Digital analysis of distant and cancer-associated mammary adipocytes

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**A B S T R A C T**

Adipocytes and cancer-associated adipocytes (CAAs) are poorly investigated cells in the tumor microenvironment. Different image analysis software exist for identifying and measuring these cells using scanned hematoxylin and eosin (H&E)-stained slides. It is however unclear which one is the most appropriate for breast cancer (BC) samples. Here, we compared three software (AdipoCount, Adiposoft, and HALO®). HALO® outperformed the other methods with regard to adipocyte identification, (> 96% sensitivity and specificity). All software performed equally good with regard to area and diameter measurement (concordance correlation coefficients > 0.97 and > 0.96, respectively). We then analyzed a series of 10 BCE samples (n = 51 H&E slides) with HALO®. Distant adipocytes were defined >2 mm away from cancer cells or fibrotic region, whereas CAAs as the first three lines of adipocytes close to the invasive front. Intra-mammary heterogeneity was limited, implying that measuring a single region of ~500 adipocytes provides a reliable estimation of the distribution of their size features. CAAs had smaller areas (median fold-change: 2.62) and diameters (median fold-change: 1.64) as compared to distant adipocytes in the same breast (both p = 0.002). The size of CAAs and distant adipocytes was associated with the body mass index (BMI) of the patient (area: rho = 0.89, p = 0.001; rho = 0.71, p = 0.027, diameter: rho = 0.87 p = 0.002; rho = 0.65 p = 0.049, respectively). To conclude, we demonstrate that quantifying adipocytes in BC sections is feasible by digital pathology using H&E sections, setting the basis for a standardized analysis of mammary adiposity in larger series of patients.

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1. Introduction

Mammary adipose tissue (MAT) represents up to 56% of the total breast volume [1] and it is composed by different cell populations such as endothelial cells, fibroblasts, immune cells, pericytes, various stem and progenitor cells in addition to adipocytes [2]. Adipocytes are normally separated from the human mammary epithelium by a layer of non-specialized stroma in which the terminal ductal lobular units are embedded. This layer is disrupted...
2. Materials and methods

2.1. Patients and slides

We selected a total of 51 H&E slides from 10 BC patients treated at the Institut Jules Bordet (Brussels, Belgium). The selection criteria were the following: 1) postmenopausal status of the patient at diagnosis, 2) estrogen receptor (ER)-positive status of the tumor, 3) availability of multiple (at least 2) FFPE blocks from primary surgery with distant adipocytes and CAAs, and 4) availability of BMI status of the patient recorded on the day of oncological surgery. BMI (kg/m²) was categorized according to World Health Organization (WHO) criteria [26]. All patients selected had an invasive breast carcinoma, five patients had an invasive breast carcinoma of no special type, commonly referred to as invasive ductal carcinoma, and five patients had an invasive lobular carcinoma. Eight patients had T2 stage and two patients had T3 stage at diagnosis. All the patients were treated with neoadjuvant endocrine therapy with letrozole followed by surgery. The age of the patients ranged from 58 to 82 years, with a mean 70.3 years. For nine patients, there were at least three evaluable slides with CAAs and distant adipocytes, and for one patient there were only two. For the first part of the study, we selected an area comprising ~100 adipocytes in three test H&E slides taken from this cohort, to compare the three software. For the second part of the study, we analyzed ~500 distant and ~500 CAAs in multiples slides per patient for 10 patients to assess intra-patient and inter-patient heterogeneity (see power calculations in Supplementary Methods). The project has been approved by the ethics committee of the Institut Jules Bordet (CE2844).

2.2. Digital pathology and software analysis

H&E stained tissue slides were scanned using a Nanozoomer digital slide scanner (t10730-12, Hamamatsu) with a 40x objective (0.228 µm/pixel). For the software comparison, the test slides were imported as PNG images into AdipoCount, Adiposoft version 1.16 and HALO® version 2.3 (Vaccuole module, Indica Labs, Corrales, CA). We compared the correct identification of adipocytes, considering visual identification as the gold standard, their areas and diameters. Computation of the area was based on the number of pixels in the object for the three software. The area-output unit was µm² for Adiposoft and HALO®, but pixels for AdipoCount. We therefore converted pixels in µm² multiplying the area in pixels obtained for each object with the area of one pixel in µm². Diameter-output unit was µm for Adiposoft and HALO®. AdipoCount did not provide the diameter. In HALO®, an algorithm estimates the centroid of the object, records 18 diameters passing through the centroid of the object at different angles, and then consider the median of those 18 diameters as the final measure. In the current version of Adiposoft, the diameter is an equivalent diameter that assumes roundness of the objects. For the second part of the project, all digital slides were imported as NDPI images into HALO® software for subsequent steps, including annotation, segmentation, count and measurements of area and diameter of adipocytes. We extracted the area and diameter of each single adipocyte for each annotated region. We did not include adipocytes with a diameter <30 µm to exclude artefacts [16]. Based on microscopic evidence and literature [27], we defined adipocytes distant from the tumor as those being at least 2 mm away from cancer cells as well as 2 mm away from fibrosis area and epithelial structures. CAAs were defined as the three first lines of adipocytes at the invasive front of the tumor, which are mostly in contact with invasive tumor cells (peri-tumoral CAAs), and as maximum 2 mm within the tumor starting from the invasive front (intra-tumoral CAAs). These criteria were established by an expert breast pathologist (D.L.) and the supervision of two breast pathologists: D.L. and M.D.S., according to the aforementioned criteria. We manually excluded adipocytes with incomplete stain of the membrane or adipocytes that touched the border of the image.

during tumor initiation/growth, bringing the adipocytes in close contact with BC cells [3]. The so-called cancer-associated adipocytes (CAAs) have unique characteristics as compared to mature adipocytes: they are characterized by fibroblast-like phenotypes, smaller size, reduced lipid content, free fatty acid release and immunomodulatory adipokine secretion [4]. Cancer cells can induce the delipidation of CAAs [5] and undergo a metabolic reprogramming with a shift from oxidative to lipid metabolism. The two main subpopulations of CAAs are peritumoral adipocytes that are located at the invasive front of the tumor and intratumoral adipocytes that infiltrate into or are engulfed by the tumor [6]. CAAs can also cause resistance to radiotherapy [6] and to chemotherapeutic drugs through different mechanisms [7,8]. All these complex interactions between CAAs and cancer cells seem to be more pronounced in obese patients [3,9].
2.3. Statistical analysis

Sensitivity (SS) and specificity (SP) of adipocyte identification were evaluated for each software. For the computation of SS and SP, we considered as true positives (TP), the adipocytes previously manually identified that software recognized correctly. False positives (FP) were fragments of cells or white areas that software recognized incorrectly as adipocytes. True negatives (TN) were fragmented cells or white areas that software did not recognize as adipocytes. False negatives (FN) were manually identified adipocytes that the software did not recognize as such. SS and SP were calculated as TP/(TP + FN) and TN/(TN + FP), respectively. The agreements regarding diameter and area were assessed using the Bland–Altman method, Passing–Bablok regression [28] and concordance correlation analyses. Bland–Altman plots represent the difference of the areas and diameters computed by each software plotted in a head-to-head comparison. In the Passing–Bablok regression lines, intercepts and slopes indicate constant and proportional bias respectively. Associations between continuous and (ordinal) categorical variables were assessed using Wilcoxon and Mann-Kendall tests. Association between adipocyte count and BMI was assessed using Mann-Kendall’s tau coefficient when BMI was considered as a categorical variable whereas Spearman correlation coefficient was used when BMI was considered as a continuous variable. McNemar’s Chi-squared test was used to assess the statistical difference between HALO® and the two other software concerning sensitivity and specificity. Statistical analyses were performed using R version 3.5.2.

Fig. 1. Comparison of adipocytes measurements by AdipoCount, Adiposoft and HALO®. Representative image of a test slide analyzed with AdipoCount (A), Adiposoft (E) and HALO® (I). Bland–Altman plots (D, G, H) showing software agreement between two software using area measurement of twenty adipocytes. For each comparison, the averaged area of each adipocyte calculated by software (x axis) is plotted against the difference between the two area measurements of the same adipocyte. The solid and dashed horizontal lines represent the overall geometric mean of the differences and the 95% confidence intervals respectively. Passing–Bablok regressions of adipocyte areas of AdipoCount vs Adiposoft (B), AdipoCount vs HALO® (C), and Adiposoft vs HALO® (F) are shown. Each comparison is represented by a scatter diagram where the regression line and 95% pointwise confidence bands are superimposed with the identity line (dashed line). The intercept and slope are reported with their 95% confidence intervals (CI). CCC: concordance correlation coefficient.
3. Results

3.1. Comparative analysis of HALO®, Adiposoft and AdipoCount for adipocyte identification and measurements

We analyzed three regions comprising ~100 adipocytes in three H&E BC slides using HALO®, Adiposoft and AdipoCount (Fig. 1 and Supplementary Figs. 1–3). We first compared the three software with regard to adipocyte identification. We obtained a high specificity and a high sensitivity with HALO® for all the tested slides (Table 1). In contrast, AdipoCount had a high number of false positives resulting in a low specificity in recognizing CAAs. This software would therefore require a post-processing step to eliminate the objects that are improperly recognized as adipocytes. Adiposoft would therefore require a post-processing step to eliminate adipocytes when the quality of the H&E stain was not optimal (Supplementary Fig. 2E). This software would therefore require a pre-processing for each slide to adjust the image threshold.

We then compared the areas and the diameters computed by the three software considering 20 individual adipocytes per slide. The results were reported in Bland–Altman plots depicting the difference between two software’s measurements against their mean. There was no major constant (intercept) or proportional (slope) drift between the three methods, as shown in the Passing–Bablok regression analysis (Fig. 1B,C,F). The concordance correlation coefficients confirmed a substantial concordance between the three methods for both area and diameter (Fig. 1B,C,F; Supplementary Fig. 1B,C,F; Supplementary Fig. 2B,C,F).

To conclude, we opted for HALO® for downstream analyses because of its higher specificity and sensitivity in adipocyte recognition and the ease of use, not needing pre- or post-processing steps.

3.2. Intra-mammary heterogeneity of CAAs and distant adipocytes

We aimed to investigate whether intra-mammary heterogeneity in the size distribution of distant adipocytes and CAAs should be taken into consideration or not. Specifically, we aimed at assessing whether a single region of at least 500 adipocytes would provide a good representation of the adipocytes (distant or CAAs) present in the tissue. To this end, we analyzed ~500 distant adipocytes and ~500 CAAs in each single digital slide taken from ten BC samples. Intra-patient variability in adipocyte size was low for both CAAs and distant adipocytes, as illustrated in Fig. 2 for one of the patients. We further generated the cumulative distribution of the adipocyte areas and diameters for the ten patients and represented the intra-mammary heterogeneity for both the CAAs and distant adipocytes by displaying the range of adipocyte measurements for each quartile (Supplementary Figs. 4 and 5). Altogether, the cumulative distribution of CAAs and distant adipocytes area in each of the 10 patients revealed small intra-patient variability in each group, which led us to further consider a single region of at least 500 adipocytes (CAAs or distant) to estimate the distribution of the adipocytes in terms of area and diameter.

3.3. Comparison of distant adipocytes and CAAs using digital pathology

We compared the diameter and area of CAAs with those from the distant adipocytes (Fig. 3 and Supplementary Table 1 for detailed comparisons at the patient-level). Across all evaluated tumors, the mean diameter was 50.0 μm for CAAs and 77.3 μm for distant adipocytes and the median diameter was 46.7 μm and 76.1 μm, respectively (unpaired Wilcoxon test, p < 0.001). The mean area was 2311.8 μm² for CAAs and 5537.4 μm² for distant adipocytes and the median area was 1881.3 μm² and 4853.0 μm², respectively (unpaired Wilcoxon test, p < 0.001). Furthermore, comparing CAAs to distant adipocytes within each tumor, we observed that CAAs had smaller areas (median fold-change: 1.64, IQR: 1.50–2.65) and diameters (median fold-change: 1.64, IQR: 1.50–1.67) as compared to distant adipocytes (paired Wilcoxon test, p = 0.002 and p = 0.002, respectively). Of interest, the reduction in adipocytes diameter and area between distant and CAAs is consistent at each quintile in all samples (paired Wilcoxon tests, p = 0.002 and p = 0.002, respectively) (Fig. 3). These results therefore highlight the consistent reduction in size between distant adipocytes and CAAs, which can be quantified using digital pathology.

3.4. Association between CAAs and distant adipocytes measurements and BMI

The distributions of adipocyte measurements for the ten patients according to the BMI are illustrated in Fig. 4. We observed a correlation between BMI as a categorical variable (lean, overweight or obese) and adipocyte measurements with regard to area (Kendall’s τ = 0.80, p = 0.004 for CAAs, and Kendall’s τ = 0.65, p = 0.021, for distant adipocytes) and diameter (Kendall’s τ = 0.75 p = 0.007, Kendall’s τ = 0.59, p = 0.035, respectively). The correlation was further confirmed when considering BMI as a continuous variable, both with regard to area of CAAs and distant adipocytes (r = 0.89 p < 0.001; r = 0.71 p = 0.027, respectively) and diameter (r = 0.87 p = 0.002; r = 0.65 p = 0.049, respectively).

### Table 1
Comparison of the counting results of AdipoCount, Adiposoft and HALO®.

| Test Image | Software | Manual Count | Software Count | TP | FP | FN | TN | Sensitivity | Specificity | p-value sensitivity | p-value specificity |
|-----------|----------|--------------|----------------|----|----|----|----|-------------|-------------|-------------------|-------------------|
| Slide 1   | AdipoCount | 100          | 123            | 87 | 36 | 13 | 13 | 87.00%      | 26.53%      | 0.002             | <0.001            |
|           | Adiposoft | 114          | 92             | 22 | 8  | 33 | 33 | 92.00%      | 60.00%      | 0.132             | <0.001            |
|           | HALO®    | 98           | 97             | 1  | 3  | 48 | 48 | 97.00%      | 97.96%      |                   |                   |
| Slide 2   | AdipoCount | 109          | 140            | 105| 35 | 4  | 23 | 96.33%      | 39.66%      |                   | <0.001            |
|           | Adiposoft | 50           | 28             | 22 | 8  | 31 | 31 | 25.69%      | 63.93%      | <0.001            | <0.001            |
|           | HALO®    | 107          | 105            | 2  | 4  | 56 | 56 | 96.33%      | 96.55%      |                   |                   |
| Slide 3   | AdipoCount | 102          | 129            | 99 | 30 | 3  | 22 | 97.06%      | 42.31%      | 0.157             | <0.001            |
|           | Adiposoft | 134          | 94             | 40 | 8  | 25 | 25 | 92.16%      | 38.46%      | 0.008             | <0.001            |
|           | HALO®    | 102          | 101            | 1  | 1  | 50 | 50 | 99.02%      | 98.04%      |                   |                   |

Manual count is the reference. FN: False negative, FP: false positive, TP: true positive, TN: true negative.
4. Discussion

So far, digital pathology has not yet been applied standardly to measure the distant adipocytes and CAAs in H&E samples from BC patients. Here, we compared different software to detect and measure mammary adipocytes. In addition, we defined criteria to distinguish CAAs from distant adipocytes in breast tumor samples. All the software we tested had a good agreement in the measurement of both the area and the diameter of the adipocytes. However, regarding the adipocyte identification, the highest sensitivity and specificity were reached by HALO®, while these were unsatisfactory for the other two software. AdipoCount further does not provide the diameter of the cells and that does not allow setting a lower range for the area in order to avoid false positives, such as interstitial optical empty spaces. Adiposoft failed to identify adipocytes when tested on slides with a lower staining quality of the fat tissue. On the basis of these results, we conducted our subsequent analyses with HALO®. In addition, we observed that ~500

![Fig. 2. Intra-mammary heterogeneity of distant and cancer-associated adipocytes. Violin plots and boxplots of adipocytes diameter and adipocytes area of three regions close to the tumor (CAAs, blue) and three regions distant (distant adipocytes, red) in three slides taken from one patient (D, H). Each scanned slide has an annotation layer that shows the selection of adipocytes close to the tumor (A, B, C) and distant from the tumor (E, F, G). CAAs: cancer-associated adipocytes.](image-url)
adipocytes (distant or CAAs) provide a realistic representation of the adipocyte measurements given the minimal intra-mammary variability. We acknowledge that there might be additional free or commercial digital pathology software able to identify and measure adipocytes, such as QuPath [29] or Visiopharm® for example, however these were not considered in this study since they did not have already existing applications for identifying and measuring adipocytes.

Delipidation and the accompanying size reduction of CAAs as compared to distant adipocytes are key features of CAAs [30,31]. However, to the best of our knowledge, the size reduction has never been quantified and evaluated across several breast tumors. Our results clearly show that CAAs are smaller than distant adipocytes, with a median 1.6-fold decrease in diameter and a median 2.6-fold decrease in area when comparing CAAs and distant adipocytes from the same tumor. While this will need to be investigated in larger series, we hypothesize that the magnitude of the decrease in size between distant and CAAs could potentially reflect the strength of the interaction between cancer cells and adipocytes. The reduction of size could be a surrogate of lipid use as energy source by cancer cells [32]. It still has to be investigated whether it is associated with specific clinical, pathological or treatment characteristics which is ongoing.

Finally, we investigated the association between BMI and the size of the CAAs and distant adipocytes. It has been demonstrated that the MAT expands preferentially through the hypertrophy than through the hyperplasia of the adipocytes [33,34]. So far, the studies that investigated the association between BMI and the size of the adipocytes in the MAT of BC patients focused on distant adipocytes only and based their measurements on a limited number of adipocytes [18]. While it is known that BMI might not be the best surrogate marker for the adiposity of a patient, it remains the most commonly used measurement in clinical practice. The hypothesis is that hypertrophy of adipocytes may be a more reliable indicator of a patient’s adiposity determining an unhealthy MAT as shown in previous studies [18]. We found a significant correlation between BMI and the diameter and area of both CAAs and distant adipocytes, with patients with a higher BMI having not only larger distant adipocytes, as previously demonstrated [16], but also larger CAAs.

5. Conclusions

While we reckon that this study is based on a relatively small number of samples and patients, this is, to the best of our knowledge, the first to investigate both CAAs and distant adipocytes by digital software analysis in BC samples. Our study sets the basis for the standardized analysis of larger cohorts of BC patients, providing a more accurate estimation of mammary adiposity. Of interest, this analysis could further be extended to other cancer types that grow...
in close contact with the adipose tissue, such as soft tissue tumors, renal cancer and upper gastrointestinal tumors.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2020.10.004.

Ethical approval and consent to participate

The project has been approved by the ethics committee of the Institut Jules Bordet (CE2844). The necessity to obtain an informed consent was waived by the ethics committee.

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