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Title:

Biopathological characterization and grade assessment of breast cancer with 3D multiparametric ultrasound combining Shear Wave Elastography and Backscatter Tensor Imaging.

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1 **Abstract:**

2 **Objective:** Despite recent improvements in medical imaging, the final diagnosis and biopathological
3 characterization of breast cancers currently still requires biopsies. Ultrasound is commonly used for
4 clinical examination of breast masses. B-mode and Shear Wave Elastography (SWE) are already
5 widely used to detect suspicious masses and differentiate benign lesions from cancers. But additional
6 ultrasound modalities such as Backscatter Tensor Imaging (BTI) could provide relevant biomarkers
7 related to tissue organization. Here we present a 3D multiparametric ultrasound approach applied to
8 breast carcinomas aiming to (i) validate the ability of BTI to reveal the underlying organization of
9 collagen fibers and (ii) assess the complementarity of SWE and BTI to reveal biopathological features
10 of diagnostic interest.

11 **Methods:** 3D SWE and BTI were performed *ex vivo* on 64 human breast carcinoma samples using a
12 linear ultrasound probe moved by a set of motors. In this paper, we present a 3D multiparametric
13 representation of the breast masses and quantitative measurements combining B-mode, SWE and
14 BTI.

15 **Results and Conclusion:** Our results show for the first time that BTI can capture the orientation of
16 the collagen fibers around tumors. BTI was found to be a relevant marker for assessing cancer stages
17 revealing a more tangent tissue orientation for *in situ* carcinomas than for invasive cancers. In
18 invasive cases, the combination of BTI and SWE parameters allowed for classification of invasive
19 tumors with respect to their grade with an accuracy of 95.7%. This demonstrates the potential of
20 such a 3D multiparametric ultrasound imaging approach for biopathological characterization of
21 breast tumors.

22 **Keywords:**

23 Backscatter Tensor Imaging, Breast cancer, Collagen fibers, Shear Wave Elastography, Tissue
24 characterization, Ultrasonic imaging

1 Introduction

2 Breast cancer is the most common cancer in the world with 2 261 000 new cases and 685 000
3 deaths in 2020 (1). In the late 1980s, general screening programs started to be implemented across
4 the world. These programs combined with medical imaging improvements have continuously helped
5 detect breast cancers at earlier stages (2).

6 One of the main remaining challenge of research in the field is to reduce the screening false
7 positive rate. Indeed, many benign tumors are classified as indeterminate (Breast Imaging-Reporting
8 and Data System - BI-RADS category 4) and therefore require a biopsy. This percutaneous tissue
9 sampling is required to establish a definitive diagnosis. It is reliable but suffers from a high rate of
10 benign results (only 28.6% lead to a diagnosis of cancer (3)) causing women unnecessary stress and a
11 possible loss of confidence in breast cancer screening.

12 Another challenge for the coming years lies in the diagnostic step. Radiologists need non-
13 invasive imaging modalities to characterize breast cancers and help them make faster treatment
14 decisions without the need to wait for the biopsy's result. In this study, we investigated how a 3D
15 multiparametric ultrasound approach could better characterize the biopathological signatures in
16 breast cancers.

17 Ultrasound imaging is commonly used for women with abnormal clinical examination, a
18 detected lesion on mammograms or with dense breasts. Several ultrasound modes have potential for
19 improving breast cancer diagnosis. Shear Wave Elastography (SWE) is already widely available in
20 clinics to differentiate benign from malignant masses (4,5) and is now evaluated for its ability to
21 characterize carcinoma invasiveness (6) and immunohistochemical subtypes (7). Other techniques
22 have been studied to assess vascularization properties using Doppler imaging, Contrast-Enhanced
23 ultrasound (CEUS) (8), Ultrasound Localization Microscopy (9) or Superb Microvascular Imaging (10).
24 Some other recent techniques intend to explore the microscopic tissue organization based on the

1 coherence of the backscattered ultrasonic wave such as Short-Lag Spatial Coherence (11) or
2 Backscattered Tensor Imaging (BTI) (12).

3 Among the different characteristics of tumors, we decided to have a particular focus on the
4 organization of the collagen fibers which plays an important role in cancer development and its
5 spread (13,14). In particular, the correlation between the collagen properties and the survival of
6 breast carcinoma patients (15,16), the invasion of cancer cells (17), and the recurrence of ductal
7 carcinoma *in situ* (DCIS) (15,17,18) have been reported by different studies. At least 8 different tumor
8 associated collagen signatures (TACS) have been characterized at different scales ranging from
9 100 μm to a few millimeters (19). However, current TACS assessment techniques rely on optical
10 imaging such as image analysis from histological staining, second harmonic generation imaging, two-
11 photon microscopy and fluorescence (20). These methods either require biopsies or are limited to
12 superficial tissues. Recent works in the magnetic resonance (MR) field have shown that diffusion
13 tensor imaging (21) correlates with collagen properties. But MR imaging (MRI) remains an expensive
14 method with low availability. More importantly, it is rarely used for breast cancer screening, as
15 opposed to ultrasound. However, to our knowledge, no work has validated the ability of ultrasound
16 to track the collagen organization in breast tumors.

17 3D ultrasound has been used to study complex tumor structures (22–24). It minimizes some
18 of the most important disadvantages of ultrasound imaging, namely the operator-dependence for
19 the positioning of the probe when selecting an imaging plane. Matrix-array probes (fully-addressed,
20 sparse or with different organizations of the transducers) are being investigated by various groups on
21 a diversity of applications (25–28). However, such probes generally lack sensitivity and require very
22 large ultrasound systems, which can be unsuitable for clinical use. To address these drawbacks, we
23 decided to investigate multiple 3D ultrasound techniques using a linear probe and a set of motors to
24 scan our volume of interest with a compact set-up (12).

1 Our approach includes 3D B-mode imaging, SWE and BTI. SWE estimates the local stiffness of
2 a medium by remotely inducing a shear wave in tissues and measuring its local speed. The stiffer soft
3 tissues are, the faster shear waves propagate through them. BTI is a modality which relies on the
4 spatial coherence of the backscattered echo of the ultrasonic wave. It was first introduced by our
5 group (29,30). BTI is used to measure the coherence (Co , i.e. the degree of organisation), the
6 fractional anisotropy (FA , i.e. the degree or anisotropy) and the local orientation (α) of a medium in
7 planes parallel to the probe (12).

8 For this study, our main objectives were to build a 3D multiparametric representation of
9 tumors using these modalities, to validate the link between BTI and collagen organization and to
10 investigate new relevant biomarkers for diagnosis, combining SWE and BTI information. This work
11 may be useful in the future for *in vivo* research, to prevent unnecessary biopsies and help clinicians
12 select the optimal treatment.

13 **Materials and Methods**

14 **Protocol for patient inclusion**

15 The data acquisition took place at the Institut Curie (Paris, France). All participants provided
16 their written and informed consent granting access to their medical data for research purposes
17 according to French regulations. An internal review committee for studies on patient data (CRI data,
18 Institut Curie) authorized relevant clinical data use and transfer. The institutional Breast Review
19 Board of the Institut Curie approved all of the procedures.

20 We included all total and partial mastectomies without neoadjuvant chemotherapy
21 performed during the seven weeks of the study, except those required by the pathologists for
22 immediate diagnosis. The tumors were retrospectively included in the study if they were detectable
23 by an experienced radiologist (A.T.) on B-mode imaging (details of this and the outlining process are
24 provided below). We thus included 65 tumors, one of which had to be removed of our study because
25 the tissue on the corresponding histological slide was too fragmented to perform our image analysis

1 (see section II D.). In conclusion, our analysis included 64 validated cases, including Ductal
2 Carcinomas In Situ (DCIS), Invasive Carcinomas of No Special Type (IC-NST), Invasive Lobular
3 Carcinomas (ILC), and other types (mucinous carcinoma, papillary carcinoma etc.). A description of
4 the cohort including information on Elston-Ellis (EE) grade, lymph node status, and Progesterone
5 receptor status, extracted from the anonymized medical reports of the pathologist team for the 52
6 validated invasive cases can be found in Table 1.

7 **Ultrasound acquisition**

8 *Acquisition setup*

9 The acquisitions were performed using a 256-channel Vantage ultrasound system
10 (Verasonics, USA) equipped with a 128-element linear transducer (L7-4, Philips – ATL, Netherlands,
11 pitch 0.298 mm, bandwidth 4-7 MHz), working at its central frequency (5.2 MHz). The elevational
12 focal distance of the probe was 25 mm. The probe was fixed to a motorized setup enabling three
13 degrees of translation (X-Y-Z) (VT 80, Physik Instrumente, Germany) and one degree of rotation (θ in
14 the XY plane) (DT 80, Physik Instrumente, Germany).

15 Breast tissue collected from patients was placed in a plastic bag (TissueSAFE®, Milestone
16 Medical, Italy) and vacuum-packed right after surgery. The imaging acquisition took place 30 min to
17 4 h after surgery (except a few cases imaged in the morning 18 h after surgery). Tumor location was
18 identified on the specimen on the basis of the patient report and palpation of the specimen. The
19 tissue was then immersed in water at room temperature to ensure the acoustic coupling and allow
20 the probe to move over the sample without touching it (Figure 1).

21 By convention, the Z-axis is the vertical axis. The X axis is aligned with the lateral dimension
22 of the probe and the Y axis corresponds to the elevation when $\theta = 0^\circ$ (initial position of the probe),
23 see Figure 1 a).

24 *Estimation of the speed of sound*

1 Before each multiparametric acquisition, the speed of sound was estimated at the center of
2 the probe using the method described by Imbault et al. (31):

3 (i) the probe emitted 101 plane waves (tilted with a 0.4° angle step from -20° to $+20^\circ$)

4 (ii) the coherence of the centerline of our image was calculated for different speed of sound
5 values.

6 The sound speed in the medium was estimated equal to the one resulting in the maximum
7 coherence during (ii). The estimated speed was then used in the beamforming process.

8 *Tomographic 3D B-mode*

9 At each position of the probe, the B-mode was acquired using coherent plane-wave
10 compounding with 101 plane waves tilted with a 0.4° angle step from -20° to $+20^\circ$. In reception, a
11 constant F-number was chosen equal to 1.5.

12 The 3D B-mode was then reconstructed averaging all the B-mode planes obtained during the
13 Backscatter Tensor Imaging (BTI) scan (see below).

14 *3D Shear Wave Elastography (SWE)*

15 The probe was centered on the estimated location of the tumor. Then, we performed three
16 linear scans at 0° , 45° and 90° (cf. **Erreur ! Source du renvoi introuvable.** b) and c)). Each scan
17 consisted in 21 to 41 acquisitions (median 27 acquisitions) depending on the tumor size, with a
18 constant 1 mm step. At each position, we generated sequentially seven shear waves linearly
19 distributed every 4 mm along the lateral axis. Each shear wave was induced by five focused acoustic
20 radiation force pulses of $80 \mu\text{s}$ at 60 V with a duty cycle of 100% and an F-number of 1.5. The shear
21 waves were imaged using ultrafast imaging with 26 frames at 2 516 Hz. A directional filter was used
22 to avoid potential reflection artefacts, as described in (32). For each frame, five plane waves were
23 emitted with a 4° step ranging from -8° to $+8^\circ$. The 3D SWE was reconstructed by averaging all the
24 SWE planes in the different orientations.

1 3D Backscatter Tensor Imaging (BTI)

2 3D BTI was performed over a cylindrical field of view (diameter 25 mm, depth 35 mm) using a
3 linear probe with an optimized acquisition methodology (12) to reduce the acquisition time
4 (~20 min).

5 3D BTI provides three features: the coherence (*Co*, i.e. the degree of organization), the
6 fractional anisotropy (*FA*, i.e. the degree of anisotropy) and the local orientation (α) of the scatterers
7 in the tissue in planes parallel to the probe. The method to obtain these features is explained in
8 details in a previous paper (12).

9 Using these features, we defined a score quantifying to which degree the tissue orientation
10 around the tumor is tangent to the tumor border (cf. **Erreur ! Source du renvoi introuvable.**). This
11 was done by comparing the orientation α measured by BTI to the local orientation of the normal to
12 the tumor border. For each voxel j outside the tumor, this local orientation of the normal to border
13 Θ_j was calculated as a contribution of the normal orientations θ_k of all border voxels k , weighted by
14 the opposite of their distance $d(j, k)$ to the voxel of interest:

$$15 \quad \Theta_j = \text{arg} \left(\sum_k e^{-d(j,k)} e^{i\theta_k} \right) \quad \text{Eq. 1}$$

16 The final score was then calculated for a given voxel outside the tumor as:

$$17 \quad (S_{\text{tangent}}^{BTI})_j = |FA_j \times \sin(\alpha_j - \Theta_j)| \quad \text{Eq.2}$$

18 where *FA* is the local fractional anisotropy used to promote the scores corresponding to high
19 anisotropy regions only, expected to correspond to fibers or oriented tissue.

20 The *FA* weighting was taken into account when averaging these scores in a ROI, by dividing
21 the sum of scores in the ROI by the sum of corresponding *FA* values. This methodology produced
22 “tangent score” maps outside the tumor with scores close to 0 for a voxel in which the BTI
23 orientation is normal to the local tumor border (“radial” organization) and to 1 when it is tangent to

1 the local tumor (“enveloping” organization). Note that this score was calculated in-plane (as BTI
2 orientations were only available in XY directions) on all Z-planes containing some part of the tumor
3 except the upper and lower planes, to avoid edge effects.

4 *Multiparametric 3D representation*

5 The 3D representations of the tumors were reconstructed from the B-mode values in the
6 entire field of view, the SWE in the tumor (*union*) and the orientation and FA of the BTI in the tumor
7 *border* using Houdini FX Version 19.0.531 (SideFX, Toronto, Ontario, Canada). The zones in italic are
8 defined below.

9 **Tumor outlines on B-mode images**

10 An experienced radiologist (A.T.) delineated the tumors retrospectively on two perpendicular
11 B-mode views with a 1 mm step on a custom Matlab-based application. The 3D volumes were
12 extracted using the “alphashape” function of Matlab (The MathWorks, Inc., Natick, Massachusetts,
13 United States). The *intersection* and the *union* of these two volumes were used as references to
14 create three additional volumes of interest named *center* (0.5 homothety of the *intersection*), *border*
15 (*union* \pm 1 mm) and *peritumoral area* (+ 5 mm around the union), see Figure 3. Lastly, the *distant*
16 *tissues* correspond to the tissues located further away than the *peritumoral area*.

17 **Histological analysis**

18 *Tumor outlines on histological sections*

19 The procedures were similar to the standard analysis performed by the Institut Curie for their
20 clinical practice. After imaging by our protocol and examination by the pathologists’ team, all tumors
21 were post-fixed in 10% formaldehyde for 36 h and processed in formalin fixed paraffin embedded
22 (FFPE) blocks. Thin sections (3 μ m) were cut and stained with Mayer’s hematoxylin (Dako,
23 Histological staining reagent S3309, Santa Clara, CA, USA), eosin (RAL Diagnostics, Martillac, France),
24 and saffron (RAL Diagnostics), and were imaged with a numeric microscope DOM-1001 (RWD, San
25 Diego, California, USA). The saffron gives an orange color to the collagen fibers.

1 For all the validated cases, one representative section was selected by a pathologist (L.D.)
 2 who validated the manually segmented tumor area. Similarly to the ultrasound, the *peritumoral area*
 3 was defined as the area around the tumor contour up to + 5 mm and the *border* corresponded to the
 4 area comprised in a 2 mm band around the edge of the tumor.

5
 6 *Orientation estimation*

7 We estimated the local orientation and intensity of the collagen fibers from histological
 8 sections using a custom image analysis pipeline described on Figure 4. First, the collagen fibers were
 9 segmented using a threshold in the hsv color space (Figure 4 a) and b)). Then we calculated the
 10 Radon transform on every patch of 1.5 mm x 1.5 mm of the image (consisting of about $10^2 - 10^3$
 11 pixels in each dimension) with an angular step of 10° . For better precision, we fitted an ellipse (33) to
 12 the polar plot of the maximum value of the Radon transform at each angle (see Figure 4 c)). From
 13 this ellipse, we extracted the measure of the local orientation (α) of the fiber (see Figure 4 d)), as the
 14 orientation of the major axis, plus 90° since the Radon transform is maximal when the original image
 15 orientation is perpendicular to the projection on a given angle. The intensity of fibers (I) was defined
 16 as half the value of the major axis.

17 Similarly to the BTI analysis described above, we defined the local orientation of the normal
 18 to border Θ_j at each pixel j (see Figure 2). Finally, we defined a local tangent score for the histological
 19 sections as:

$$20 \quad (S_{tangent}^{histo})_j = |I_j \times \sin(\alpha_j - \Theta_j)| \quad \text{Eq. 3}$$

21 The global tangent score is given by the mean of all the $S_{tangent}^{histo}$ of all the pixels of a selected
 22 zone of the histological section divided by the sum of all intensity values, with n the number of pixels:

$$23 \quad S_{tangent}^{histo} = \frac{\sum_j (S_{tangent}^{histo})_j}{n \sum_j I_j} \quad \text{Eq. 4}$$

1 **Statistical analysis**

2 Statistical analysis was performed using the R software. We considered statistical tests not
3 significant (n.s.) if $p \geq 0.05$ and we used the following notation to indicate the degree of
4 significance: " * " when $p < 0.05$, " ** " when $p < 0.01$, " *** " when $p < 0.001$, " >*** " when
5 $p < 0.0001$. The correlation between the collagen tangent score assessed on the histological
6 sections and the BTI tangent score was calculated using the Pearson's correlation.

7 To evaluate the predictive power of the measured SWE- and BTI-derived parameters for the
8 different available biopathological outcomes, we used MANOVA and ANOVA tests as follows: The
9 analysis was done in several steps.

- 10 • First, correlated parameters were excluded from the analysis: only one measurement
11 was kept for each pair of parameters returning a significant ($p < 0.05$) Pearson
12 correlation value and an absolute correlation coefficient above 0.8.
- 13 • Then, only normally distributed parameters were kept by excluding all variables for
14 which the Shapiro-Wilk test returned a significant p-value ($p < 0.05$). The multivariate
15 normality of the remaining set of parameters was tested using the *mshapiro* test in R.
- 16 • Finally, a MANOVA test with the Pillai algorithm was used to evaluate the predictive
17 power of the remaining parameters for the different outcome variables. Prior to the
18 MANOVA test, the following hypotheses were checked: the Shrout & Fleiss intraclass
19 correlation (ICC) using the *ICC* function in the *hmisc* library, and the homogeneity of
20 the covariance matrix with the *boxM* method in *heplots* library. Results are reported
21 only for single variables or sets of variables for which these hypotheses were verified
22 and the MANOVA p-value was significant.

23 The biopathological outcome variables used in the analysis are the ones described in Table 1.
24 This statistical analysis was done on invasive cases only, as well as on IC-NST cases only. *In situ* and
25 invasive cases could not be included in the same analysis because different biopathological variables

1 are assessed for each. Input parameters included relative differences between BTI- and SWE-based
2 parameters as well as the tangent BTI scores, calculated in different tumor zones as defined above. In
3 the rest of this manuscript, only outcome variables for which significant results were obtained in the
4 ANOVA are reported. For these outcome variables, only the parameters returning those significant
5 predictive p-values in the ANOVA test were kept to build a combined classifier. For this purpose, a
6 decision tree classification was run to evaluate the classification accuracy reached. Decision trees
7 were run in R using the *rpart* library with the 'class' method and 'gini' algorithm. Classification error
8 (CE: % of cases accurately classified) and residual mean deviance (RMD) values are reported. Results
9 are reported only for the parameter sets yielding significant outcomes.

10 Pairwise comparisons for parameters selected as described in previous paragraphs (hence
11 normally distributed variables) were done using the Welch Two sample t-test (function *t.test* in R).

12 **Results**

13 **3D multiparametric representation**

14 Our technique allowed for a full 3D representation of the three different modalities for each
15 tumor. An example of different possible representations is shown in Figure 5.

16 **Multiparametric contrast in and around the tumor**

17 The boxplots on Figure 6 show the comparison of SWE, BTI FA and BTI Co in the different tumor
18 zones defined above. Average values are reported across the whole of the corresponding 3D volume,
19 to avoid any bias due to specific ROI selection within that volume. SWE values were higher in the
20 *peritumoral* area and in the *union* (mean 3.69 m/s and 3.24 m/s respectively) than in the tumor
21 *center* and *distant tissues* (mean 3.12 m/s and 2.78 m/s respectively). Differences were statistically
22 significant with $p < 0.05$ except between the *union* and *distant tissues* (cf. Figure 6). BTI Co was higher
23 in the *peritumoral* area and *distant tissues* (mean 0.487 and 0.487 respectively) than inside the
24 tumor (mean 0.428 and 0.403 for *union* and *center* respectively). Differences were statistically
25 significant with $p < 0.05$ except between the *peritumoral* area and *distant tissues* (cf. Figure 6). On the

1 contrary, the BTI FA was higher inside the tumor (mean 0.360 and 0.349 for *center* and *union*
2 respectively) than outside (mean 0.293 and 0.303 in *peritumoral area* and *distant tissues*
3 respectively). Differences were statistically significant with $p < 0.05$ except between the *union* and the
4 *center*, and the *peritumoral area* and *distant tissues* (cf. Figure 6).

5 6 **Statistical analysis**

7 *BTI correlates with collagen orientation*

8 Figure 7 shows that the tangent scores calculated with the BTI ($S_{tangent}^{BTI}$) and with the
9 histological sections ($S_{tangent}^{histo}$) were correlated. The Pearson correlation coefficient between the two
10 variables was equal to 0.47 with a significant p-value ($p = 0.000147$). As an illustration, the case
11 shown on **Erreur ! Source du renvoi introuvable.** yielded BTI-derived and histological scores within
12 1% of each other.

13 *Predictive power for the different outcome variables*

14 The variables kept for the statistical analysis were:

- 15 • the average SWE value in *union* (SWE_{union_mean})
- 16 • the mean BTI fractional anisotropy value in *distant tissues* ($BTI_FA_distant_tissues$)
- 17 • the relative difference between mean BTI coherence values in the *peritumoral* region
18 and *union* ($BTI_CO_peritum_union$)
- 19 • the score evaluating the tangent orientation of fibers at the tumor border evaluated
20 using BTI ($S_{tangent}^{BTI}$ in the *border*).

21 *Identification of the histological type*

22 For the outcome histological type, only one parameter within the subset of selected
23 parameters (uncorrelated and normally distributed) yielded a significant p-value in the ANOVA test
24 (MANOVA $p = 0.0255$) : the $S_{tangent}^{BTI}$ score (ANOVA $p = 0.02242$) in the *border*. Indeed, $S_{tangent}^{BTI}$ in the
25 *border* was significantly lower for lobular carcinomas (ILC, mean 0.563) compared to *in situ* ductal

1 carcinomas (DCIS, mean 0.669, t-test p=0.049) and other types (mean 0.684, t-test p=0.046). Invasive
2 carcinomas (IC_NST) also yielded lower score values (mean 0.626) than DCIS but this difference was
3 not significant (cf. Figure 8 a)).

4 *Identification of the progesterone status*

5 For RP, the only parameter yielding a significant p-value in the ANOVA test (MANOVA
6 p=0.02136) was $S_{tangent}^{BTI}$ (ANOVA p=0.01205) in the *border*. Indeed, this score was significantly
7 higher for RP+ masses (mean 0.644) compared to RP- masses (mean 0.527, t-test p=0.0052) (cf.
8 **Erreur ! Source du renvoi introuvable.** b)).

9 *Identification of the grade*

10 Statistically significant results using MANOVA and ANOVA tests were obtained when
11 evaluating the predictive value for the EE Grade of *SWE_union_mean*, *BTI_CO_peritum_union*, and
12 $S_{tangent}^{BTI}$ in the *border* (MANOVA p= 0.03726*).

13 Pairwise analysis with the Welch's t-test showed that *SWE_union_mean* was significantly
14 higher for grade 2 (mean 3.65 m/s) than for grades 1 (mean 2.88 m/s, t-test p=0.001579) and 3
15 (mean 2.78 m/s, t-test p=0.02963) (see Figure 8 c) left). In contrast, *BTI_tangent_border* was
16 significantly higher for grade 1 (mean 0.701) than for grade 2 (mean 0.582, t-test p=0.008891). It was
17 also higher for grades 1 than grades 3 (mean 0.610) but this difference was not significant (see
18 **Erreur ! Source du renvoi introuvable.** c) middle). Finally, *BTI_Co_peritumoral_union* was
19 significantly higher for the IC-NST of grade 1 (mean 0.165) compared to grade 2 (0.0728, t-test
20 p=0.02667) and grade 3 (mean 0.0299, t-test p=0.002353). The difference between grade 2 and
21 grade 3 was not significant (see Figure 8 c) right).

22 *Multi-parametric classifier*

23 Following these results, a decision tree was trained using these three parameters to classify
24 the tumors in terms of their EE grade, both when considering all invasive cases (DCIS excluded) and

1 when considering only IC_NST cases. This was done using the *rpart* library in the R software version
2 4.2.2 (34). Results provided in Table 2 report the Classification Error (CE) as the percentage of cases
3 inaccurately classified, the Residual Mean Deviance (RMD), and the Sensitivity (Se) and Specificity
4 (Sp) for each grade defined as:

$$5 \quad RMD = \frac{\sum tree\ residuals}{(N_{observations} - N_{tree\ nodes})} \quad Eq. 5$$

$$6 \quad \text{and} \quad Se = \frac{TP}{P}, Sp = \frac{TN}{N} \quad Eq. 6$$

7 With TP the number of cases of a given category classified as that category and P the actual
8 number of cases of that category, and with TN the number of cases not belonging to a given
9 category, not classified as that category, and N the actual number of cases not belonging to that
10 category.

11 Results in Table 2 show that the model was able to separate the three categories with a low
12 proportion of errors in both cases. The method also returns the relative importance to the model of
13 each variable in %, which was, for *SWE_union_mean*, *BTI_CO_peritum_union* and
14 *BTI_tangent_border*, 44%, 30% and 26% respectively in the case with all the invasive cases, and 41%,
15 33% and 26% respectively in the case of IC-NST only.

16

17 Discussion

18 In this study, we investigated three different ultrasound modalities (B-mode, SWE and BTI)
19 on 64 human *ex vivo* breast carcinomas. We reconstructed a 3D multiparametric representation of
20 tumors, making it possible to display the overall B-mode, the SWE in the tumor and the BTI
21 orientation to visualize at a glance all the parameters. The important differences in average values in
22 different regions of the tumors and their periphery (cf. **Erreur ! Source du renvoi introuvable.**)
23 highlight the potential interest of such a multiparametric approach as an assistance to the detection
24 and/or outline of tumors on B-mode imaging.

25 The tissue stiffness was found to be higher in the *peritumoral* area than in the tumor and in

1 more distant tissues. This result is coherent with nanomechanical tests performed on human biopsies
2 and animal models (35) and known as the “stiff rim” in SWE (36). Although this can sometimes be
3 artefactual, the post-processing used in this work should help reduce reflection artefacts at tumor
4 boundaries. In this study we did not assess SWE heterogeneity or anisotropy, although the setup
5 would have allowed such a study. Indeed we averaged data obtained at different probe orientations,
6 in order to maintain high contrast and voxel isotropy despite the tomographic procedure. This was a
7 strategic choice to optimize the robustness of our SWE maps. In the future, the study of SWE
8 anisotropy could be included, but it was not within the scope of this work. It should be noted also
9 that the methodology used in this work to evaluate the tumor’s or peritumoral area’s stiffness is
10 somewhat different from previously published studies(37,38), in that the whole predefined region
11 was used to compute an average value, regardless of the appearance or extent of the stiff region,
12 and without selecting a representative stiff ROI. In particular, the peritumoral area was chosen to be
13 a 5mm dilation of the tumor contour, which can extend largely beyond the stiff rim area often
14 observed and include lower rigidity areas. This can explain the relatively lower values obtained here,
15 with Young’s modulus values reaching 40 kPa at most. This methodology was used for reproducibility
16 and to allow direct comparison between the different tumors. Finally, the excision process may
17 impact the tissue’s mechanical properties due to the absence of perfusion or metabolic activity for
18 example. Specific in-vivo assessment would therefore be necessary to provide biomarkers directly
19 applicable to in-vivo tumors.

20 For the first time, BTI was assessed on a large number of tumors. BTI yielded lower
21 Coherence values inside the tumor compared to the *distant tissues* (cf. **Erreur ! Source du renvoi**
22 **introuvable.** middle). Simulation of the spatial coherence in human tissue models predicted such a
23 low coherence in tumors (39). We additionally showed that the BTI-FA was higher inside the tumor
24 than outside (cf. **Erreur ! Source du renvoi introuvable.** right). This may be related to a more
25 anisotropic organization within the tumor. BTI-derived tissue orientation also correlated with the
26 collagen orientation assessed on stained histological sections. Here, the global score from the 3D

1 imaging was compared to a score derived on a single histology slice chosen to be representative of
2 the whole tumor after observation of all available histology slices. Comparing matched local
3 orientation scores would have been more precise, but maintaining an accurate spatial matching
4 between the images specimen and histology slices was extremely difficult in this context, and tumor
5 boundaries were not included in all slices. Further work should be conducted to confirm this initial
6 result by looking at the 3D collagen structure of the tumor using more sections and/or other
7 techniques (such as second-harmonic generation microscopy). If this result is validated, BTI could
8 become a tool to assess *in vivo* some of the TACS. It may also be used to track the development of
9 the different TACS during tumor growth in animal models. Here, the tangent BTI score at the tumor
10 border was useful to assess the histological type of tumors, the RP response of IC-NST masses as well
11 as to differentiate grade 1 and 2 masses (cf. **Erreur ! Source du renvoi introuvable.**). BTI indeed
12 measured a more “enveloping” organization of tissues around the tumor for *in situ* carcinoma in
13 comparison with invasive cancers. This result is coherent with the appearance of TACS-3 at invasive
14 stages (40). The absence of differentiation with grade 3 may be due to a lower number of cases in
15 this category, as well as to larger and more heterogeneous peritumoral areas including some healthy
16 tissue. For EE grade characterization, BTI and SWE appeared complementary, with SWE
17 differentiating grades 2 from other grades, BTI tangent score grades 1 from the others and BTI
18 coherence grades 1 and 3. The combination of these three parameters reached high accuracy using a
19 simple decision tree to classify invasive and IC-NST tumors according to their grade. The amount of
20 data did not allow here the use of a training/testing split, or of some more performant classifiers
21 such as random forests, and these results cannot support a conclusion as to the predictive power of
22 this combination of parameters. Nevertheless these are encouraging results, as such scores could
23 help the clinicians in their treatment choices without the need for a biopsy. Obviously, this method is
24 far from being able to completely replace biopsies. However, small improvements could quickly
25 replace some of them. For example, in the case of multiple lesions, multiparametric imaging could
26 give indicators of how different the tumors are and therefore whether one biopsy is sufficient or

1 whether several are necessary.

2 These results will need to be confirmed on larger cohorts, and *in vivo*. Besides, the tumor
3 segmentation in this study was the work of a single operator, although expert in the field. The results
4 presented therefore include some operator-dependency, which represents one main limitation of
5 this study. However, we took steps to reduce this dependency by outlining the same tumor twice in
6 different directions. The different outer and inner tumor regions were then defined on the basis of
7 the *intersection* and *union* respectively of the two outlined contours, i.e. regions not taking into the
8 variability between the two outlines so as to limit the impact of the single-operator outlining.

9 Partial and full mastectomies were used in this work. This did not lead to any modifications of
10 our acquisition protocol, since the tissue sample distance to the probe could be adjusted in every
11 case, by moving the probe up and down in the water. The proportion of skin and fat to water
12 between the probe and the mass varied slightly, and was partly accounted for by measuring and
13 adapting the speed of sound to each case, but the main difference lay in the width of the tissue
14 sample, which did not affect the imaging. The tumor masses imaged were included within large
15 margins of the tissue samples.

16 This study was a pilot experiment, aiming to investigate if one or several of the proposed
17 parameters could be useful for breast tumor characterization. It will lay the foundation for larger
18 studies focusing on the most promising parameters highlighted here, applied to masses *in vivo*. Some
19 of the results obtained here may not apply or may produce less clear outcomes *in vivo*, because of
20 the presence of blood which could affect BTI and SWE measurements, and because the tissue
21 structure may be affected by the surgery. The amount of tissue present around the tumor could also
22 lead to slightly different results, although a large amount of tissue, fat and skin was generally present
23 around the specimen used here. The full multiparametric acquisition took typically 45 min here. This
24 duration could also be reduced using SSD or M2 disks. In such a case, it is expected that several
25 minutes without motion would still be required. Nevertheless, electrocardiography (ECG) and

1 respiratory triggering could reduce motion artifacts. For *in vivo* human studies, a matrix-array probe
2 may still be needed to reduce the acquisition time to an acceptable acquisition time (41,42).
3 Quantitative ultrasound (QUS) and other ultrasound modes (such as Doppler) (43) could be added to
4 the parameters presented in this paper to investigate new composite biomarkers. In the long term, a
5 large retrospective multiparametric 3D study could be used to train a model able to assist the
6 clinicians in setting an efficient and early diagnosis, in a fully non-invasive way.

7

8 **Conclusion/Summary**

9 In this paper, we performed a 3D multiparametric ultrasound acquisition consisting in B-
10 mode, SWE and BTI on 64 *ex vivo* human breast cancers. We showed that the stiffness, and the BTI
11 parameters (coherence, fractional anisotropy and local orientation) provide relevant information for
12 the characterization of breast tumors, in terms of their microenvironment organization, the cancer
13 stage (*in situ* vs invasive), the RP status and the tumor grade. In particular, we demonstrated that the
14 BTI-based measure of tissue orientation was correlated to the collagen organization in tumors.

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18 Bettencourt-Schueller. The work was performed with the support of the Inserm Technology Research
19 Accelerator (Inserm ART) in Biomedical Ultrasound, Paris, France.

20 **Data Availability Statement**

21 Anonymized data corresponding to the ultrasound acquisitions and histology slices will be
22 available upon request

23 **Conflict of Interest Statement**

1 M.T. is co-inventor of patents describing the BTI method (44).

2

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13

14 **Figure Caption List**

15 Figure 1: Ultrasound acquisition setup. a) the breast tissue is placed in a vacuum bag and immersed
16 in water. The linear-array probe is attached to a motorized setup to scan the volume of interest b)
17 and c) linear scans are performed at different probe angles for both BTI and SWE (0° and 90° shown
18 here).

19 Figure 2: Methodology for calculating tangent scores for tissue orientation around the tumor. For a
20 given pixel j , a global orientation of the normal (thick green arrow) to the tumor outline (blue line)
21 was calculated as the sum of all normal angles to the border (thin green arrows), weighted by the
22 distance of the corresponding border pixel to pixel j . The tangent score was given by the sinus of this
23 angle to the BTI evaluated orientation α , weighted by the fractional anisotropy at pixel j . This
24 methodology was used for both BTI- and histology-derived scores.

25 Figure 3: Description of the tumor delineation process. First, the radiologist outlined the tumor
26 independently in two perpendicular directions in 1mm-spaced planes. Then we created the volumes
27 (represented in a plane here for illustration purposes) corresponding to the intersection (black) and to
28 the union (green) of the two initial volumes. The center (grey) was defined performing a 0.5
29 homothety of the intersection (B is the barycenter of the intersection). The peritumoral area (grey

1 and white lines) was arbitrarily defined as the volume surrounding the union + 5 mm. Finally, the
2 border (purple) corresponds to the volume at ± 1 mm around the limit of the union. The distant
3 tissues correspond to the tissues located further away than the peritumoral area.

4 Figure 4. Local fiber orientation measurement on histological slices: a) the collagen fibers were
5 extracted from the microscopy image by a color threshold. b) the Radon transform was performed on
6 1.5 mm x 1.5 mm patches of the mask obtained. c) In each patch, the local orientation was estimated
7 from the polar plot of the maxima obtained by the Radon transform, by fitting an ellipse to the
8 maximum values at each orientation and extracting the orientation of its major axis. c) these values
9 were used to derive a local orientation map where fibers were present, to be used as α in the
10 estimation of the tangent histological score. The local fiber intensity I was estimated as half the value of the major
11 ellipse axis.

12 Figure 5: 3D multiparametric representation. This figure shows an example of multiparametric views
13 of an IC-NST (grade 3, ER+, HER2+). a) SWE overlaid onto the B-mode on a horizontal cut. The green
14 line represents the outlined volume union. b) BTI orientation (threshold BTI FA > 0.2) represented with
15 the Matlab "streamline" function and colored according to the Co level. c) 3D B-mode cut to reveal the
16 tumor (darker area in the center). d) 3D multiparametric representation with the overall B-mode, the
17 SWE in the tumor (same color scale as in a.) and the BTI orientation in the border.

18 Figure 6: Boxplots comparing the distribution of average values measured in the different tumor zones
19 for SWE, BTI Co and BTI FA. Results of the statistical analysis for the Welch Two sample t-test (when
20 normally distributed as per the Shapiro-Wilk test) or the paired Wilcoxon signed-rank test.

21 Figure 7: Tangent scores in peritumoral area. This figure plots tangent scores calculated from the BTI
22 as a function of the tangent score calculated from the fibers detected on the histological sections as
23 described on Fig 3. N=64 tumors.

24 Figure 8: Illustration of statistically significant findings. Boxplots comparing a) the distribution of BTI
25 tangent scores in the border for masses of different histological type, b) the distribution of BTI tangent
26 scores for masses of positive and negative RP status (Estrogen receptor) and c) from left to right, the

1 distribution of values of masses graded 1, 2 and 3 for SWE mean values in the union, BTI tangent scores
 2 and BTI coherence difference between the peritumoral area and union. These correspond to the three
 3 variables showing significant differences between grade groups in multivariate analysis.

4
 5

6 **List of tables**

7 Table 1. Summary of the distribution of tumor characteristics in the cohort imaged

8	Total number of cases included		64 (inc. 3 men)
9	Age	Mean (years)	62.8
		Range (years)	40 - 88
10	Histological type	IC_NST	34 (53.1%)
		DCIS	12 (18.8%)
11		ILC	9 (14.1%)
		Other	9 (14.1%)
12	Tumor size	Median (mm)	16
		Interquartile (mm)	15
13	Specimen type	Full mastectomy	13 (20.3%)
		Partial mastectomy	51 (79.8%)
14	for invasive carcinomas :		
	Grade (EE)	1	10 (19.2%)
15		2	36 (69.2%)
		3	6 (11.5%)
16	Progesterone status*	Positive (RP+)	27 (79.4%)
		Negative (RP-)	7 (20.6%)

**assessed for IC_NST only*

17 Table 2. Results of classification trees for the identification of the EE grade

	Minimum splits	Grade 1		Grade 2		Grade 3		CE (%)	RMD
		Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)		
DCIS excluded	10	70	93	94	75	50	98	5.2	0.18
IC_NST only	5	67	100	67	86	80	92	4.3	0.089

18