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Assessment of mammary adiposity in breast cancer using digital pathology

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Summary

Increased adiposity is a significant risk factor for many cancers, including breast cancer. Patients with breast cancer who are overweight or obese have an increased risk of recurrence, and breast cancer-related mortality. While several hypotheses have been proposed to explain the biological mechanisms that drive the obesity-breast cancer interconnections, progress in our understanding of the adipose tissue tumor microenvironment and its relevance to breast cancer initiation and progression or the emergence of resistance to therapy has been limited.

In parallel to major breakthroughs on the increased adiposity as a risk and prognostic factor for several diseases and cancers, researchers have begun to utilize digital pathology to characterize the tumor microenvironment in diverse types of adipose tissue-rich neoplasia. Pioneering studies have uncovered the existence of a peculiar type of adipocytes named cancer-associated adipocytes in the microenvironment of tumors that grow in close contact with adipose tissue. At the time this thesis was started, only a few studies with small cohorts of patients and often, a limited number of adipocytes analyzed, mostly distant from the tumor, have shown the importance of the adipose tissue tumor microenvironment. However, an in-depth knowledge was currently lacking for breast cancer.

The work presented here harnesses the strength of digital pathology and state of the art of adipocyte measurement tools to analyze adipocytes in the adipose tissue microenvironment of breast cancer. Beyond providing histopathological criteria for adipocyte analysis, these results suggest that the analysis of mammary cancer-associated adipocytes is feasible using image analysis software. Moreover, our analyses reveal that cancer-associated adipocytes are smaller than distant adipocytes, reflecting the delipidation process undergone by cancer-associated adipocytes. The body mass index of the patient is associated with the size of distant and cancer-associated adipocytes giving new insights in the evaluation of mammary adiposity of a patient.

The data presented here are too preliminary to derive practice-changing evidence but provide the basis for performing adipocyte analysis on larger number of patients and support the concept that the evaluation of adipose tissue should be taken into account in breast cancer management.

Sommario

L'obesità è un fattore di rischio significativo per molti tumori, compreso il carcinoma della mammella. I pazienti con carcinoma della mammella che sono sovrappeso o obesi hanno un aumentato rischio di recidiva e di mortalità correlata al tumore. Sebbene siano state proposte diverse ipotesi per descrivere i meccanismi biologici alla base dell'interazione tra obesità e carcinoma della mammella, i progressi nella comprensione del microambiente tumorale e del ruolo degli adipociti presenti in esso e della loro rilevanza per lo sviluppo e la progressione tumorale o per l'emergere di resistenze alle terapie sono ancora limitati.

Parallelamente alle importanti scoperte sull'obesità come fattore di rischio e fattore prognostico per diverse malattie e tipi di tumore, i ricercatori hanno iniziato a utilizzare la patologia digitale per caratterizzare il microambiente tumorale. Studi pionieristici hanno scoperto l'esistenza di un tipo particolare di adipociti chiamati adipociti associati al tumore presenti nel microambiente di tumori che crescono a stretto contatto con il tessuto adiposo. Nel momento in cui è stata scritta questa tesi, solo pochi studi con piccole coorti di pazienti e spesso, un numero limitato di adipociti analizzati, per lo più distanti dal tumore, hanno mostrato la rilevanza del tessuto adiposo e degli adipociti come componente del microambiente tumorale. Tuttavia, allo stato attuale manca ancora una conoscenza approfondita del ruolo degli adipociti nel carcinoma della mammella.

Lo studio presentato in questa tesi descrive le potenzialità della patologia digitale e fornisce una rappresentazione dello stato dell'arte sui software con algoritmi dedicati per la misurazione degli adipociti, con una attenzione per gli adipociti presenti nel microambiente di carcinomi mammari. Oltre a stabilire criteri istopatologici per l'analisi degli adipociti, i risultati presentati dimostrano che è possibile effettuare l'analisi degli adipociti associati al tumore come di quelli distanti, utilizzando un software di patologia digitale. Inoltre, abbiamo mostrato che gli adipociti associati al tumore sono più piccoli degli adipociti distanti, riflettendo il processo di delipidazione subito dagli stessi. Infine, l'indice di massa corporea del paziente è associato alle dimensioni degli adipociti distanti e associati al tumore, fornendo nuove prospettive per la valutazione dell'adiposità mammaria di un paziente.

I dati qui presentati, sono preliminari per un cambiamento diretto nella pratica clinica, ma forniscono i criteri per eseguire l'analisi degli adipociti su coorti più ampie di pazienti ed evidenziano l’importanza della valutazione del tessuto adiposo nella gestione del carcinoma della mammella.

List of Abbreviations

ASCs	Adipose-Derived Stromal/Stem cells
AT	Adipose Tissue
BC	Breast Cancer
BMI	Body Mass Index
BMAT	Bone Marrow Adipose Tissue
CAFs	Cancer-Associated Fibroblasts
CLS	Crown-Like Structure
CT	Computerized Tomography
DCIS	Ductal Carcinoma In Situ
E1	Estrone
E2	17-b-Estradiol
ECM	Extracellular Matrix
ER	Estrogen Receptor
EU	European Union
FFAs	Free-Fatty Acids
HER2	Human Epidermal Growth Factor Receptor 2
H&E	Hematoxylin and Eosin
ICIs	Immune Checkpoint Inhibitors
IGF-1	Insulin-Like Growth Factor 1
IL-6	Interleukin 6
INF-γ	Interferon Gamma
MAT	Mammary Adipose Tissue
MRI	Magnetic Resonance Imaging
pCR	Pathological Complete Response
PR	Progesterone Receptor
SAT	Subcutaneous Adipose Tissue

TAMs	Tumor-Associated Macrophages
TILs	Tumor Infiltrating Lymphocytes
TME	Tumor Microenvironment
TNBC	Triple Negative Breast Cancer
TNF-α	Tumor Necrosis Factor Alpha
US	United States
VAT	Visceral Adipose Tissue
WAT	White Adipose Tissue
WHO	World Health Organization

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Introduction

Breast cancer incidence and current management: a short overview

Breast cancer (BC) is the most commonly diagnosed cancer in Western Countries. To give an order of magnitude, in the European Union (EU), over 355,000 women are estimated to be diagnosed with BC in 2020[1]. BC also represents the first cause of cancer-related death in women with more than 90,000 deaths every year in the EU[2]. Mortality rates vary worldwide, and they have decreased in the last decades with the advent of more accurate prognostic predictors and with the introduction of novel treatments. Despite that, a considerable percentage of patients, between 20% and 30%, still fail to respond to treatments and die as a consequence of the development of distant metastases[3].

BC is a heterogenous disease with distinct clinical and molecular subtypes. The traditional BC management involves the evaluation of several clinico-pathological variables such as age, tumor size, axillary lymph node involvement, histopathological features (especially histological grade and lympho-vascular invasion) and key markers such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67. Taken together this information allows to identify three clinical phenotypes with different behaviors and treatment strategies: hormone receptor positive (HR^+, ER^+ and/or PR^+), HER2 positive ($HER2^+$) and triple negative (TNBC: ER^-, PR^-, $HER2^-$) BC. Patients with ER^+ BC generally benefit from endocrine therapy. Similarly, patients with $HER2^+$ BC may benefit from anti-HER2 targeted therapy. Instead, the standard treatment for TNBC is based on chemotherapeutic agents. There are no targeted treatments available for this tumor type, at least in the early setting. There exist four molecular subtypes based on gene expression profile that partially overlap with the above-mentioned clinical subtypes: Luminal A, Luminal B, $HER2^+$, and triple negative. This last molecular subtype, in turn, has been divided into seven molecular subtypes by Lehman and colleagues: immunomodulatory, mesenchymal, mesenchymal stem-like, luminal androgen receptor, unstable, basal-like 1 and basal-like 2 subtypes[4]. Thereafter, a refined sub-classification of the previous one was still proposed by Lehman and colleagues identifying 4 molecular subtype: basal-like 1, basal-like 2, luminal androgen receptor and mesenchymal[5]. These molecular subtypes differ with regard to their patterns of gene expression, clinical features, response to treatment, and prognosis[6–9]. In the last years,

prognostic tests based on multigene signatures such as MammaPrint® (Agendia) and the Oncotype DX® Breast Recurrence Score (Exact Sciences Corp.) have been introduced[10]. Large-scale studies have shown that these tests can potentially help to guide treatment[11,12].

BC not only consists of neoplastic cells, but also of significant alterations in the surrounding stroma or tumor microenvironment (TME). These alterations in various cells of the TME such as lymphocytes, fibroblasts, endothelial cells, adipocytes and other cells types, are now recognized as a critical element for BC development and progression, as well as potential therapeutic targets[13]. For instance, the evaluation of tumor infiltrating lymphocytes (TILs) in the TME has added important prognostic and predictive information to current BC management[14–16]. The recent introduction of immunotherapy, especially immune checkpoint inhibitors (ICIs), for the treatment of metastatic TNBC, has shown the important role of the TME in developing and modulating an anti-tumor response[17]. ICIs are novel agents that boost the immune system including the immune cells infiltrated in the TME[18,19]. Other cells present in the TME such as cancer-associated fibroblasts (CAFs), have been described in previous studies and are under investigation as potential target for cancer therapy[20]. More recently, adipocytes are gaining increasing attention due to the fact that they represent a major component of the TME in several types of cancer, specifically in BC. Also, the breast is a unique structure mostly composed of adipose tissue (AT) and adipocytes can interact with tumor cells and other cells from the TME[21]. The author will refer along the text to the white adipose tissue (WAT), without mentioning other types of AT, since WAT is the most represented AT in the body and the most studied as involved in several diseases including cancer[22].

The impact of obesity on breast cancer

Obesity is a complex preventable disease and a global public health problem. Over a third of the world's population is overweight or obese and a recent study estimated that by 2030 nearly 1 in 2 adults will have obesity in the United States (US)[23,24] and 1 in 3 adults in the EU[25]. Moreover, obesity and even more severe obesity are more prevalent in women than men in the US[24]. Obesity is typically defined as an excessive fat accumulation and it is categorized according to the World Health Organization (WHO) criteria in terms of Body Mass Index (BMI, kg/m^2). Overweight individuals have a BMI ranging from 25 to 29.9 kg/m^2 and obese individuals have a BMI greater than or equal to 30 kg/m^2[26]. Obesity is frequently subdivided into sub-categories with extreme or severe obesity defined by a BMI ≥ 40 kg/m^2[26] .

Obesity is recognized as one of the major risk factors for several types of cancer, including BC, especially in the post-menopausal HR^+ population[27,28]. The effect of obesity on BC risk differs according to menopausal status and disease subtypes. Several studies have shown a stronger association between increased BMI and a higher incidence of post-menopausal HR^+ BC (33% increase of BC risk per 5 kg/m^2 BMI increment) whereas the risk for post-menopausal ER^- and TNBC seems to be minimally or inversely associated[29–31]. By contrast, an inverse association between obesity and pre-menopausal HR^+ BC was observed (8-10% decrease of BC risk per 5 kg/m^2 BMI increment) whereas pre-menopausal TNBC showed a higher BC risk[29,32,33]. Overweight and obesity not only impact the risk of developing BC, but also the prognosis and treatment efficacy of patients with BC. Obesity is a prognostic factor for worse clinical outcome independent from the menopausal status[34,35]. A large meta-analysis showed that obesity at the time of BC diagnosis was associated with an increased risk of total mortality and with an increased risk of BC mortality[36]. The increased mortality risk in obese women was mostly observed for HR^+/luminal subtypes[37], while there are contrasting results regarding the increased mortality in TNBC obese women[38,39]. In contrast, a recent study showed that obesity was associated with worse prognosis in all molecular subtypes[40]. Furthermore, a few studies have shown that obesity was associated with an increased rate in distant, but not loco-regional recurrences[34,41]. A list of the most relevant studies evaluating the impact of the obesity on BC risk and prognosis is reported in Table 1.

Obesity also affects response to therapeutic treatments. Although some studies have shown that obese patients are often under treated due to the concerns about potential treatment toxicities[42], several pre-clinical studies have demonstrated that the TME can contribute to treatment resistance in obese patients[43–47]. A retrospective analysis of a large clinical trial showed that obese patients treated with taxane-based regimens had a worse outcome compared to lean patients, while no differences were observed in patients treated in the arms without taxanes[48]. Furthermore, another study showed that there might be a change in pCR rate according to BMI in patients with TNBC and high TILs levels treated with neoadjuvant chemotherapy. A higher pCR rate was observed in lean patients in comparison to overweight/obese patients[49]. Finally, there is some evidence that obesity might also affect endocrine therapy response. An exhaustive review of Jiralerspong and Goodwin summarized the main findings of several clinical trials showing a trend of less efficacy of anastrozole in post-menopausal overweight/obese women compared to lean women that it was not observed for letrozole[28].

Most of the studies that evaluated the links between obesity and BC risk/BC outcomes used BMI as main measure of patient's adiposity. BMI is easy to measure, inexpensive, strongly correlated with body fat at population level and provides standardized definitions for lean, overweight and obese categories[50]. By contrast, it is considered a surrogate marker of adiposity because it does not reflect accurately the body composition of an individual, failing to distinguish between muscle mass and fat mass[51]. In addition, it does not consider fat localization, age and ethnic differences. For these reasons, the impact of patient's adiposity on BC risk and BC outcomes might be greater. Complementary measures of assessment of body fat distribution have been evaluated and have gained interest in the last two decades: waist circumferences, waist to hip ratio, waist to height ratio, dual energy x-ray absorptiometry, bioelectric impedance, computerized tomography (CT) and magnetic resonance imaging (MRI)[52–54]. All these methods add specific information regarding body fat distribution compared to BMI, but they have some limitations such as the cost, the under or the overestimation of a specific fat depot, and the lack of standardization. A recent observational study showed that information on muscle quantity and adiposity from clinically acquired CT scans provide significant prognostic information that outperforms BMI[55]. CT and MRI are now considered to be the most accurate methods for measuring body

composition (fat and muscle mass), but their use is still limited to research purposes[56]. Lastly, it was observed, in several studies, that the size of the mammary adipocytes was strongly correlated with BMI[57–59]. Thus, histological measuring of adipocytes, might be a valid indicator of patient's adiposity as well as giving information on health status and type of AT expansion.

Overall, obesity has a significative impact not only on BC risk and mortality, but also on therapeutic management. To date, current BC treatments do not consider the patient's adiposity. Therefore, there is an urgent need to find novel treatments and to optimize the existing treatments according to patient's adiposity, together with more meaningful assessments of body (fat) composition at individual level.

	Author (year, journal)	Study type and population	Focus	Results
Obesity and BC risk				
	Park et al.[31] 2020, Breast Cancer Res Treat	1,418,180 premenopausal and 4,854,187 postmenopausal Korean women. All BC subtypes	To examine the association between obesity and BC	In postmenopausal women, there was a positive association between obesity and BC risk (HR: 1.28, $CI_{95\%}$: 1.25 – 1.32). An inverse association was present between obesity and BC risk in premenopausal women (HR: 0.95, $CI_{95\%}$: 0.91 – 0.98)
	Pierobon et al.[33] 2012, Breast Cancer Res Treat	Meta-analysis of case-case and case-control studies 24,479 BC 3,845 TNBC	To analyze the association between TNBC and obesity	There was a greater risk in pre-menopausal women of developing a TNBC than non-obese women (OR: 1.43, $CI_{95\%}$: 1.23 – 1.65)
	Suzuki et al.[29] 2009, Int J Cancer	Meta-analysis of cohort and case control studies. Pre- and postmenopausal BC patients. All BC subtypes	To clarify the association between BMI and the incidence of BC defined by ER/PR status of the tumors	In premenopausal women there was a 20% lower risk for the development of ER^+PR^+ BC. In postmenopausal women there was 82% increased risk for ER^+PR^+ BC. Each increment in the BMI by 5 kg/m^2 was associated with 10% lower risk of premenopausal and 33% increased risk of postmenopausal BC
	Renehan et al.[32] 2008, The Lancet	Meta-analysis of prospective studies of incidence cases of 20 cancer types 23,909 postmenopausal BC women 7,930 premenopausal BC women	To determine the risk of cancer associated with a 5 kg/m^2 increase in BMI	A 5 kg/m^2 increase in BMI was weaker associated with post-menopausal BC (RR: 1.12, $IC_{95\%}$: 1.08 – 1.16)
Obesity and BC prognosis				
	Lohmann et al.[40] 2021, J Natl Cancer Inst	Meta-analysis of 27 studies 108,908 BC women All BC subtypes	To evaluate the association of BMI with outcomes in non-metastatic BC across the spectrum of IHC defined BC subtypes (HR^+HER2^-, $HER2^+$, and TNBC) in women receiving adjuvant therapies	Obesity was associated with modestly worse DFS and OS in all BC subtypes. ER^+/PgR^+ HER2- (DFS, HR: 1.26, CI_{95}: 1.13 – 1.41; OS, HR: 1.39, CI_{95}: 1.20 – 1.62) $HER2^+$ Any ER/PgR (DFS, HR: 1.16, CI_{95}: 1.06 – 1.26; OS, HR: 1.18, $CI_{95\%}$: 1.05 – 1.33) TN (DFS, HR: 1.17, $CI_{95\%}$: 1.06 – 1.29; OS, HR: 1.13, $CI_{95\%}$: 1.13 – 1.53)

	Author (year, journal)	Study type and population	Focus	Results
Obesity and BC prognosis				
	Pan et al.[37] 2014, J Clin Oncol	Meta-analysis of 21 pooled trials (EBCTCG data) 80,000 BC women All BC subtypes	To investigate the association between BMI and the risk of mortality	BC specific mortality is worse in premenopausal obese patients with ER^+ BC (RR: 1.34, $CI_{95\%}$: 1.22 – 1.4); no differences in postmenopausal patients
	Chan et al.[36] 2014, Ann Oncol	Meta-analysis 213,075 BC women All BC subtypes	To explore the associations between BMI and the risk of total and cause-specific mortality, overall and in women with pre- and postmenopausal BC	Obesity is associated with poorer OS in pre (RR: 1.75, $CI_{95\%}$: 1.26 – 2.41), and postmenopausal BC women (RR: 1.34, $CI_{95\%}$: 1.18 – 1.53) and is associated with poorer BC survival (global RR: 1.35, $CI_{95\%}$: 1.24 – 1.47)
	Ewertz et al.[34] 2011, J Clin Oncol	18,967 BC women (DBCG data), All BC subtypes	To characterize the impact of obesity on the risk of BC recurrence and death	The risk of developing distant metastases after 10 years was increased by 46% (HR: 1.46, $CI_{95\%}$: 1.11 – 1.92), and the risk of dying as a result of BC after 30 years was increased by 38% for obese BC women (HR: 1.38, $CI_{95\%}$: 1.11 – 1.71)
	Protani et al.[35] 2010, Breast Cancer Res Treat	Meta-analysis Number of patients: not specified All BC subtypes	To determine the effects of obesity on survival in newly diagnosed BC patients	Obese BC women have a poorer OS and BCSS compared with non-obese women (HR: 1.33, $CI_{95\%}$: 1.21 – 1.47), (HR: 1.33, $CI_{95\%}$: 1.19 – 1.50)

Table 1. Meta-analysis and national studies investigating obesity-breast cancer risk and obesity-breast cancer prognosis.

The larger and most relevant studies are reported in the table.
Abbreviations: BCSS, breast cancer–specific survival; BMI, body mass index; DBCG, Danish Breast Cancer Cooperative Group, EBCTG, Early Breast Cancer Trialists Collaborative Group; ER, estrogen receptor; HR, hazard ratio; IHC, immunohistochemically; RR, risk ratio; TN, triple-negative; OR, odds ratio; OS, overall survival

Molecular mechanisms linking obesity to breast cancer

The molecular mechanisms and the pathways involved in BC development and progression have not been fully elucidated yet in adipose-rich women. Several studies have hypothesized and described possible mechanisms[60–62]. Some of these mechanisms are shared by other cancer types and diseases linked to obesity. The main candidate mechanisms are discussed below, except for the role of TME and adipocytes in obesity-associated chronic inflammation that will be discussed in a separate paragraph.

Estrogens. 17-b-estradiol (E2) and estrone (E1) are the two main estrogens produced in pre-menopausal and post-menopausal women. Aromatase is the key enzyme presents in AT, that mediates the conversion of androgens to estrogens and its activity is higher in the expanded AT of obese women[63]. AT contributes up to 100% of circulating estrogens in post-menopausal women whereas the ovaries are the major source of estrogens in pre-menopausal women[64]. Estrogens are one of the major factors for post-menopausal ER^+ BC risk. In particular, a recent study showed for the first time how E2 and E1 act in pre and post-menopause. The pro-inflammatory effect of higher levels of E1 that occur in post-menopausal and obese women are unopposed by the anti-inflammatory effects of E2, predominant in pre-menopausal women[65]. Therefore, the declining E2:E1 ratio after menopause and the increased inflammatory cytokine environment, in obesity, might in part explain why obese women are exposed to an increased risk of developing ER^+ BC after menopause and not before. Another hypothesis is that obese women have a significant disruption in the menstrual cell cycle resulting in less exposure to estrogens than normal weight women, therefore leading to a decreased ER^+ BC risk[66,67].

Adipokines. Adiponectin and leptin are the two major adipokines secreted by adipocytes. In normal condition, they contribute to maintain a metabolic homeostasis. In obese individuals, metabolic homeostasis is commonly disrupted with an increase of leptin, not counterbalanced by an increase of adiponectin[68]. Adiponectin is protective against cancer, having anti-inflammatory, anti-proliferative, insulin-sensitizing properties and promoting a healthy AT expansion[61,68]. By contrast, leptin whose levels are correlated to the fat mass, activates several signaling pathways such as JAK/STAT, MAPK/ERK, and PI3K/AKT involved in tumor

development and progression[69]. In addition, leptin, at high concentration, can increase aromatase activity fostering higher level of estrogens in obese women[70].

Insulin-like growth factor (IGF-1) pathway. Several metabolic pathways are dysregulated in obese women. In particular the IGF-1/IGF-1R pathway seems to be one of the most relevant in contributing to breast carcinogenesis[71]. IGF-1 levels are increased in obese women as a consequence of hyperinsulinemia, a common condition in obese women affected by metabolic syndrome[72]. Moreover, IGF-1 is tightly linked to ER increasing the transcriptional activity of ER and it also can act in synergy with estradiol inducing a mitogenic response in breast epithelial cells[73]. A recent study has not only shown that there was an association between higher levels of IGF-1 and BC development, but also, that IGF-1 was likely to be a cause of the disease[74].

Metabolic Inflammation. Most obese women present with a systemic and chronic low-grade inflammation termed metabolic inflammation. Obese MAT is characterized by several immune-metabolic changes including hypertrophy of adipocytes and the presence of crown-like structures (CLS)[75]. CLS are defined by the presence of $CD68^+$ macrophages surrounding necrotic adipocytes. CLS are considered as a histologic marker of AT inflammation and are associated with obesity, post-menopausal status[76], aromatase activity[77,78] and insulin resistance[79]. The incidence of CLS in BC patients is ~50% and it is higher in overweight and obese women with an incidence of 53-70% and 75%-90%, respectively[75,76,80]. Of interest, the presence of CLS has been described in a not negligible percentage, around 34-39%, of lean women defined as metabolically-obese normal-weight women[76,78]. Furthermore, a study showed that the presence of CLS was higher in patients who received neoadjuvant chemotherapy compared to patients who did not receive neoadjuvant chemotherapy[76]. Most of the studies evaluated CLS on mastectomy specimens of BC patients[76,81]. On the contrary, Vaysse et al, evaluated CLS on both lumpectomy and mastectomy specimens, and found CLS, mainly in overweight/obese women with low grade tumors treated with breast conservative surgery[58]. Overall, the presence of CLS in MAT may contribute to generate a pro-inflammatory environment favorable to BC initiation and progression. Moreover, the hypoxia in expanding MAT may trigger inflammatory responses[82], suppressing the synthesis of anti-inflammatory adipokines such as adiponectin[83] and increasing the synthesis of pro-inflammatory cytokines such as interleukin-6 (IL-6)[84]. Iyengar and colleagues reported that

MAT inflammation was associated with short overall survival and short recurrence-free survival in BC[79]. A recent study showed that the presence of free fatty acids (FFAs), often elevated in the blood of obese women, increased the risk of ER^+ BC in the obese postmenopausal population through ERα and mTOR signaling pathways[85].

Overall, the local and systemic alterations induced by obesity may influence BC development and progression through direct effects on cancer epithelial cells as well as indirect effects on the TME.

Obesity and tumor microenvironment

The TME influences cancer development and progression through a bidirectional and dynamic crosstalk between cells constituting the TME and cancer cells. Mammary TME consists of 1) a cellular compartment including cancer cells, adipocytes, fibroblasts, immune cells such as macrophages and lymphocytes, endothelial cells, pericytes, and, 2) a non-cellular compartment mainly constituted of extracellular matrix (ECM)[86]. Adipocytes are a key player orchestrating local changes in the TME of BC and they will be discussed in a separated paragraph. The other main players are the following:

Tumor-associated macrophages (TAMs). TME defines remodeling of both infiltrating and resident macrophages into TAMs. Up to 90% of macrophages in obese TME are associated with adipocytes hypertrophy and adipocytes necrosis[87,88]. There are two main classically-described phenotypes of macrophage activation: M1 and M2[89]. M1 macrophages respond to a series of stimuli including tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) secreting specific molecules with pro-inflammatory properties, anti-cancer activity and associated to insulin resistance. Instead, M2 macrophages have different surface markers and respond to another spectrum of stimuli such as IL-4 and IL-10 resulting in anti-inflammatory activity, immune tolerance, insulin sensitivity and promoting tumor development and progression [72]. Besides the classical model of M1/M2 macrophage activation, recent studies have revealed a more complex range of macrophage activation states, and the existence of different macrophage profiles based on BMI, location of AT depots and between non-cancer and cancer patients[49,90]. For instance, subcutaneous adipose tissue (SAT) of obese individuals exhibits a mixed phenotype characterized by the simultaneous presence of markers that generally belong to M2- and M1- macrophages [91]. Another study showed that obese MAT of non-cancer patients had an increased density of M2-biased or TAM-like macrophages promoted by the remodeling of ECM that occurs in obese individuals[92]. Moreover, Tiwari and colleagues, showed that in MAT of obese TNBC patients, the predominant macrophages phenotype was composed by a mixed phenotype with pro-inflammatory and pro-tumorigenic characteristics called metabolically activated macrophages[93].

Tumor-infiltrating lymphocytes (TILs). A subpopulation of BC is able to mount an anti-tumor immune response. One of the discriminant features is the ability of T lymphocytes to infiltrate the tumor. TILs of the stromal compartment are generally quantified on hematoxylin-eosin (H&E) stained slides, and the result is a continuous variable expressed as percentage of the stromal surface area occupied by TILs[16,94]. TILs were more frequently observed in high-grade lesions, in TNBC and $HER2^+$ BC[95,96]. A low TILs infiltrate has been associated with poor outcome in TNBC patients[96,97]. A large pooled analysis has demonstrated that higher levels of TILs predict pathological complete response (pCR) to neoadjuvant chemotherapy in patients from all molecular subtypes, but longer survival only in TNBC and $HER2^+$ BC[95]. In addition, a recent study showed that a subgroup of systemically untreated patients with early stage TNBC having an excellent prognosis was identified by a TILs cut-off of 30%[98]. In consideration of the consistent findings, TILs have now been integrated in the new WHO classification of breast tumors[99]. Furthermore, recent experimental and human data have revealed that the adiposity of a subject can influence the TME inducing T-cell dysfunction[100].

Cancer-associated fibroblasts (CAFs). Fibroblasts are one of the most abundant cell types in tumor stroma and they can become CAFs in the TME. CAFs residing in the TME are heterogeneous cells with different functions (pro or anti-tumor activities) and different surface markers. CAFs can originate from a wide range of cells including CAAs and adipose-derived stromal/stem cells (ASCs), especially in obese patients[101]. CAFs are the major contributor to the remodeling and to the stiffness of ECM observed in tumors[20]. CAFs positively influence the proliferation and metabolism of cancer cells through oxidative stress. CAFs promote angiogenesis, tumor initiation and tumor progression[20,102]. Furthermore, Costa and colleagues identified four CAF subpopulations, according to the expression levels of specific fibroblast markers in a series of BC. They discovered as one of these CAF subpopulations named CAF-S1 might be involved in creating an immunosuppressive environment in BC[103]. Another work showed that some CAFs subpopulations may induce an immune-suppressive TME promoting $PD\text{-}1^+$ TAMs[104].

Mammary adipose tissue and adipocytes

Adipose tissue is no longer seen as a mere energy depot. It has been recognized to be involved in various biological processes including energy metabolism, neuroendocrine function and immune function. In general, in obese individuals, there is a pathological expansion of WAT that occurs mainly in response to overnutrition. AT presents a considerable heterogeneity across fat depots consisting in different anatomic locations and distinct functions[22]. SAT, the largest fat depot present in the body, and visceral adipose tissue (VAT) are the most well characterized fat depots with regard to the mechanisms of expansion (hypertrophy vs. hyperplasia) and the contribution to metabolic disease.

Mammary adipose tissue is a specialized fat depot that can represent up to 56% of the volume of the non-lactating breast tissue and 35% of lactating breast tissue[105]. It is constituted by adipocytes, the most abundant cell type, and by a stroma-vascular fraction that includes immune cells, fibroblasts, pericytes, endothelial cells, ECM, precursor of adipose cells (pre-adipocytes and ASCs), and nerve fibers[106]. MAT undergoes physiological remodeling in the context of pregnancy, lactation, post-lactation involution and age-related involution[22,107]. It can also undergo pathological remodeling: in obesity, where MAT becomes dysfunctional and does not expand properly to store the energy excess, and in BC initiation and progression where adipocytes become able to communicate with cancer cells and turn into the so-called cancer-associated adipocytes (CAAs), establishing a bidirectional cross-talk with cancer cells[108]. Although it is still under investigation, an interaction between adipocytes and cancer cells might already occurs at pre-invasive stage of BC as it has been hypothesized that the breast-associated adipocytes secretome of obese individuals might be conducive to tumor development and progression[109,110]. In a recent work, we showed the association between adipocyte size and the risk of a subsequent ipsilateral invasive BC after primary DCIS supporting the role of adipocytes in promoting BC[111].

Initially, adipocytes are separated from the normal mammary epithelium by a fibrous layer that during tumor initiation is disrupted bringing the adipocytes in direct contact with cancer cells. The invasive front (IF) of the tumor is a crucial region where adipocytes engage cancer cells in a direct interaction. CAAs located at the IF are defined as peritumoral (Fig. 1) whereas CAAs that are recruited and engulfed by the tumor are defined as intratumoral[112].

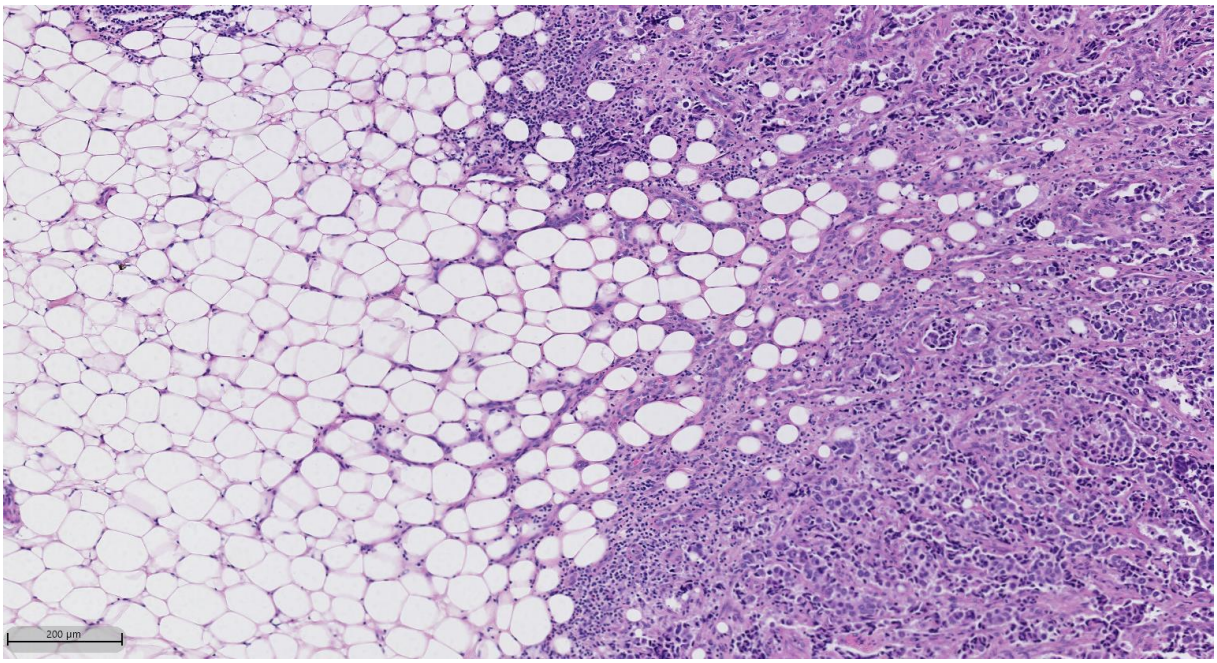

Figure 1. Adipocytes located at the invasive front of the tumor.

H&E slide scanned at 40x magnification.

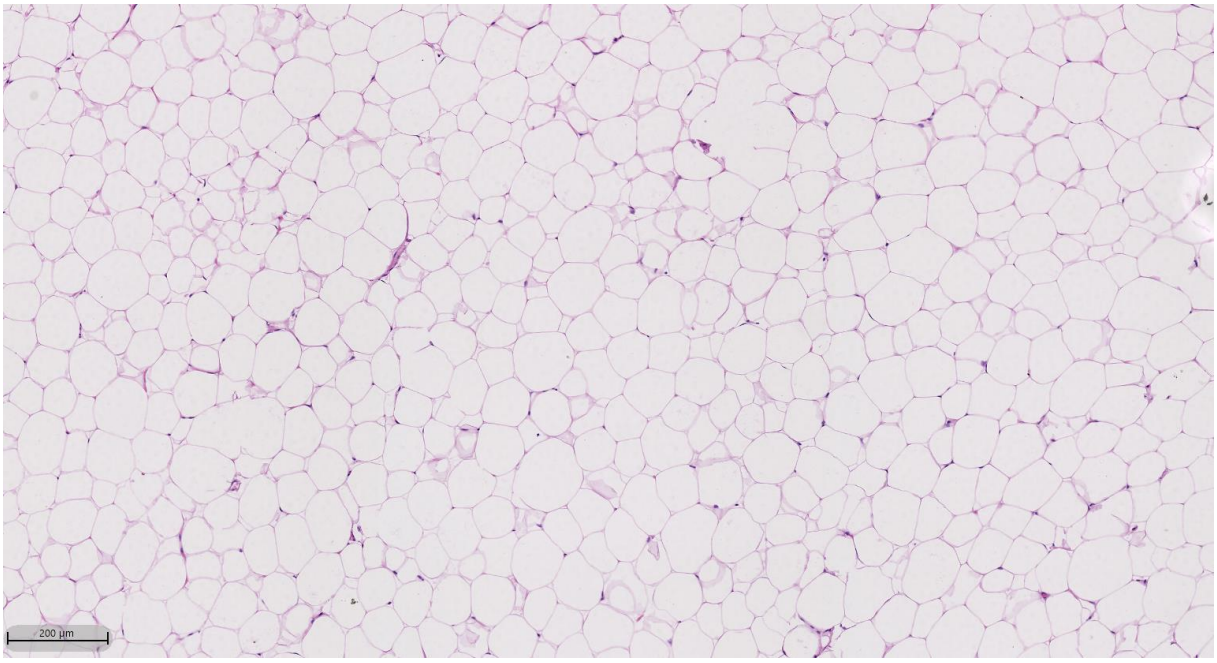

Figure 2. Adipocytes located at a distance > 2 mm from the tumor.

H&E slide scanned at 40x magnification.

CAAs have distinct phenotypes with respect to their normal counterpart that generally is at a certain distance from the tumor(Fig. 2). CAAs are smaller, with smaller lipid droplets, aberrant secretion of inflammatory cytokines such as TNF-α, IL-6 and IL-1β as well as proteins involved in ECM remodeling like matrix metalloproteinase 11(MP11)[113]. The hallmark of CAAs is the

delipidation, a process that through the release of FFAs fuels cancer cells growth. Cancer cells, in turn, undergo a metabolic reprogramming with a dramatically increased of use of lipids [114]. In addition, the metabolic symbiosis between adipocytes and cancer cells enhances both ketogenesis in adipocytes and ketolytic activity in BC cells[115]. Ultimately, CAAs may acquire a fibroblast-like phenotype with migratory and invasive abilities and complete disappearance of adipocyte markers contributing to the so-called desmoplastic reaction – dense fibrous tissue surrounding the tumor[116]. The transfer of FFAs to cancer cells occurs through three main proteins: CD36 [fatty acid translocase (FAT)], fatty acid binding protein 3 (FABP3) and fatty acid binding protein4 (FABP4)[71]. FFAs can be used by cancer cells as building blocks for newly synthetized membrane phospholipids[117]. In obesity, the CAAs-cancer cells cross-talk might be amplified, as it was observed that CAAs seem to be more prone to release lipids to cancer cells[114]. Moreover, the amount of lipid transfer might be different according to the tumor subtype, as a recent *in vitro* study showed that TNBC cell lines had the highest lipid transfer[118]. CAAs has been described to influence BC therapy response through several mechanisms. For example, major vault protein (MVP) is a transport-associated protein present in cancer cells, whose expression is upregulated by adipocytes, especially in obese individuals, and promotes chemotherapy resistance[44]. AT hypoxia is another mechanism of resistance to chemotherapy and also to anti-angiogenic drugs[47,112]. Furthermore, CAAs might promote a radioresistant phenotype in BC cells through the enhancement of IL-6 expression[119].

Collectively, all these findings suggest an important role for CAAs in BC evolution and response to therapeutic agents, that should be further investigated.

State of the art in adipocytes measurements

Adipose tissue homeostasis is orchestrated in part by the balance between adipocyte hypertrophy and pre-adipocyte hyperplasia. Both hypertrophy and hyperplasia are related to AT expansion and are known to be involved in a wide range of diseases including several types of cancer[109,120]. Therefore, determining the changes in AT volume deriving from hypertrophic or hyperplastic adipocytes, along with the specific location of AT growth, is important to understand the mechanisms driving obesity and cancer. Since the present work focuses on adipocytes measurements in MAT of BC, an overview of the available techniques for counting and measuring adipocytes in AT samples will first be provided.

Microscopic measurements on conventional histological preparations or fresh-cut AT, automatic counting or sizing of osmium tetroxide-fixed fat cells or collagenase-isolated unfixed adipocytes in a suspension using a Coulter electronic counter are some of the methods that have been described[121–126]. Although no gold standard technique exists for adipocyte counting and size measurements, manual count and manual tracing of cross-sectional area are considered the reference techniques[127]. The major limitation is that these procedures are tedious and extremely time-consuming if performed on a large number of adipocytes. Therefore, several image analysis software have been developed for counting and measuring adipocytes in an automated way. These software rely mainly on histological images. Others automated image analysis tools use cell suspension but the major limitations are the complexity in preparing the suspension and the complete loss of the histopathological context. Instead, one of the advantages of using histological samples is the preservation of AT architecture that is crucial to study the AT TME in cancer and other diseases. In addition, preparation of histological samples have a low cost and histological samples are available for retrospective analysis and other types of analysis such as immunohistochemical staining. On the other hand, some of the possible limitations are the poor fixation and the damaged AT that might result in distorted measurements of adipocytes (Fig. 3)[124,128].

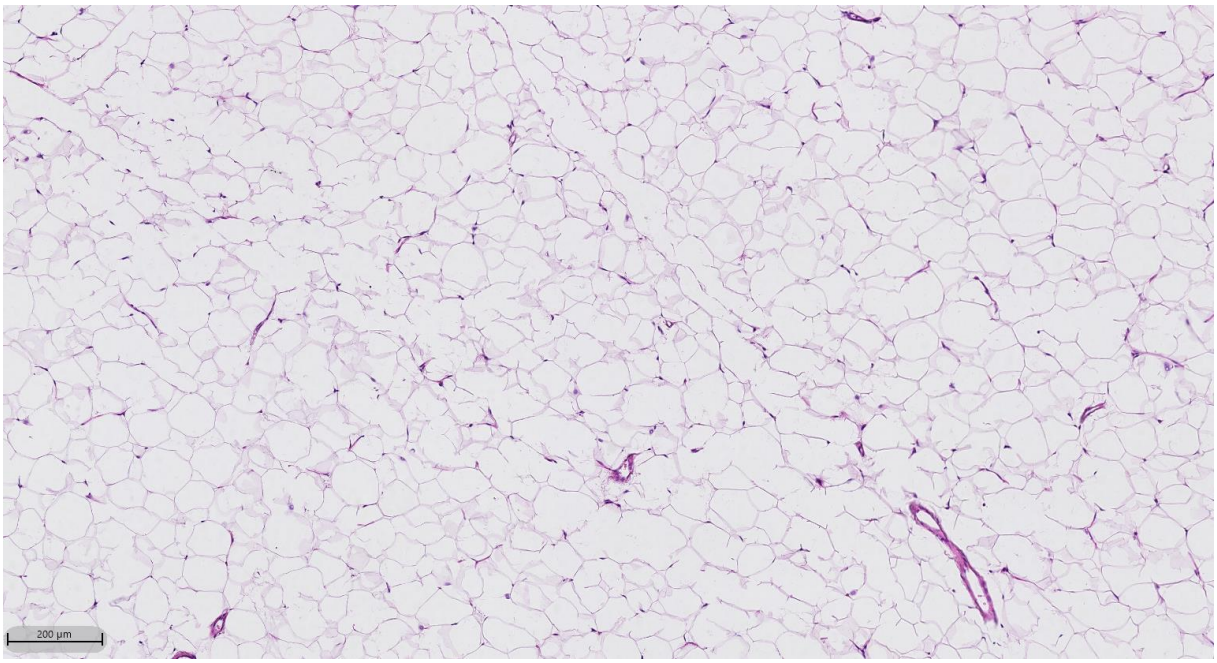

Figure 3. Example of damaged adipose tissue not suitable for adipocyte analysis.

H&E slide scanned at 40x magnification.

There exist open-source software dedicated to adipocyte analysis such as AdipoCount[129], Adiposoft[127] developed as a plug-in for ImageJ[130], QuPath[131] and others, or proprietary software including HALO® (Indica Labs, Corrales, US) and VisioPharm® (Hørsholm, DK). All these software or at least their modules/plug-ins, are built for adipocyte analysis and the majority has been tested on AT of non-cancer patients or obese individuals where the AT is more regular. Few studies tested these software in cancer patients in whom the remodeling of AT driven by cancer is extremely higher and disordered, and adipocytes are intermixed to cancer cells and other cell types. Moreover, most of these studies focused on adipocytes located far from the IF of the tumor. The studies that evaluated adipocyte size in MAT of BC patients are listed in Table 1. Most of the studies are performed on a limited number of adipocytes (~30) due to the inherent limitation of the manual analysis and on adipocytes distant from the tumor. The work of Vaysse et colleagues is the first in the BC field that used an automated software algorithm on a large number of adipocytes[58].

Overall, there is a lack of standardization for measuring adipocytes and a lack of criteria for the histopathological identification of CAAs in BC patients.

Author (year, journal)	Study population/tissue type and location	Software/Number of adipocytes	Focus	Results
Greenlee et al.[132] 2019, Cancer Prev Res	Hispanic/Latin women (n = 137) Breast WAT (distant)	Linear Dimensional Tool in Canvas 11 (ACS Systems of America, Inc.) 30 or more adipocytes per patient	Association of breast WAT inflammation with adipocyte size, body composition, serum markers	WAT inflammation was associated with BMI, adipocyte size, smoking history and neoadjuvant chemotherapy, ALT
Iyengar et al.[80] 2018, Cancer Prev Res	Taiwanese women (n = 72) Caucasian women (n = 237) Breast WAT (distant)	Linear Dimensional Tool in Canvas 11 (ACS Systems of America, Inc.) 30 or more adipocytes per patient	Association of breast WAT inflammation with adipocyte size, body composition and serum markers	WAT inflammation in Taiwanese women was associated with BMI, adipocyte size, CRP. triglycerides, insulin. Taiwanese women have larger breast adipocytes and lower BMI on average than Caucasian women
Vaysse et al.[58] 2017, NPJ Breast Cancer	Women (n = 107) Breast WAT (distant)	Adiposoft ~ 300 adipocytes per patient	Association of breast WAT inflammation with adipocyte size, body composition and serum markers	WAT inflammation correlated with BMI, adipocyte size, CRP (pre-menopausal), triglycerides (post-menopausal)
Iyengar et al.[78] 2017, Cancer Prev Res	Normal weight women (n = 72) Breast WAT (distant)	Linear Dimensional Tool in Canvas 11 (ACS Systems of America, Inc.) 30 or more adipocytes per patient	Association of breast WAT inflammation with aromatase level, adipocyte size, circulating inflammatory factors	WAT inflammation correlated with aromatase activity, adipocyte size, leptin, insulin, triglycerides
Dobbs et al.[126] 2016, Int J Cancer	Women (n = 22) Breast WAT (distant and adjacent to BC)	Confocal fluorescence microscopy and in-house computerized algorithm in MATLABVR ® (R2011b, MathWorksV®). Number of adipocytes: not specified	Assessing whether confocal microscopy can identify changes in adipocyte morphology and size near the margins of BC and pre-invasive lesions	Adipocytes adjacent to tumor margins are smaller in area compared to adipocytes far from the margins of BC and compared to adjacent to non-neoplastic collagenous stroma

Author (year, journal)	Study population/tissue type and location	Software/Number of adipocytes	Focus	Results
Iyengar et al.[76] 2015, Cancer Prev Res	Women (n = 237) Breast WAT (distant)	Linear Dimensional Tool in Canvas 11 (ACS Systems of America, Inc.) 30 or more adipocytes per patient	Association of breast WAT inflammation with BMI and menopause	WAT inflammation was significantly associated with menopausal status and BMI
Morris et al.[63] 2011, Cancer Prev Res	Obese women (n = 30) Breast WAT (distant)	Linear Dimensional Tool in Canvas 11 (ACS Systems of America, Inc.) 18 – 42 adipocytes per patient	Aromatase activity Adipocyte size Serum inflammation marker	The severity of breast inflammation correlated with both BMI and adipocyte size

Table 2. Clinical studies evaluating adipocytes in breast cancer patients.

Abbreviations: BC, breast cancer; BMI, body mass index; WAT, white adipose tissue; CRP, c-reactive protein; ALT, alanine aminotransferase

Aims and Objectives

The previous paragraphs summarized the major findings of the most influential studies which have shaped our understanding of the association of BC and obesity from different perspectives.
In line with the above, the overarching goal of the present research is the application of digital pathology to quantify mammary adiposity in the context of BC by focusing on the most abundant cell type of the adipose tissue, the adipocytes.

The present work was a pilot study to evaluate the feasibility of using digital pathology to measure adipocytes in H&E stained slides of breast tumour and to define criteria for subsequent analyses. We conducted our analysis on ten patients from a cohort of 66 post-menopausal ER^+ BC patients. At the time this work was completed, the analysis of the entire cohort was ongoing. Furthermore, several translational clinical trials developed from our group that were starting to enroll patients or that were in the last phase of approval, will use the adipocyte analysis developed in this work.

Our aims and objectives were as follows:

1. To test the accuracy of multiple software of digital analysis in order to identify the most suitable software tools for adipocyte analysis in BC.
2. To develop histopathological criteria for the recognition of distant and CAAs.
3. To evaluate possible associations between distant and CAAs and patient's adiposity measured as BMI.

The results presented in this thesis have been published on *The Breast*[133]

Digital analysis of distant and cancer associated mammary adipocytes

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Abstract

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 ten BC samples (n = 51 H&E slides) with HALO®. Distant adipocytes were defined as >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.

Introduction

Mammary adipose tissue (MAT) represents up to 56 percent of the total breast volume[134] and it is composed of different cell populations such as endothelial cells, fibroblasts, immune cells, pericytes, various stem and progenitor cells in addition to adipocytes[109]. Adipocytes are normally separated from the human mammary epithelium by a layer of specialized stroma in which the terminal ductal lobular units are embedded. This layer is disrupted during tumor initiation/growth, bringing the adipocytes in close contact with BC cells[87]. 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 acids release and immunomodulatory adipokine secretion[21]. Cancer cells can induce the delipidation of CAAs[70] 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[112]. CAAs can also cause resistance to radiotherapy[112] and to chemotherapeutic drugs through different mechanisms[44,135]. All these complex interactions between CAAs and cancer cells seem to be more pronounced in obese patients[87,136].

Obesity has been become increasingly prevalent in most parts of the world over the last decades[137]. Recent projections estimate that nearly 1 in 2 adults will probably be obese by 2030 in the US, and this proportion will even be more accentuated in women[23]. Obese patients mostly have a global dysfunctional state mainly characterized by metabolic and immunologic alterations. This leads to an increased risk for developing various cancers among which BC, especially estrogen receptor (ER)-positive BC in the post-menopausal population[65,138]. Additionally, obesity has been shown to negatively impact the prognosis of BC patients[28,36]. Iyengar and colleagues have observed that the majority of the obese BC patients have a chronic inflammatory state in the MAT[76]. This is characterized by the hypertrophy of adipocytes, the formation of Crown-Like Structures (CLS), which are dying or dead adipocytes surrounded by macrophages, elevated levels of the aromatase enzyme[78], and the presence of inflammatory cytokines such as IL-6[58]. Inflammation of the MAT could still be further modulated by physical activity, diet and drugs although data are limited so far[117,139]. Recently, Iyengar et al. demonstrated that even a subgroup of lean patients can present a status of chronic inflammation associated with the MAT[78], emphasizing the limitation of BMI to accurately capture patient's and mammary adiposity. Tools and methods are therefore needed to correctly identify and measure adipocytes and CAAs in order to quantify and characterize mammary adiposity. Intra-mammary comparison of the size of distant adipocytes and CAAs could further be used to quantify the magnitude of adipocyte delipidation.

So far, most studies that aimed to measure mammary adipocytes only considered the largest diameter of a relatively small number of carefully selected adipocytes distant from the tumor, without considering CAAs[58,75,76,78,140]. Acknowledging the limitation in the number of evaluable adipocytes with this manual/semimanual methods, several image analysis software have been developed to automize this process. An important challenge for

these software is to analyze CAAs, since they are close to or inter-mixed with other cell types. So far, extensive intra-mammary comparative studies between distant and CAAs using digital pathology have to the best of our knowledge not been performed yet.

In this study, we first compared the characteristics and outputs of three main image analysis software for identifying and measuring adipocytes (AdipoCount[129], Adiposoft[127], and HALO®). Then, using the best platform, we analyzed a series of BC tissues to measure distant adipocytes and CAAs and their potential association with the body mass index (BMI) of the patient.

Materials and Methods

Patients and slides

We selected a total of 51 H&E slides from ten 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) formalin-fixed paraffin-embedded 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/m^2) was categorized according to WHO criteria[26]. We considered only post-menopausal BC ER+ starting from the evidence that obesity is more prevalent in post-menopausal women and the link obesity-BC seems to be tighter in this population. 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 at diagnosis and two patients T3. 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).

Digital Pathology and software analysis

H&E stained tissue slides were scanned using a Nanozoomer digital slide scanner (c10730-12, 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 (Vacuole 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® and 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[76]. Based on microscopic evidence and literature[126], 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). The areas to be analyzed were drawn by E.I. under 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.

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 recognized 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[141] 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.

Results

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 Fig. 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 was characterized by a high number of false negatives,

since it failed to detect 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.

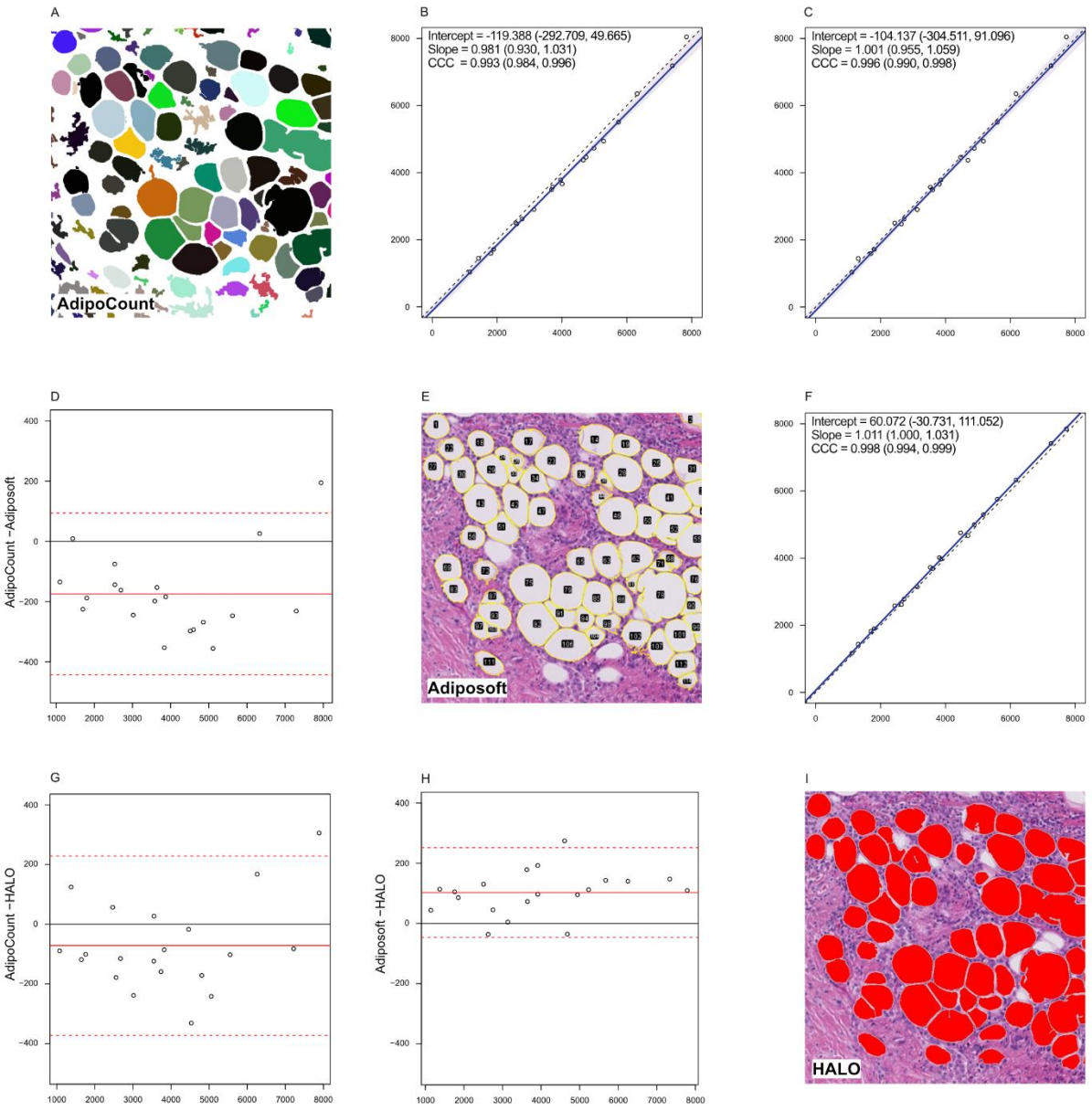

Figure 4. Comparison of adipocytes measurements by AdipoCount, Adiposoft and HALO.

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.

Test Image	Software	Manual Count	Software Count	TP	FP	FN	TN	Sensitivity	Specificity	p-value sensitivity	p-value specificity
Slide 1		100									
	AdipoCount		123	87	36	13	13	87.00%	26.53%	0.002	<0.001
	Adiposoft		114	92	22	8	33	92.00%	60.00%	0.132	<0.001
	HALO®		98	97	1	3	48	97.00%	97.96%	-	-
Slide 2		109									
	AdipoCount		140	105	35	4	23	96.33%	39.66%	1	<0.001
	Adiposoft		50	28	22	81	39	25.69%	63.93%	<0.001	<0.001
	HALO®		107	105	2	4	56	96.33%	96.55%	-	-
Slide 3		102									
	AdipoCount		129	99	30	3	22	97.06%	42.31%	0.157	<0.001
	Adiposoft		134	94	40	8	25	92.16%	38.46%	0.008	<0.001
	HALO®		102	101	1	1	50	99.02%	98.04%	-	-

Table 3. Comparison of the counting results of AdipoCount, Adiposoft and HALO®.

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

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 Fig. 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.

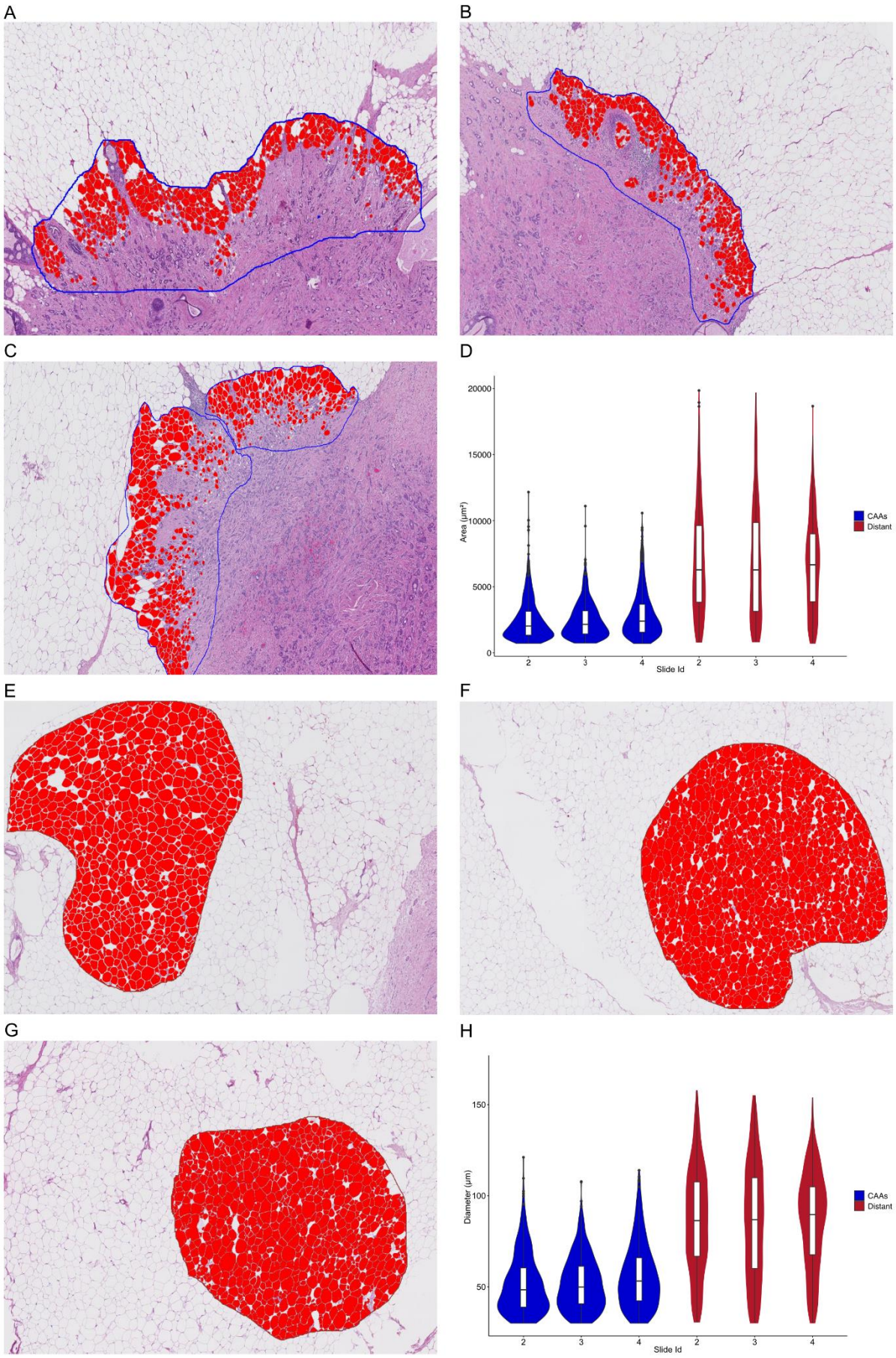
A
B
C
D
20000
15000
10000
5000
0
Area (µm²)
2
3
4
Slide Id
CAAs
Distant
E
F
G
H
150
100
50
Diameter (µm)

Figure 5. 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.

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 μm^2 for CAAs and 5537.4 μm^2 for distant adipocytes and the median area was 1881.3 μm^2 and 4853.0 μm^2, 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: 2.62, IQR: 2.15 – 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 quantile 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.

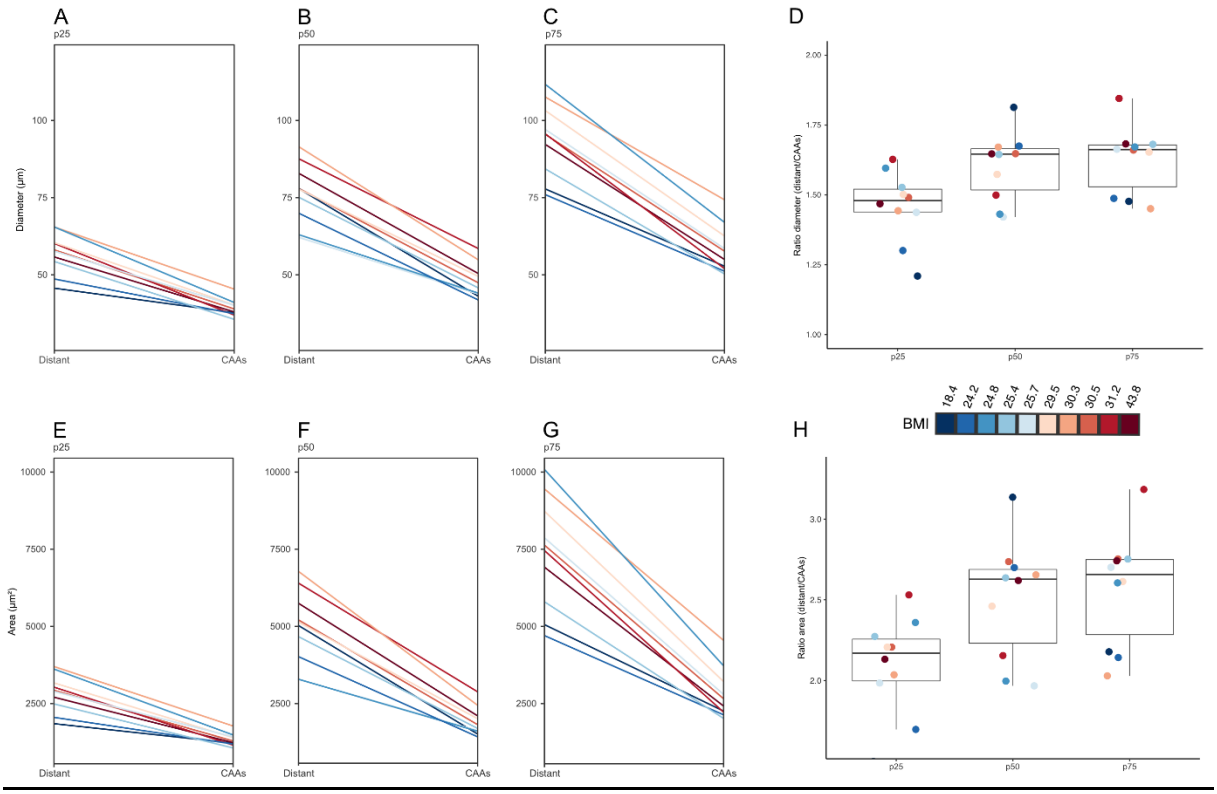

Figure 6. Plots of the size difference between distant and cancer-associated adipocytes.

Parallel plots (A, B, C) with associated boxplots (D) show the variations of adipocytes diameters between distant and cancer-associated adipocytes at 25th percentile (A), 50th percentile (B), and 75th percentile (C). Below, parallel plots (E, F, G) with associated boxplots (H) show the variations of adipocyte areas between distant and CAAs at 25th percentile (E), 50th percentile (F), and 75th percentile (G). Each line and each dot correspond to a patient and are colored according to ascending BMI. For each percentile, the p-value from the paired Wilcoxon tests was 0.002 for both diameters and areas. CAAs: cancer-associated adipocytes.

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 Figure 4. We observed a correlation between BMI as a categorical variable (lean, overweight or obese) and adipocyte measurements with regard to area (Kendall's tau = 0.80, p = 0.004 for CAAs, and Kendall's tau = 0.65, p = 0.021, for distant adipocytes) and diameter (Kendall's tau = 0.75 p = 0.007, Kendall's tau = 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 (rho = 0.89 p = 0.001; rho = 0.71 p= 0.027, respectively) and diameter (rho = 0.87 p= 0.002; rho = 0.65 p= 0.049, respectively).

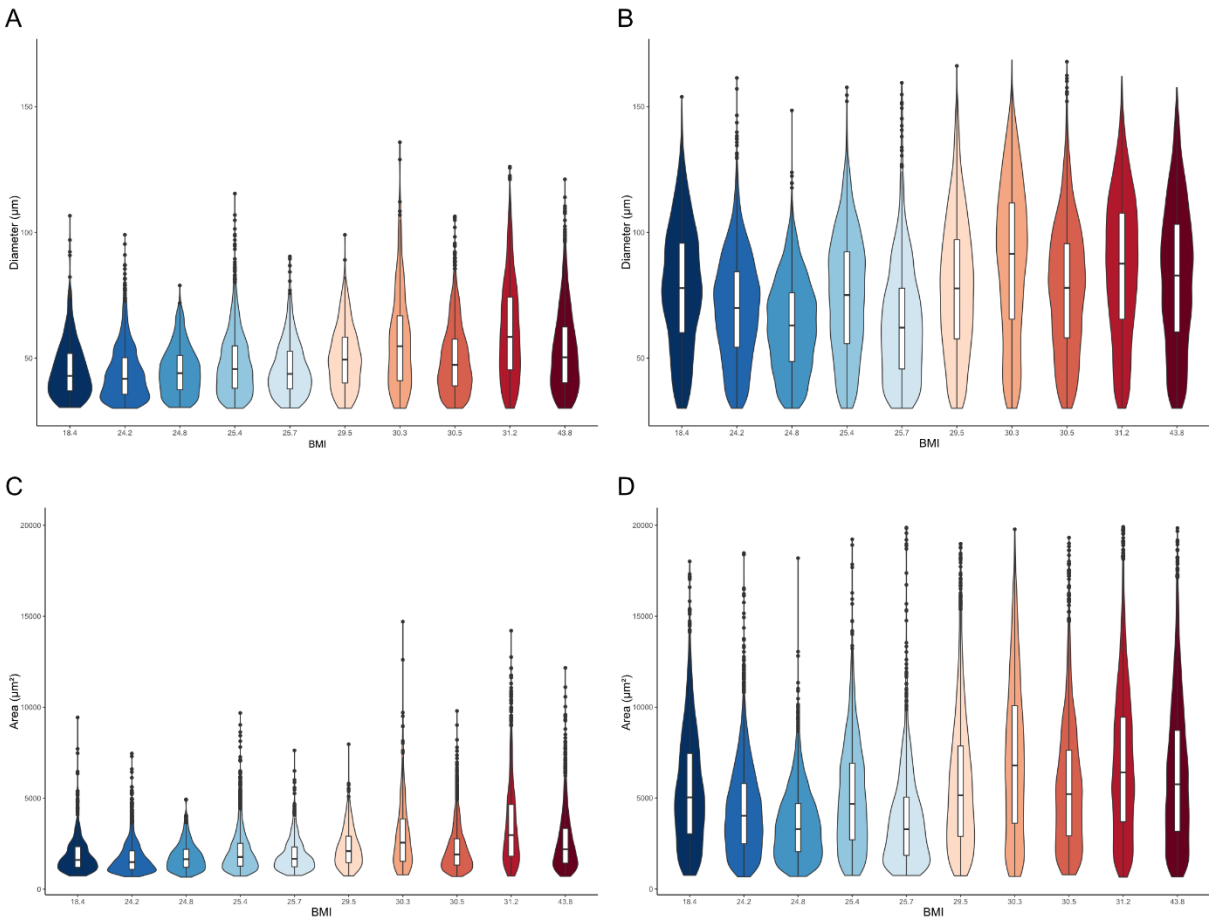

Figure 7. Distribution of adipocyte sizes in ten patients according to BMI.

Violin plots (A, B) including boxplot that represents the 25th, median and 75th percentiles of diameters for CAAs (A) and distant adipocytes (B) in ten patients ordered by ascending BMI. Violin plots (C, D) including boxplot that represents the 25th, median and 75th percentiles of areas for CAAs (C) and distant adipocytes (D) in ten patients ordered by ascending BMI. The violin plots outlines illustrate kernel probability density, i.e. the width of the colored area represents the proportion of the adipocyte diameters (A, B) and adipocytes areas (C, D). The same color code as in Fig. 3 has been used.

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 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[131] 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[113,142]. 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[143]. 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[144,145]. 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[58]. 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[58]. 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[76], but also larger CAAs.

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.

Supplementary Methods

Statistical analysis

A sample size analysis and a power calculation were performed: 1a) to determine the sample size to assess sensitivity and specificity of the software for recognizing and counting the adipocytes, 1b) to determine the number of adipocytes for the software comparison regarding areas and diameters, and, 2) to compare the distribution of the diameter and area of distant and CAAs according to their means. Concerning the sensitivity and specificity analyses a sample size of 150 objects, of which 100 are adipocytes lead to a power of 90% and 70% with a 0.05 significance level (two-sided) to show a 15% difference in sensitivity and specificity respectively given a proportion of discordant pairs of 20% according to the tests for paired sensitivities PASS procedure. For the comparison of the software regarding adipocyte diameter and area, measuring 20 adipocytes results in 84% power with a 0.05 significance level (one-sided) according to the Lin's concordance correlation coefficient PASS procedure. For point 2, a sample size of 500 adipocytes in each of the two categories distant and CAAs should achieve a power of 90% at a level of significance of 0.05 (two-sided) to detect a difference in means of 0.1 given a standard deviation of 0.4 and 0.5 for log transformed area distribution in CAAs and distant adipocytes respectively according to the two-sample T-tests allowing unequal variance PASS procedure. In term of variance, the same sample size should achieve a power of 90% at level of significance of 0.05 in a F test to detect a ratio of 0.74 from CAAs to distant adipocytes when setting their variances to 0.4 and 0.5 respectively in the tests for two variances PASS procedure. Computation were performed using PASS version 20.0.2.

Supplementary Figures

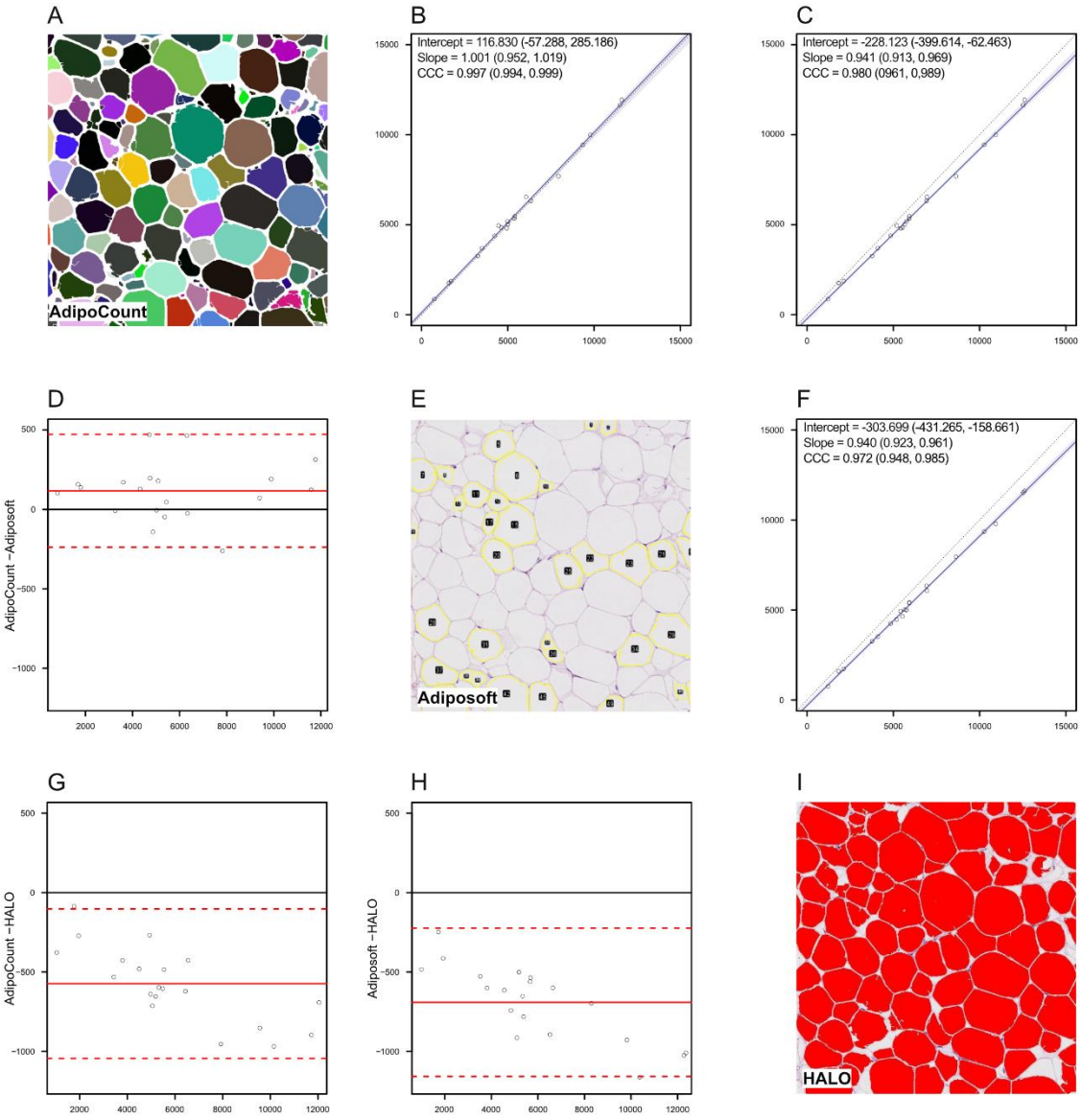

Figure 8. Comparison of adipocytes measurements by AdipoCount, Adiposoft and HALO®.

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 regression 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.

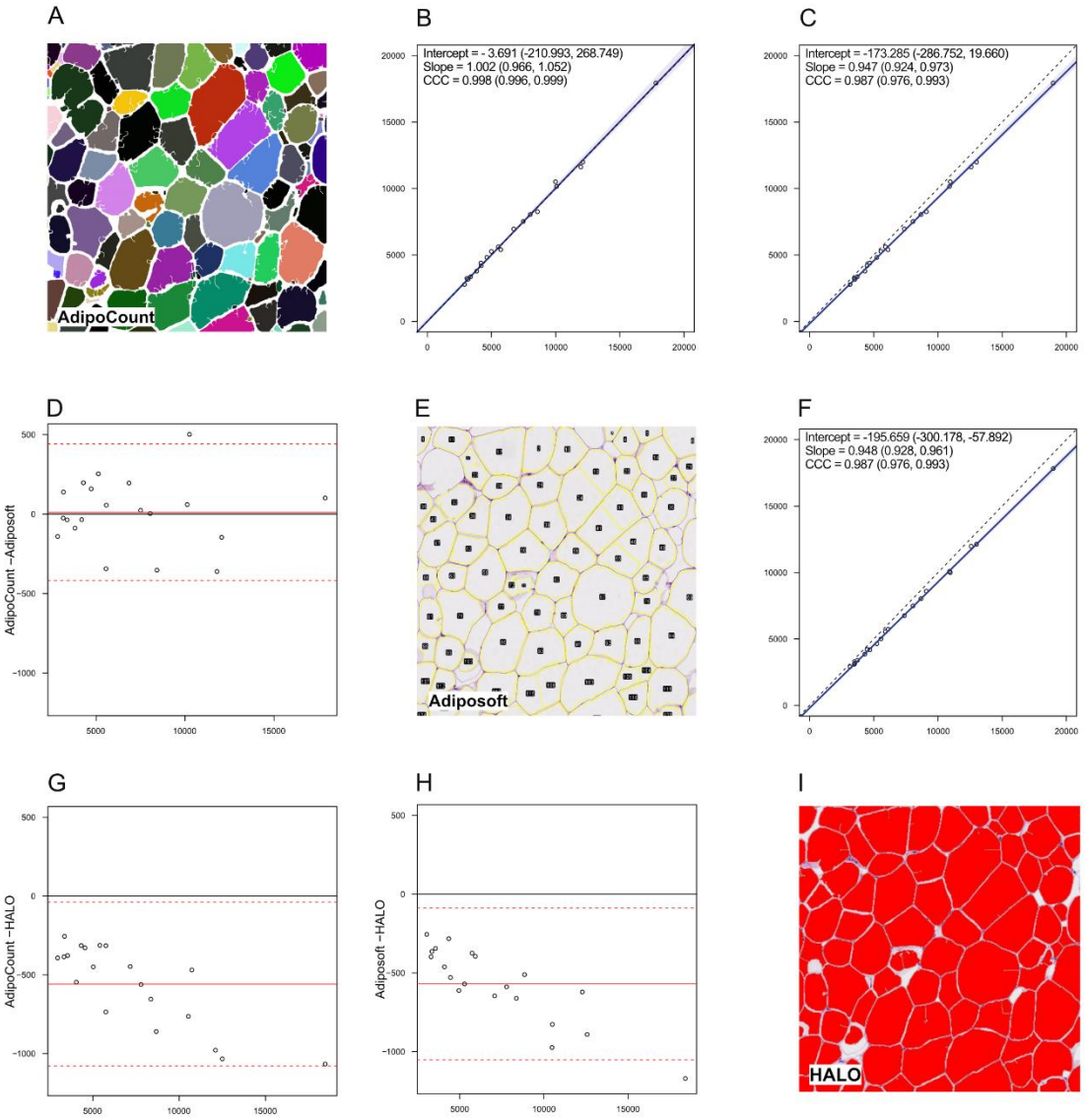

Figure 9. Comparison of adipocytes measurements by AdipoCount, Adiposoft and HALO®.

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 regression 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

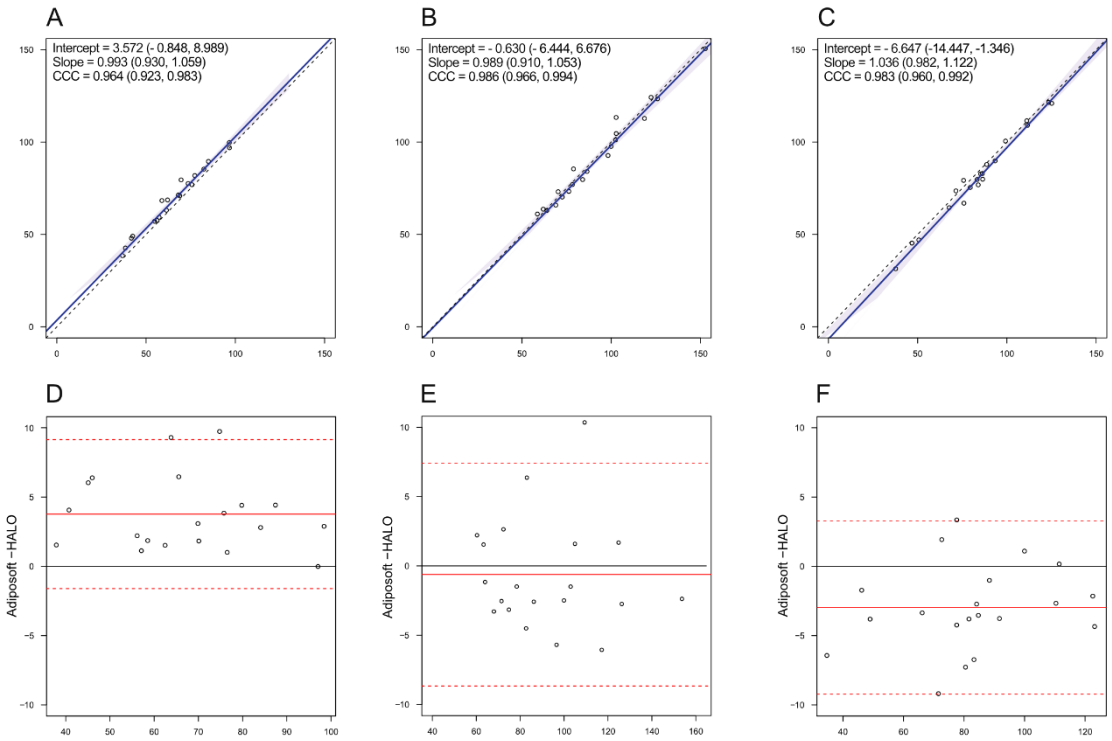

Figure 10. Comparison of adipocytes measurements by Adiposoft and HALO®.

Passing–Bablok regression (A,B,C) of adipocyte diameters of Adiposoft vs HALO® of three test slides. 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. Bland–Altman plots (D,E,F) showing software agreement between two software using diameter measurement of twenty adipocytes. For each comparison, the averaged diameter of each adipocyte calculated by software (x axis) is plotted against the difference between the two diameter 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.

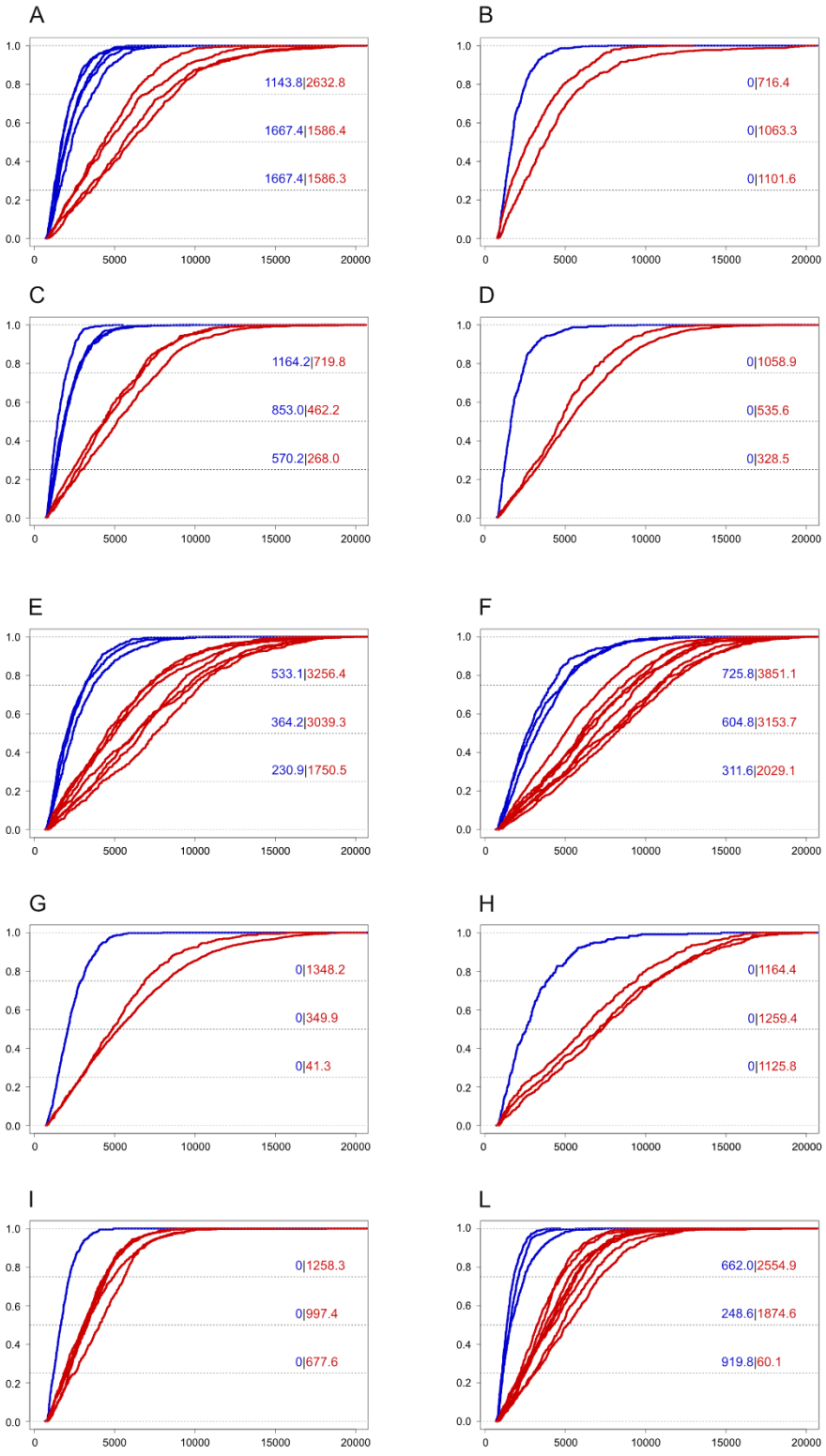

Figure 11. Cumulative distribution of CAAs and distant adipocytes area.

Each panel is dedicated to a patient, each curve represents an area of 500 adipocytes. Blue and red curves depict the cumulative distributions of area for CAAs and distant adipocytes respectively. The horizontal dashed lines represent the 25th, median and 75th percentiles from bottom to top respectively. Values provided on each dashed line provide the maximal range observed among the CAAs (blue value) and the distant adipocytes (red value) for the percentile of interest.

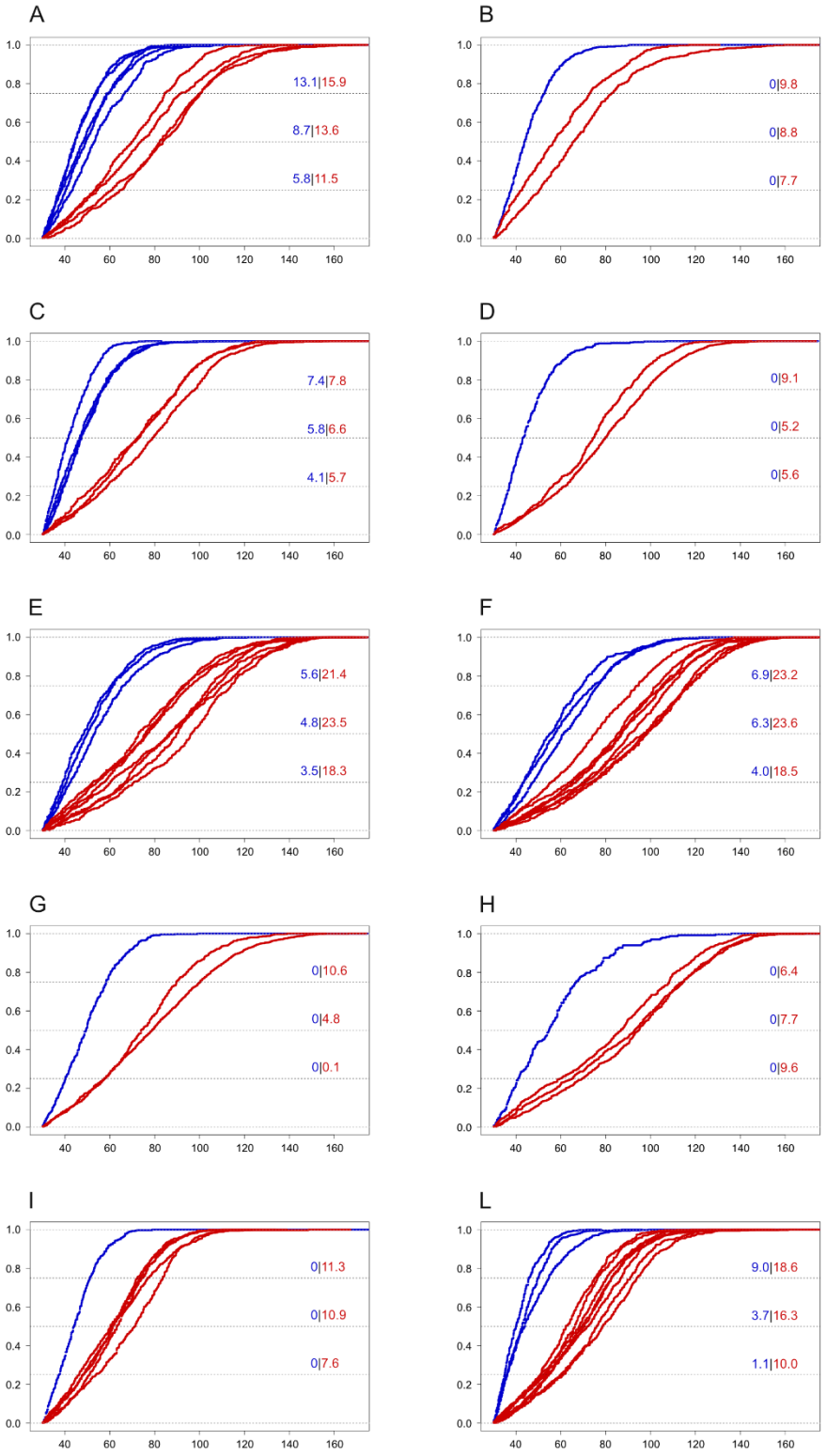

Figure 12. Cumulative distribution of CAAs and distant adipocytes diameter.

Each panel is dedicated to a patient, each curve represents an area of 500 adipocytes. Blue and red curves depict the cumulative distributions of diameter for CAAs and distant adipocytes respectively. The horizontal dashed lines represent the 25th, median and 75th percentiles from bottom to top respectively. Values provided on each dashed line provide the maximal range observed among the CAAs (blue value) and the distant adipocytes (red value) for the percentile of interest.

Discussion

The inflammatory TME of BC is an important driving factor for the progression of the disease and may become a potential target for new and personalized treatments, especially in overweight and obese patients. Most of the theoretical basis for studying the role of adipocytes originates from epidemiological and clinical studies that documented worse outcomes in obese BC patients, with potential differences according to the molecular subtype of tumor and the menopausal status of the patient[30,32]. Although adipocytes may be one of the mediator between obesity and BC, the specific mechanisms still remain to be elucidated[117]. It has already been described that mammary adipocytes tend to be larger (hypertrophied) in obese as compared to lean patients. Dirat and colleagues also reported the plasticity of these cells, by describing for the first time the existence of the so-called CAAs in human cancers[113]. However, so far, there are no defined criteria and methods to quantify mammary adipocytes and CAAs in BC samples. For this reason, we conducted our research on a small group of post-menopausal ER^+ BC women, with the main aim of evaluating the feasibility of using digital pathology to measure adipocytes and CAAs in BC. The analysis of these ten patients sets the standard for digital analysis of adipocytes in all our future studies. At the time of writing, we are concluding the analysis on the entire cohort from which these ten patients are taken, and we will submit the results for publication soon.

The results of the present research are threefold. First, we identified the best software to identify and measure adipocytes in the MAT of BC. We observed that one of the most important limitation was the low quality of the H&E staining or the damaged AT as previously described. For that reason, to have an accurate representation of AT and minimize bias, it is important to exclude slides with damaged AT. In addition, we identified a number of 500 adipocytes as sufficient number to have a good representation of the adiposity of a patient minimizing the intra-patient and intra-slide heterogeneity. We established important criteria for the recognition of CAAs, defining them as located in the first 3-5 lines from the IF and no more than 2 mm deeper in the tumor tissue. We consider that our criteria have a potential impact on other research projects. Furthermore, no previous studies directly measured CAAs at the IF of BC through image analysis software. To our knowledge, we are the first to perform this type of analysis in BC comparing distant and CAAs. Second, we demonstrated that CAAs

are smaller than distant adipocytes, reflecting the delipidation process undergone by CAAs. Third, BMI of the patient was associated with the size of distant adipocytes and CAAs. Of interest, we observed that adipocyte distributions were characterized by a certain overlap between them, especially in the same BMI category, supporting our concept that BMI might not be a reliable estimator of fat cells properties. Moreover, we did not find any difference in adipocytes size according to the histology (ductal vs lobular, results not shown), possibly also due to the small number of the samples that were evaluated. Therefore, open questions remain, such as whether obesity amplifies the metabolic crosstalk between CAAs tumor cells in all BC subtypes and histological subtypes and what the determinants are of this adipocytes size reduction.

There are some limitations in this study that could be addressed in future research. First, the limited small sample size that prevented us to investigate additional associations with clinical and pathological variables. However, we are currently exploring these association on the entire cohort and the results will be presented soon. Second, we did not perform any immunohistochemical or histochemical analysis on our samples. Hence, we identified CAAs exclusively according to morphological and topographic criteria. Third, our cohort was composed of post-menopausal ER^+ BC women treated with neoadjuvant therapy with letrozole. Therefore, we cannot exclude an interaction of letrozole with adipocytes in determining a change in their size. In addition, we also cannot exclude an interaction of other types of treatment used by the patients with adipocytes size. The analysis of the pre-treatment biopsies was not possible due to small quantity of AT presented on those samples. Also, the intra-tumoral population of adipocytes was not considered because it was not present in all the samples, but we will further explore it in future studies. In order to have sufficient MAT for this type of analysis, future studies on mammary adipocytes could benefit from adaptations in the surgical breast pathology pipeline. Finally, we recognize the exploratory nature of this work; however, our findings will be the basis to extend the application of digital pathology to measure adipocytes on large clinical trials.

Conclusions and Future Perspectives

In parallel to the present research, I took part in another work where we assessed breast adipose features using digital pathology in a case-control study nested population-based

ductal carcinoma in situ (DCIS) cohort[111]. We showed that women with DCIS and large adipocytes size might have an increased risk of subsequent ipsilateral invasive BC compared to women with DCIS and smaller adipocytes. Although BMI was missing for several patients, this study demonstrated that adipocyte size associated to the presence of CLS, could be a reliable indicator of mammary adiposity and MAT inflammation. This finding might also be relevant for the group of metabolically obese normal-weight women in which BMI is not able to discriminate the inflamed MAT[78]. Taken together, these findings have important implications showing that an altered AT micro-environment preexisting to the tumor might already be an active contributor to BC initiation adding insights to improve the follow-up of women with DCIS.

Another important aspect that should be considered for the future is our currently poor understanding of adipocyte heterogeneity and functional specialization. A few studies, mostly in mice, through single-cell genomics analyzed normal AT identifying an homogenous cell population of ASCs[146] whereas instead there seems to be different subpopulations of pre-adipocytes and mature adipocytes[147–149]. Currently, none of the large-scale molecular characterization studies of BC have considered patient's adiposity. To overcome this lack of knowledge, our research group have recently started a clinical trial (National Library of Medicine [NLM], NCT04200768)[150] with the aims, using single-nuclei and lipidomics data, to characterize the micro- and macroenvironment of BC according to patient's adiposity in different histological and molecular subtypes. Furthermore, we will use multi-level adiposity measurements among which the measurement of adipocytes size through digital pathology that it was validate with the work described in this thesis. Of interest, we will conduct our analysis also on normal mammary tissue samples as controls in order to dissect the transcriptome of mammary adipocytes (progenitors, mature and CAAs) also according to the presence or not of cancer.

Another study has been recently started from our research group (National Library of Medicine [NLM], NCT04531696)[151]. This study consists on extensive post-mortem multi-level and multi-region sample analysis of women who died for metastatic BC in order to unravel BC evolution, biology, heterogeneity, and treatment resistance. One of the novelty of this study is that digital pathology and molecular analyses of adipocyte will be performed on the different metastatic lesions collected during the autopsy. The initial pilot phase with the

analysis of the first two patients is completed and we were able to perform CAAs analysis on various metastatic sites.

Finally, we believe that our analysis can be expanded to the AT TME of other cancer types. For example, several studies described bone marrow adipose tissue (BMAT) as a niche for tumor cells, for both solid and hematologic malignancies[152,153]. Bones are one of the favorite site of metastases in HR^+ BC, therefore it will be important to study how adipocytes of BMAT contributes to creating a unique metastatic niche[154].

To conclude, while the work presented here aims to characterize mammary adipocytes in BC patients using digital pathology, future studies with larger number of patients combining this type of analysis with multi-omics approaches are necessary to provide further insights in the link between obesity and BC.

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Statements

Personal Contribution

The author of this doctoral thesis was involved in the concept and design of the present study, sample analysis by digital pathology, statistical analysis, interpretation of the data, and writing of the manuscript.

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Conflicts of Interest

The author of this doctoral thesis and all the contributing authors declare no conflicts of interest.

List of Publications

Almekinders MM, Schaapveld M, Thijssen B, Visser LL, Bismeijer T, Sanders J, **Isnaldi E**, Hofland I,Mertz M, Wessels LFA, Broeks A, Hooijberg E, Zwart W, Lips EH, Desmedt C, Wesseling J. Association of Breast Adipocyte Size and Cyclooxygenase-2 expression with Risk of Subsequent Ipsilateral Invasive Breast Cancer in Patients with Ductal Carcinoma In Situ. Npj Breast Cancer 2021;7:31. https://doi.org/10.1038/s41523-021-00232-w

The abovementioned research paper represented a part of the research focus of my PhD.

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