecules were also promising, indicating that these molecules should be targets for further studies in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A.L. Santoro, M.N. Eberlin, V.P. Andrade

Development of methodology: A.L. Santoro, J.T. Silva

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.L. Santoro, P.H. Vendramini, M.B.C. Lemos, V.P. Andrade

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.L. Santoro, R.D. Drummond, J.T. Silva, M.B.C. Lemos, V.P. Andrade

Writing, review, and/or revision of the manuscript: A.L. Santoro, R.D. Drummond, L. Juliano, M.B.C. Lemos, M.N. Eberlin, V.P. Andrade

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.L. Santoro, S.S. Ferreira, V.P. Andrade

Study supervision: L. Juliano, M.N. Eberlin, V.P. Andrade

Acknowledgments

The authors would like to thank the Biobank from the AC Camargo Cancer Center for providing breast cancer samples and sample preparation and the Axon Science for technical assistance for MS validation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 21, 2018; revised November 8, 2019; accepted December 23, 2019; published first January 7, 2020.

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Breast Cancer Molecular Subtypes: DESI-MSI Profiles


Molecular Imaging of the Tumor Microenvironment Reveals the Relationship between Tumor Oxygenation, Glucose Uptake, and Glycolysis in Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Molecular imaging approaches for metabolic and physiologic imaging of tumors have become important for treatment planning and response monitoring. However, the relationship between the physiologic and metabolic aspects of tumors is not fully understood. Here, we developed new hyperpolarized MRI and electron paramagnetic resonance imaging procedures that allow more direct assessment of tumor glycolysis and oxygenation status quantitatively. We investigated the spatial relationship between hypoxia, glucose uptake, and glycogenesis in three human pancreatic ductal adenocarcinoma tumor xenografts with differing physiologic and metabolic characteristics. At the bulk tumor level, there was a strong positive correlation between ¹⁸F-FDG-PET and lactate production, while PO₂ was inversely related to lactate production and ¹⁸F-2-fluoro-2-deoxy-D-glucose (¹⁸F-FDG) uptake. However, metabolism was not uniform throughout the tumors, and the whole tumor results masked different localizations that became apparent while imaging. ¹⁸F-FDG uptake negatively correlated with PO₂ in the center of the tumor and positively correlated with PO₂ on the periphery. In contrast to PO₂ and ¹⁸F-FDG uptake, lactate dehydrogenase activity was distributed relatively evenly throughout the tumor. The heterogeneity revealed by each measure suggests a multimodal molecular imaging approach can improve tumor characterization, potentially leading to better prognostics in cancer treatment.

Significance: Novel multimodal molecular imaging techniques reveal the potential of three interrelated imaging biomarkers to profile the tumor microenvironment and interrelationships of hypoxia, glucose uptake, and glycogenesis.

Introduction

Cancer cells acquire aberrant biochemical pathways to support their uncontrolled growth. The abnormal energetic demands of malignant cells differ significantly from normal cells to support metabolic activities, which generate building blocks for further growth in anabolic processes (1). Tumors adapt to their microenvironment by altering their metabolism in response to hypoxia, nutrient starvation, immune surveillance, and other stressors (2–4). These alterations, known as metabolic reprogramming, provide a clear biochemical phenotype for detection and grading and may guide potential treatment options (5). The unique microenvironment of tumors offers opportunities for targeted therapeutic strategies. Several inhibitors that preferentially target these bioenergetic pathways are now in preclinical and clinical trials (6). As development of these inhibitors progresses, metabolic imaging may become increasingly relevant for pharmacodynamic validation of drug action beyond its current role in developing diagnostic and prognostic information (7, 8).

The current standard for cancer diagnosis, staging, and treatment management by metabolic imaging is ¹⁸F-2-fluoro-2-deoxy-D-glucose (¹⁸F-FDG)-PET, which monitors the uptake and subsequent phosphorylation of the nonmetabolizable glucose analogue fluorodeoxyglucose. Because the upregulation of glucose transport is a common feature of many cancers and is frequently used to monitor treatment prognosis, FDG-PET has found widespread use in cancer diagnosis and staging (5). As FDG-PET probes only the first steps of glucose uptake and phosphorylation and is insensitive to the exact chemical nature of the tracer, other methods that analyze the enzymatic reactions downstream of entry in metabolism are advantageous. ¹³C magnetic resonance spectroscopy has been suggested as an alternative but suffers from low sensitivity. To bring the signal of the tracer to a level that can be detected by MRI, it is necessary to use the process of hyperpolarization to transfer the larger spin polarization of the unpaired electron on a paramagnetic molecule to the ¹³C-labeled tracer under nonequilibrium conditions.

Hyperpolarized MRI has several theoretical advantages over FDG-PET for metabolic imaging (9, 10). Hyperpolarized MRI can be particularly advantageous in organs such as the brain that have a naturally high glucose uptake, which hinders utilizing ¹⁸F-FDG-PET imaging approaches due to the large background from normal...
tissue (5). Hyperpolarized pyruvate in particular has proved useful as the pyruvate to lactate kinetics of conversion measures flux through the critical switching point from glycolysis to the tricarboxylic acid cycle (11).

Given that FDG-PET is already in widespread clinical use and has proven effective in multiple clinical trials, the question naturally arises about what new information hyperpolarized MRI can bring. Although FDG-PET and pyruvate hyperpolarized MRI in theory measure two distinct metabolic processes, glucose uptake and the Warburg effect, they share common regulation points through transcriptional control of both glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA) by hypoxia-inducible factor 1 (HIF1) and cMyc. Both processes are known to exist in the same tumor microenvironment and hence the delivery of a bolus of pyruvate or $[^{15}F]$fluoro-2-deoxy-D-glucose (FDG) is affected similarly in both cases by the tumor vasculature and tumor interstitial pressure.

Prior studies have probed the relationship between FDG-PET uptake and hyperpolarized MRI. In voxel-wise comparisons of rat hepatocellular carcinomas, Menzel and colleagues found no significant correlation between lactate to pyruvate ratios in hyperpolarized MRI and standardized uptake values (SUV) in FDG-PET (9). On the other hand, a strong positive voxel-wise and patient-wise correlation was found between FDG uptake and lactate/pyruvate in sarcomas but not carcinomas in canine cancer models (10). Differences in vasculature along with differences in intrinsic metabolism were speculated to be responsible for these differences. To provide better insight into these discrepancies, we compared FDG-PET and hyperpolarized MRI measurements in three well-characterized pancreatic ductal adenocarcinoma (PDAC) mouse xenograft models along with pO2 imaging through electron paramagnetic resonance (EPR). We show that the successful comparison of multimodal modalities allows us to monitor tumor physiology in a complemental manner, and provides insights into detailed assessment of in vivo tumor metabolism and microenvironment.

Materials and Methods

Animal studies

All of the animal experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources, and experimental protocols were approved by the Animal Care and Use Committee, NCIC-CCR-ACUC, ref. 12. The human pancreatic tumor cell lines, Hs776t, and SU.86.86 cells were obtained from Threshold Pharmaceuticals, and MiaPaCa2 cells were purchased from the ATCC. All cell lines were authenticated by IDEXX RADIL utilizing a panel of microsatellite markers, and tested Mycoplasma negative by Frederick National Laboratory for Cancer Research. Hs776t, MiaPaCa2, and SU.86.86 pancreatic tumor–bearing mice (n = 9 each) were created by injecting 3 × 106 cells subcutaneously into the right hind legs of athymic nude mice.

Hyperpolarized $^{13}$C MRI

Hyperpolarized $^{13}$C MRI experiments were performed on a 3T MRI Scanner (MR Solutions Inc.) using a 17-mm diameter home-built $^{1}$H/$^{13}$C coil. A 96 mmol/L hyperpolarized [1-13C] pyruvate solution from a Hypermag NMR Polarizer (Oxford Instruments) was administered via a tail vein cannula (12 µL/g body weight). $^{13}$C two-dimensional spectroscopic images were acquired with a 32 × 32 mm2 field of view in a 8-mm axial slice through the tumor.

18F-FDG-PET

Tumor-bearing mice were injected with 100 µCi of $^{18}$F-FDG in PBS via a tail vein cannula under anesthesia. Sixty minutes after $^{18}$F-FDG administration, a PET scan was conducted using a BioPET/CT ( Bioscan Inc.) under anesthesia with 1.5% isoflurane with a nominal resolution of 0.375 mm × 0.375 mm × 0.375 mm.

EPR imaging

A 300 MHz pulsed EPR imaging scanner with a tailer designed parallel coil was used for oxygen imaging using OX063 as the paramagnetic tracer to obtain pO2 maps with a resolution of 0.4375 mm × 0.4375 mm × 0.4375 mm.

Results

Molecular imaging establishes a relationship between tumor oxygenation, glucose uptake, and glycolysis at the bulk tumor level in PDAC xenografts

pO2, FDG uptake, and lactate/pyruvate ratios for MiaPaCa2, Hs776t, and SU.86.86 xenografts were correlated at the whole-tumor level (Fig. 3A–C). All three cell lines were derived from PDAC tumors and have a largely similar genetic background (13), but differ strongly in the tumor microenvironment. Similar to previous reports, we found a strong global inverse relationship between tumor oxygenation and lactate/pyruvate, a measurement of LDH activity with a saturating bolus of pyruvate (14). At the whole-tumor level, FDG uptake was strongly correlated with lactate/pyruvate and inversely correlated with pO2. Out of the three xenografts, SU.86.86 most closely resembled normal tissue in all measures, in-line with its higher degree of vascularization and differentiation. The cardiac ordering for each measure (SU.86.86>MiaPaCa2>Hs776t for pO2 and the reverse Hs776t>MiaPaCa2>SU.86.86 for FDG uptake and lactate/pyruvate) was in overall agreement with the corresponding biomarkers (Fig. 2A and B). GLUT1 transporter and, to a lesser degree, hexokinase-2 were expressed more highly in Hs776t, which showed elevated FDG uptake and retention relative to the other two PDAC xenografts. Similarly, the xenograft with the highest median pO2 value, SU.86.86, also showed the highest expression levels of the angiogenesis biomarker CD31, while the highest expression levels of HIF1 were found in Hs776t, the cell line with the lowest median pO2 value. The ordering of LDH enzyme expression matched the ordering of lactate/pyruvate, as expected by the relationship between lactate/pyruvate ratios and pyruvate to lactate enzymatic flux (15).

Strong correlations existed between bulk measurements of pO2, FDG uptake, and lactate/pyruvate in xenografts from different PDAC cell lines when values were summed across the entire tumor (Fig. 1C). While these correlations are useful in establishing a correspondence between different metabolic properties of the tumor in bulk, the advantage of imaging studies is that they can address the inherent heterogeneity of tumors to reveal how metabolic processes are linked to the tumor environment, which varies considerably within the tumor.

Multimodal imaging reveals the intrinsic heterogeneity of the physiologic and metabolic aspects of tumors, linked to the tumor environment

Representative 0.46-mm slices from EPR pO2 imaging of MiaPaCa2, SU.86.86, and Hs776t xenografts were selected (Fig. 3A). In contrast to hyperpolarized MRI and FDG-PET images, which have a variegated
the well-oxygenated region outside the hypoxic fraction that is predominantly in the tumor core. Correlations between pO2 values and FDG uptake calculated from a voxel-by-voxel comparison for each slice indicated that FDG uptake was negatively correlated with pO2 in the center of the tumor and positively correlated with pO2 on the exterior regions.

Quantitatively, there was a modest but consistent pixel-wise correlation between both the pyruvate and lactate signals from hyperpolarized MRI and FDG-PET (Fig. 4A and B). The interpretation of this relationship is complicated by the fact that lactate production reflects both the uptake of pyruvate into the tissue and its metabolic conversion; the lactate signal is highly correlated with pyruvate as the variance appeared, indicating substantial tumor heterogeneity on at least the millimeter scale, the only noticeable feature of the pO2 images was a contiguous hypoxic volume near the center of the tumor, whose size was reflective of the known angiogenic potential of the cell lines from which they were derived (Fig. 3A). Partially due to this relative smoothness, there was a modest correlation on a voxel-by-voxel basis between pO2 levels and FDG-PET (Fig. 3B). On larger distance scales there was a rough inverse correlation between pO2 and FDG uptake; metabolic activity in general was highest in the interior of the tumor where pO2 was at its lowest point (Fig. 3C). This was also observed in coregistered images, which overlaid the hypoxic fraction, the radioinsensitive region of the tumor where pO2 was less than 10 mmHg with the FDG uptake (Fig. 3A). Although the relationship is not precise, a significant fraction of highly metabolically active cells lay in

Figure 1.
Multimodal imaging identifies the relationships between tumor oxygenation, glucose uptake, and glycolysis at the whole-tumor level. A, pO2 maps derived from EPR oximetry. 13C two-dimensional spectroscopic images, and 18F-FDG PET images for the cell lines indicated (top to bottom). B, Median pO2 lactate/pyruvate (lac to pyr). 18F-FDG SUV uptake, and hypoxic fraction (<10 mmHg) on a whole tumor basis for the indicated PDAC cell lines. C, Correlation between the indicated measures when considered on a whole tumor basis (n = 9; error bars, SD). *, P < 0.05, t test.

Figure 2.
Protein expression levels of key proteins associated with metabolism support multimodal imaging results. A, Expression levels of CD31, HIF1, LDHA, GLUT1, hexokinase-2, and MCT1 from tumor extracts. B, These expression levels were determined by Western blot analysis. Error bars, SD (n = 3). *, P < 0.05, t test.
in pyruvate transport and bolus delivery is much larger than the difference in LDH activity. Because FDG and pyruvate uptake are dependent on the same vascular system for delivery, they can be expected to be correlated to some degree. Using the lactate/pyruvate ratios reduces this dependence, for in simplified models with first order kinetics, the ratio is strictly equal to the rate of the conversion of pyruvate to lactate (16). Using lactate/pyruvate to adjust for uneven uptake of pyruvate, LDH activity was distributed mostly uniformly throughout the tumor with higher activity in the tumor interior. On a quantitative level, this was seen by comparing each modality to a distribution that was completely uniform throughout the tumor, using the Kullback–Leibler divergence metric (KL) to measure how far each modality was from a uniform reference image. A KL of zero in this case indicated an image that was completely uniform; higher values indicated heterogeneity. Lactate/pyruvate was notably closer to uniform than either lactate alone or pyruvate alone or FDG uptake or pO2 levels (Fig. 4C). While hypoxia and FDG-PET uptake appeared to be partially linked at least on a gross-anatomic level, the higher uniformity of lactate/pyruvate suggested LDH activity was driven by other factors.

Discussion

Availability of new clinical imaging modalities naturally raises the question of what comparative advantage the new methods have against the existing. FDG-PET is well-established clinically and is an essential technique for tumor diagnosis and treatment planning. However, FDG-PET only probes the first stage of glycolysis, glucose uptake, and is unable to probe deeper into the metabolic and physiologic differences that distinguish tumors from normal tissues and tumor from each other. These metabolic and physiologic differences in the tumor microenvironment can potentially be targeted in multiple ways. Hypoxia in particular is an important marker for predicting radiation sensitivity and can serve as guide for directing treatment but has not yet found widespread usage due to the difficulties in imaging hypoxic regions (17). However, while glucose uptake is not a direct marker for hypoxia, they share a pathway through HIF activation of the GLUT1 transporter (15). Similarly, the regulation of GLUT1 and hexokinase-2 is tied to the regulation of other metabolic genes through Ras and other oncogenes (18).

Figure 4. Registered FDG-PET and oximetry images highlight the inherent tumor heterogeneity within the tumors. A, Registered slices from FDG-PET and EPR oximetry from the central region of the tumor. Each 64 × 64 slices was 0.375 mm in thickness; the slice number is indicated in white at the bottom left corner of each image. The hypoxic fraction (pO2 < 10 mmHg) is outlined in blue on the FDG images. B, Left, mean FDG uptake values for the hypoxic (red) and nonhypoxic fraction (green) of xenografts from different PDAC cell lines for the slices defined in A. Slice 32 corresponds to the center of the tumor. Right, correlation between pO2 values and FDG uptake calculated from a voxel by voxel comparison for each slice from registered images. FDG-PET uptake was negatively correlated with pO2 in the center of the tumor and positively correlated with pO2 on the surface. C, Mean intensities from a 10 × 10 voxel patch at the center of each tumor.

Quantitatively, there was a modest but consistent pixel-wise correlation between both the pyruvate and lactate signals from hyperpolarized MRI and FDG-PET (Fig. 4A and B). The interpretation of this relationship is complicated by the fact that lactate production reflects both the uptake of pyruvate into the tissue and its metabolic conversion; the lactate signal is highly correlated with pyruvate as the variance...
and general metabolism. Previous preclinical glucose uptake through FDG-PET may serve as a surrogate for hypoxia (19). The data for this hypothesis is however limited and metabolism exists with minor locoregional differences in some cases (19). The correlation between whole-tumor measurements. The FDG-PET and pO2 maps is largely independent with a modest correspondence when evaluated to supply metabolic demand for proteins and nucleotides, leading to a positive correlation between FDG uptake and pO2, as the effects of regions, at the rim of the tumor, rapid growth stimulates glycolysis caused by differences in metabolism. In the tumor core where the metabolic building blocks produced by glycolysis is low. High oxygen phosphorylation serves as the main energy source as the demand for oxygen consumption, would be helpful in establishing the validity of these models and largely orthogonal to both FDG-PET and pO2, refs. 20, 21).

References


On the basis of this linked regulation, it has been proposed that glucose uptake through FDG-PET may serve as a surrogate for hypoxia and general metabolism. Previous preclinical in vivo imaging studies have suggested that a correlation between hypoxic regions and glucose metabolism exists with minor locoregional differences in some cases (19). The data for this hypothesis is however limited and conflicting (11), and in the case of hypoxia, complicated by the difficulties of accurately measuring PO2 (19). We found each measure is largely independent with a modest correspondence when evaluated on a voxel by voxel basis, although a considerable correlation exists between whole-tumor measurements. The FDG-PET and PO2 maps showed a similar geometric distribution within the tumor, possibly due to a higher metabolic demand under hypoxic conditions. The positive and negative correlations between PO2 and FDG uptake seen in Fig. 2B and C are likely driven by differing rates of oxygen consumption caused by differences in metabolism. In the tumor core where the correlation is negative, the tumor is likely quiescent and oxidative phosphorylation serves as the main energy source as the demand for metabolic building blocks produced by glycolysis is low. High oxygen consumption in these regions creates a state of consumptive hypoxia. While poor perfusion would also explain the low PO2 observed in the tumor core, it would also limit FDG uptake as O2 and glucose are expected to perfuse approximately equally. In the more invasive regions, at the rim of the tumor, rapid growth stimulates glycolysis to supply metabolic demand for proteins and nucleotides, leading to a positive correlation between FDG uptake and PO2, as the effects of consumptive hypoxia are limited. It should be noted however, that this study was limited to imaging of a series of less differentiated xenografts from cell lines of a single cancer type and caution should be applied in generalizing this model. Higher resolutions studies of a more diverse cell lines, as well additional measurements of perfusion and oxygen consumption, would be helpful in establishing the validity of the model for tumor metabolism in general (Supplementary Fig. S1; refs. 20, 21).

The lactate/pyruvate ratio, which serves as a surrogate for LDH activity, is distributed relatively uniformly throughout the tumor in these models and largely orthogonal to both FDG-PET and PO2, with a modest correlation to each measure as shown in Fig. 2B and C. Hyperpolarized MRI therefore offers additional information that cannot be inferred from either FDG-PET or measures of hypoxia and may prove a useful clinical adjunct to either, although the limited resolution in human subjects remains obstacle. As clinical imaging has been transforming conventional medicine, mapping metabolic activity noninvasively can potentially contribute to better prognostics, further tumor characterization, and earlier response monitoring in cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
This research was supported by the Intramural Research Program of the NCI, NIH. This project has been funded in whole or in part with federal funds from the NCI, NIH, under contract no. HHSN261200800001E.

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Received March 19, 2019; revised November 2, 2019; accepted March 25, 2020; published first April 3, 2020.

References