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Confocal fluorescence microscopy to evaluate changes in adipocytes in the tumor micro-environment associated with invasive ductal carcinoma and ductal carcinoma *in situ*

Jessica Dobbs, Dongsuk Shin, Savitri Krishnamurthy, Henry Kuerer, Wei Yang, Rebecca Richards-Kortum

Corresponding author: Rebecca Richards-Kortum Address: 6500 Main Street, Rice University, Houston, TX 77030 Fax: 713-348-5877 E-mail: <u>rkortum@rice.edu</u>

Keywords: cancer associated adipocytes, breast cancer, confocal fluorescence microscopy

Abbreviations:

- CAAs: cancer associated adipocytes
- DCIS: ductal carcinoma in situ
- FOV: field of view
- H&E: hematoxylin and eosin
- IDC: invasive ductal carcinoma
- IL-6: Interleukin-6
- MMP-11: matrix metalloproteinase-11
- PBS: phosphate buffered saline
- ROI: region of interest

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Novelty: We developed a computerized algorithm to identify and quantitatively characterize adipocytes in confocal fluorescence microscopy images of fresh breast tissue. Images were acquired from fresh breast tissue prior to standard histologic preparation and fixation and the algorithm was used to analyze characteristics of adipocytes at sites adjacent to and far from lesion margins in neoplastic and non-neoplastic tissue.

Abstract

Adipose tissue is a dynamic organ that provides endocrine, inflammatory, and angiogenic factors, which can assist breast carcinoma cells with invasion and metastasis. Previous studies have shown that adipocytes adjacent to carcinoma, known as cancer associated adipocytes (CAAs), undergo extensive changes that correspond to an "activated phenotype," such as reduced size relative to adipocytes in non-neoplastic breast tissue. Optical imaging provides a tool that can be used to characterize adipocyte morphology and other features of the tumor microenvironment. In this study, we used confocal fluorescence microscopy to acquire images of freshly excised breast tissue stained topically with proflavine. We developed a computerized algorithm to identify and quantitatively measure phenotypic properties of adipocytes located adjacent to and far from normal collagen, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). Adjpocytes were measured in confocal fluorescence images of fresh breast tissue collected from 17 patients. Results show that adipocytes adjacent to neoplastic tissue margins have significantly smaller area compared to adipocytes far from the margins of neoplastic lesions and compared to adipocytes adjacent to non-neoplastic collagenous stroma. These findings suggest that confocal microscopic images can be utilized to evaluate phenotypic properties of adjpocytes in breast stroma which may be useful in defining alterations in microenvironment that may aid in the development and progression of neoplastic lesions.

Introduction

The development of breast cancer is a complex process that primarily occurs in ductal and lobular epithelial tissue ^{1, 2}. However, many recent studies have suggested that breast stromal tissue also plays a dynamic and influential role in breast tumor development and progression ³⁻⁶, and that stromal cell proliferation is a major contributor to increased breast cancer risk ⁷. Stromal collagen and adipose tissue in particular are of interest for their role in breast disease progression ⁸⁻¹³. It was recently reported that high mammographic density (associated with a 4-to-6-fold increase in breast cancer risk) is associated with high collagen density ⁷, which has been shown to directly promote proliferation of mammary epithelial cells *in vitro* and in murine models ⁹.

Adipose tissue, the predominant component of benign breast tissue, also functions as a dynamic organ that assists breast carcinoma cells with invasion and metastasis by providing endocrine, inflammatory, and angiogenic factors ^{12, 14-16}. Previous studies have shown that co-culturing adipocytes and cancer cells results in a decrease in adipocyte markers and an overexpression of matrix metalloproteinase-11 (MMP-11), an extracellular matrix-remodeling proteinase associated with tumor invasion, and interleukin-6 (IL-6), a cytokine associated with inflammation ¹². These studies characterize the relationship between invading carcinoma cells and adipocytes surrounding carcinoma, commonly referred to as cancer-associated adipocytes (CAAs), as a crosstalk cycle, in which adipocytes undergo de-differentiation and contribute to abnormal proliferation of tumor cells, which leads to tumor growth and increased invasive potential ^{11, 17, 18}.

Previous research on the role of CAAs and tumor progression also shows that CAAs adjacent to neoplastic tissue undergo extensive phenotypic changes, particularly reduced size relative to

adipocytes in non-neoplastic breast tissue ^{12, 18}. Tan et al. found that adipocytes near the invasive front of breast tumors tend to be smaller in size than those located further from the tumor ¹¹. Adipocytes co-cultured with breast cancer cells underwent lipolysis and developed a fibroblast-like phenotype in which the lipid vesicles greatly decreased in size and the cells took on a spindle-shaped appearance ¹². These physical changes observed in adipocytes in the tumor microenvironment correspond to an "activated phenotype" ¹², which could indicate adipocytes that are participating in crosstalk with neoplastic cells and confer increased potential for invasion to the tumor microenvironment ¹¹.

The clinical standard for evaluating breast lesions is histologic assessment, which requires extensive tissue preparation and staining with hematoxylin and eosin (H&E). However, the standard histologic preparation is not optimal for evaluation of adipose tissue, because fixation and sectioning tissue specimens can cause adipocyte cell membranes to tear and adipose tissue to slough off or dissolve. Confocal microscopy is an emerging image acquisition technique that could potentially address the current limitations of histologic assessment and has been used in several previous studies to acquire *in vivo* images ¹⁹⁻²¹ of fresh tissue specimens in real time ²²⁻²⁶. Work from Schiffhauer et al. and Abeytunge et al. demonstrated that confocal reflectance microscopy and confocal fluorescence microscopy, can acquire images of fresh, unsectioned breast tissue that are visually similar to histologic slides with H&E staining ^{22, 23}. Recent studies from our group showed that confocal fluorescence microscopy images of breast tissue acquired in less than ten minutes contain sufficient detail to identify neoplasia and non-neoplasia in unsectioned breast tissue specimens ²⁵ and to estimate tumor cellularity in core needle biopsies ²⁶. Currently available endomicroscopy platforms for *in vivo* imaging, such Optiscan FIVE1

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(Optiscan, Melbourne, Australia) and Cellvizio[®] (Mauna Kea Technologies, Paris, France), demonstrate that there is an opportunity to apply confocal microscopy for *in vivo* breast imaging in a clinical setting ¹⁹⁻²¹.

The objective of this study is to determine if confocal microscopy can identify and characterize changes in adipocyte phenotype near the margins of invasive breast cancer and pre-invasive lesions. Although the reduced size of CAAs adjacent to breast tumors has been qualitatively observed, this phenomenon has not been quantitatively evaluated. In this study we used confocal fluorescence microscopy to acquire images of adipose tissue located near neoplastic tissue as well as adipocytes near non-neoplastic collagenous stroma in fresh, unfixed breast tissue. The findings from this study support further use of confocal microscopy as a point of care tool to image breast tumors and surrounding adipose tissue and to explore whether features of adjacent adipocytes can predict the invasive potential of early breast cancers.

Material and Methods

Breast tissue acquisition and preparation

Human breast tissue specimens were acquired through a protocol approved by the institutional review boards at The University of Texas MD Anderson Cancer Center and Rice University. All specimens were acquired from patients who gave informed consent to participate in the study. Patients were eligible for the study if they were undergoing a total or segmental mastectomy for breast cancer. Fresh tissue specimens were acquired from residual resected breast tissues that were not required for clinical diagnosis. Two tissue specimens measuring approximately 15 x 15 x 2-7 mm were acquired from each patient within 30 minutes of resection, including one grossly normal specimen and one grossly abnormal specimen. Each tissue specimen was

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stained with a solution of 0.01% proflavine in 1X phosphate buffered saline (PBS) for 1 minute, washed in 1X PBS, and then immediately imaged using a confocal fluorescence microscope. Proflavine has been used in previous studies as a fluorescent contrast agent to stain nuclei in breast tissue, oral mucosa, esophageal tissue, cervical tissue, and sarcoma ²⁴⁻³³. Proflavine also stains the membranes of adipocyte cells ²⁵.

Image acquisition and evaluation

Fluorescence confocal images were acquired with a benchtop confocal microscope (Vivascope 2500, Caliber I.D.). Following topical application of proflavine, specimens were positioned on the microscope stage and imaged with 2.1 ± 0.4 mW power at 488 nm laser excitation using a 30X water immersion objective lens with a numerical aperture of 0.8. The confocal microscope has a 750 x 750 μ m² field of view (FOV) with 1.0 μ m lateral resolution and 5.0 μ m axial resolution. Images were acquired at a focal plane depth approximately 60 μ m beneath the tissue surface from a composite grid of contiguous sites with a total area of 12.2 x 12.2 mm²⁵. After image acquisition, tissue specimens were submitted for routine histologic preparation and fixation with H&E staining.

A breast pathologist reviewed the confocal and H&E stained images to provide a diagnosis for each tissue specimen based on standard histologic criteria ³⁴. We used images with H&E staining to identify regions with adipose tissue surrounding invasive ductal carcinoma (IDC), ductal carcinoma *in situ* (DCIS), and stromal collagen with no neoplastic cells (collagen), and located the same regions in confocal images. In these images, we defined regions of interest (ROIs) in confocal images consisting of two to four contiguous FOVs, originating at the margin of

IDC, DCIS, or collagen without neoplasia and extending 2 - 3 mm into adipose tissue (Figure 1); 2 -3 mm is a narrow tumor resection margin typically used in breast-conserving surgeries ³⁵.

Adipocyte segmentation algorithm and measurement of adipocyte area

We developed a computerized algorithm to segment adipocytes and characterize adipocyte morphology in confocal images of breast tissue, as shown in Figure 2. Beginning with an unprocessed confocal image containing adipocytes (Figure 2A), the algorithm applied adaptive histogram equalization to enhance the brightness of the adipocyte cell membranes (Figure 2B). Since proflavine preferentially stains nuclear material and does not stain lipid droplets, adipocytes appeared as unstained areas surrounded by non-specifically stained cell membranes in confocal images ^{25, 33}. Edge detection and edge enhancement functions were applied to the enhanced image (Figure 2C). The enhanced confocal image was converted to a binary image with a user-defined threshold based on relative staining intensity of adipocyte cell membranes (Figure 2D). The resulting binary image (Figure 2D) was combined with the adipocyte cell membranes previously segmented by edge detection and enhancement (Figure 2C) using a logical OR operation (Figure 2E). A morphological closing function performed dilation followed by erosion with a disk-shaped structuring element to fill the gaps where edge detection and enhancement do not completely segment adipocyte cell membranes (Figure 2F). In order to segment adipocytes enclosed by adipocyte cell membranes, a complement function reversed the background and segmented areas (colored black and white, respectively) in the binary image (Figure 2G). A morphological opening function performed erosion followed by dilation with a disk-shaped structuring element to remove objects smaller than adipocytes; the structuring element was designed to remove regions that were smaller than 1.5X the area of a

large mammary carcinoma cell ³⁶ (Figure 2H). To avoid measuring areas of adipocytes which were cut off by the border of the FOV, a Matlab image processing function (imclearborder, Mathworks[®]) was applied to delete objects connected to the borders of the image (Figure 2I). Following segmentation of adipocytes, an ellipse was fitted to each adipocyte to approximate its cross-sectional area. The mean ellipse area was calculated for each consecutive FOV in ROIs.

After processing, each FOV was manually reviewed to determine if the algorithm accurately segmented adipocytes compared to visual examination. We developed additional manual interactive functions to address cases in which the algorithm results did not agree with visual assessment. For example, in FOVs with very weakly-stained cell membranes, clusters of cells were occasionally incorrectly segmented as a single adipocyte. Additionally, out of focus cell membranes in a FOV sometimes caused an individual adipocyte to be separated into multiple fragments. In these cases, the interactive functions were used to separate clusters of cells into individual adipocytes or to connect fragments of a cell into a single adipocyte.

We compared the morphology of adipocytes at the margins of IDC and DCIS to those extending into surrounding stroma. We also compared the morphology of adipocytes at these ROIs to adipocytes surrounding stromal collagen in areas that did not contain neoplasia. Statistical comparisons were made using a Student's t-test for samples with unequal variances.

Results

Adipocytes were measured in 389 FOVs within 106 ROIs in composite confocal fluorescence images of breast tissue specimens collected from 17 patients. A summary of FOVs, ROIs, patients and diagnoses are shown in Table 1.

Figure 3 shows representative ROIs that consist of 4 contiguous FOVs from specimens with IDC (Figure 3A), DCIS (Figure 3B), and collagen in a non-neoplastic region (Figure 3C). FOVs labeled with I are located adjacent to IDC, DCIS, or collagen; the adjacent sets of contiguous FOVs (Figure 3A-C: II-IV) extend into adipose tissue. FOVs labeled with IV are located between 2 and 3 mm from the lesion edge (Figure 3A-C:I) and represent a theoretical narrow resection margin ³⁵. Figure 3D shows mean adipocyte ellipse area measured in each FOV (Figure 3A-C) using the segmentation algorithm. Adipocytes in FOVs adjacent to IDC and DCIS (Figure 3A,B:I; Figure 3D) have a mean area of approximately 5,000 μ m², while adipocytes in the FOV adjacent to benign collagen (Figure 3C) have a mean area of approximately 9,000 μ m². Mean adipocyte area increases to over 24,000 μ m² in FOVs 2-3 mm from the margin of the IDC lesion and to 12,000 μ m² for the DCIS lesion (Figure 3A,B: II-IV; Figure 3D). In contrast, mean adipocyte area stays relatively constant over the distance from the interface with collagen in the nonneoplastic region.

Figure 4 shows the mean adipocyte area versus distance from the margin for all 389 FOVs. Figure 4A shows that on average adipocytes adjacent to the margin of IDC are smaller than those adjacent to benign collagen, whereas the mean size of adipocytes is similar at 2-3 mm from the margin. Although the number of samples is smaller, a similar trend is seen for DCIS, with small adipocytes near the margin, increasing in size at 2-3 mm distant from the margin. Figure 4B shows the same mean adipocyte area versus distance from the margins, but data have been normalized by the mean value from the FOV immediately adjacent to IDC, DCIS, and benign collagen. Adipocytes show the greatest increase in mean area in FOVs identified in IDC specimens: adipocytes in contiguous FOVs extending into adipose tissue have an average of 2.0X (range: 0.77-4.7X) the mean area of adipocytes in FOVs adjacent to IDC margins (Figure 4B). In tissue with DCIS, adipocytes in FOVs extending into adipose tissue have an average of 1.8X (range: 0.86-2.5X) the mean area of adipocytes in FOVs adjacent to DCIS margins. In benign stroma, adipocytes in FOVs extending into adipose tissue have an average of 1.4X (range: 0.55-3.8X) the mean area of adipocytes in FOVs adjacent to benign collagen.

Figure 5 shows box and whiskers plots summarizing the distribution of mean adipocyte ellipse area in FOVs versus distance from the margin (Figure 5A) and the distribution of adipocyte area normalized by the mean value from the FOV immediately adjacent to IDC, DCIS, and collagen (Figure 5B). Significant differences in mean area are identified with asterisks (*) and the dagger symbol (†). Adipocytes in FOVs adjacent to IDC and DCIS have significantly lower mean area than adipocytes in FOVs adjacent to benign collagen (p < 0.001). There was no significant difference in mean area between adipocytes measured 2-3 mm away from benign collagen and IDC or between adipocytes 2-3 mm from benign collagen and DCIS. In addition, adipocytes in FOVs adjacent to margins of IDC or DCIS have significantly lower mean area than in FOVs located further from the associated lesion margins (2.2X-fold increase in area corresponding to p < 0.001 for IDC, 2.0X-fold increase in area corresponding to p < 0.01 for DCIS). Adipocytes in FOVs adjacent to benign collagen also have significantly lower mean area than adipocytes 2-3 mm into adipose tissue (1.2X-fold increase in area corresponding to p < 0.01 for DCIS). Adipocytes 2-3 mm into adipose tissue (1.2X-fold increase in area corresponding to p < 0.01 for DCIS).

Discussion

In this study, we developed a computerized algorithm to identify and quantitatively characterize adipocytes in confocal fluorescence microscopy images of fresh breast tissue.

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Images were acquired from fresh breast tissue prior to standard histologic preparation and fixation and the algorithm was used to analyze characteristics of adipocytes at sites adjacent to and far from lesion margins in neoplastic and non-neoplastic tissue. We found that adipocytes adjacent to neoplastic tissue margins are significantly smaller in area compared to adipocytes far from the margins of neoplastic lesions and compared to adjacent to non-neoplastic collagenous stroma.

Our observations are consistent with previous studies of histologic images of breast lesions by Tan et al. and Dirat et al., which have qualitatively observed that adipocytes adjacent to neoplastic breast lesions show phenotypic changes, such as smaller area ^{11, 12}. These studies also suggest that adipocytes adjacent to neoplastic breast lesions are elongated in shape; although we measured cell eccentricity as a metric of adipocyte shape, we did not observe significant differences in adipocyte shape adjacent to neoplastic or non-neoplastic lesions.

A strength of our study is that we demonstrate that adipocytes can be evaluated in confocal fluorescence images of fresh tissue specimens. Early computational methods to approximate adipocyte cross-sectional area include manual cell counting and measurements from cell suspensions and H&E stained histologic sections ^{37, 38}. Additionally some previous studies have demonstrated automated algorithms for adipocyte segmentation in cell suspension and H&E-stained histologic slides ³⁹⁻⁴¹. Björnheden et al. developed a method to determine human adipocyte size in a cell suspension ³⁹. Chen et al. and Osman et al. demonstrated computerized method to determine adipocyte size in histologic slides of murine tissue or human tissue ^{40, 41}. In our study we evaluated adipocyte cross-sectional area in fresh tissues, which did not undergo

fixation and preparation that could alter adipocyte phenotype. While frozen section and touch preparation are currently used to prepare tissue for evaluation, these techniques can cause distortion of breast tissue specimens with adipose tissue, particularly in cases in which core needle biopsies are obtained.

This study has some limitations. The computerized algorithm incorporates a user-defined threshold to convert images to binary in order to segment and measure adipocytes. The variable threshold was needed to account for differences in fluorescence intensity, which are based on several factors, including the variation in illumination power used for image acquisition and the presence of background fluorescence from out of focus signal. Additionally the histologic types of breast malignancies represented in this study only included IDC and DCIS and there were very few tissue specimens acquired from patients with DCIS lesions. Future studies should investigate adipocyte phenotype adjacent to a greater variety of breast malignancies. Additional work is also needed to elucidate the molecular basis for the changes in adipocyte characteristics associated with IDC and DCIS observed in this study.

Our findings support previous observations that there is a change in adipocyte phenotype at the margins of neoplastic breast lesions, including IDC and DCIS^{11, 12}. We demonstrate that adipocyte parameters could potentially characterize the microenvironment of early neoplastic breast cancers and suggest potential for lesion growth and local invasion. However, additional work is needed to analyze adipocyte phenotypes in breast tissue acquired from a larger cohort of patients and in a wider range of neoplastic and non-neoplastic breast lesions.

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scientific advisor. The other authors declare that they have no conflicts of interest.

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Tables

Table 1: Summary of patients from which tissue specimens were acquired and regions of interest (ROIs) adjacent to lesion margins consisting of 2-4 contiguous FOVs, with corresponding histologic diagnoses.

Diagnosis	Patients	ROIs	FOVs
IDC	17	49	168
DCIS	2	9	31
Collagen	17	48	190
All	17	106	389

Figure Legends

Figure 1: Schematic of the procedure used to identify regions of interest (ROIs) within adipose tissue at the margin of invasive tumors. Regions with adipose tissue adjacent to invasive tumors were identified in images of fixed tissue specimens stained with hematoxylin and eosin using standard histologic criteria (top left). The same regions were identified in the corresponding image acquired with confocal fluorescence microscopy (top right). White squares in upper right image indicate 4 contiguous FOVs adjacent to an invasive tumor margin. Scale bar is 750 µm. In the bottom image, boxes with dashed lines indicate four contiguous 750 x 750 µm FOVs next to the invasive tumor margin and extending up to 3 mm into adipose tissue. Scale bar is 3 mm.

Figure 2: Adipocyte segmentation algorithm. A: Original confocal fluorescence image. B: Adaptive histogram equalization to enhance brightness of adipocyte cell membranes. C: Adipocyte cell membrane edges detected and enhanced. D: Image A is converted to binary. E: Binary image D and enhanced edges in image C are combined. F: Closing function is applied to fill gaps in adipocyte cell membranes. G: Background (black) and segmented areas (white) are

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reversed to create the complement of F. H: Opening function is applied to remove objects smaller than 1.5X the area of a large mammary carcinoma cell. I: Objects touching the image border are removed. Scale bar is 100 µm.

Figure 3: Representative regions of interest (ROIs) consisting of 4 contiguous FOVs located adjacent to the margins of invasive ductal carcinoma (A), ductal carcinoma *in situ* (B), and benign collagen (C). I: 750 x 750 μm FOVs located adjacent to non-adipose tissue margins. II-IV: contiguous FOVs extending up to 3 mm into adipose tissue. Scale bar is 100 μm. (D) Mean adipocyte ellipse area measured for each FOV shown above using the adipocyte segmentation algorithm.

Figure 4: A: Mean adipocyte ellipse area measured for each FOV, measured adjacent to the lesion edge (I) and extending into adipose tissue (II-IV) for tissue adjacent to IDC, DCIS and benign collagen. B: Mean adipocyte ellipse area measured for each FOV extending into adipose tissue (II-IV), normalized to the FOV adjacent to the lesion edge (I); ellipse area measured in tissue adjacent to IDC, DCIS and benign collagen.

Figure 5: Box and whiskers plot showing mean adipocyte ellipse area, measured adjacent to the lesion edge (I) and extending into adipose tissue (II-IV), for ROIs adjacent to IDC, DCIS, and benign collagen. Asterisks (*) above the boxes indicate significant differences between the mean ellipse area measured for the site adjacent to the lesion edge (I) and sites extending into adipose tissue (II-IV); * = p < 0.05; ** = p < 0.01; *** = p < 0.001. The symbols (†) indicate significant differences between the mean ellipse area measured to collagen the mean ellipse area measured for the mean ellipse area measured in sites adjacent to collagen compared to sites adjacent to IDC and DCIS (+++ = p < 0.001).



Schematic of the procedure used to identify regions of interest (ROIs) within adipose tissue at the margin of invasive tumors. Regions with adipose tissue adjacent to invasive tumors were identified in images of fixed tissue specimens stained with hematoxylin and eosin using standard histologic criteria (top left). The same regions were identified in the corresponding image acquired with confocal fluorescence microscopy (top right). White squares in upper right image indicate 4 contiguous FOVs adjacent to an invasive tumor margin. Scale bar is 750 µm. In the bottom image, boxes with dashed lines indicate four contiguous 750 x 750 µm FOVs next to the invasive tumor margin and extending up to 3 mm into adipose tissue. Scale bar is 3 mm. 227x190mm (150 x 150 DPI)



equalization to enhance brightness of adipocyte cell membranes. C: Adipocyte cell membrane edges detected and enhanced. D: Image A is converted to binary. E: Binary image D and enhanced edges in image C are combined. F: Closing function is applied to fill gaps in adipocyte cell membranes. G: Background (black) and segmented areas (white) are reversed to create the complement of F. H: Opening function is applied to remove objects smaller than 1.5X the area of a large mammary carcinoma cell. I: Objects touching the image border are removed. Scale bar is 100 μm. 239x271mm (150 x 150 DPI)



Representative regions of interest (ROIs) consisting of 4 contiguous FOVs located adjacent to the margins of invasive ductal carcinoma (A), ductal carcinoma in situ (B), and benign collagen (C). I: 750 x 750 μ m FOVs located adjacent to non-adipose tissue margins. II-IV: contiguous FOVs extending up to 3 mm into adipose tissue. Scale bar is 100 μ m. (D) Mean adipocyte segmentation algorithm.

251x320mm (150 x 150 DPI)





A: Mean adipocyte ellipse area measured for each FOV, measured adjacent to the lesion edge (I) and extending into adipose tissue (II-IV) for tissue adjacent to IDC, DCIS and benign collagen. B: Mean adipocyte ellipse area measured for each FOV extending into adipose tissue (II-IV), normalized to the FOV adjacent to the lesion edge (I); ellipse area measured in tissue adjacent to IDC, DCIS and benign collagen. 311x149mm (150 x 150 DPI)



Box and whiskers plot showing mean adipocyte ellipse area, measured adjacent to the lesion edge (I) and extending into adipose tissue (II-IV), for ROIs adjacent to IDC, DCIS, and benign collagen. Asterisks (*) above the boxes indicate significant differences between the mean ellipse area measured for the site adjacent to the lesion edge (I) and sites extending into adipose tissue (II-IV); * = p < 0.05; ** = p < 0.01; *** = p < 0.001. The symbols (†) indicate significant differences between the mean ellipse area measured in sites adjacent to collagen compared to sites adjacent to IDC and DCIS (+++ = p < 0.001). 151x144mm (150 x 150 DPI)