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Influence of neoadjuvant chemotherapy on diffuse reflectance spectra of tissue in breast surgery specimens

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1 Introduction

Diffuse reflectance spectroscopy (DRS) is an optical technology that measures the composition and morphology of tissue, based on absorption and scattering of light. A DRS measurement of tissue can be used to discriminate tissue types, as the optical characteristics differ between tissue types. The ability to discern healthy tissue from tumor tissue could potentially improve intraoperative margin evaluation during breast-conserving surgery and reduce the number of re-excisions, which are currently reported to be as high as 20% of patients.

Previous research showed the potential of DRS to provide surgical guidance during breast-conserving surgery based on the ability of DRS to discriminate healthy breast tissue from tumor tissue. The reported accuracies for discriminating healthy breast tissue from tumor tissue reached up to 92% in vivo, albeit that these results were obtained in patients who did not have any "neoadjuvant chemotherapy." In addition to the resection margin assessment, DRS technology has also been used for chemotherapy monitoring. Several studies indicate that the effect of chemotherapy can be measured with optical technologies. The fact that the optical properties of tissue alter due to chemotherapy is not surprising, as effects of chemotherapy are seen in tumor tissue as well as in healthy breast tissue. However, currently, it is unclear if the contrast between healthy tissue and tumor tissue is affected by chemotherapy. It is important to consider the effect of chemotherapy on the DRS measurements, since there is an increasing tendency to treat breast cancer patients with chemotherapy before surgery as there are many benefits for the patient.

A preliminary study on the influence of neoadjuvant chemotherapy by our group did not detect neoadjuvant chemotherapy effect in the some of the datasets due to the small number of measurements in those datasets. These findings may indicate that assessment of the resection margin with DRS is also feasible in the growing population of breast cancer patients who receive neoadjuvant chemotherapy. However, it is possible that we did not detect neoadjuvant chemotherapy effect in the some of the datasets due to the small number of measurements in those datasets.

Keywords: neoadjuvant chemotherapy; diffuse reflectance spectroscopy; margin assessment.

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find any significant differences between patients with and patients without neoadjuvant chemotherapy; however, the number of patients included in that study was limited \((n = 9)\), as well as the number of measurements per patient.\(^{27}\)

Since our ultimate goal is to use the DRS technology during surgery for the detection of tumor deposits at the resection edge, it is essential to investigate if the changes induced by the neoadjuvant chemotherapy will affect the DRS measurements of the different tissue types. If the DRS measurements are profoundly different, then this might also affect the optical contrast between tumor tissue and healthy tissue types. This implies that, for the classification of DRS measurements of patients with neoadjuvant chemotherapy, the models based on the measurements of patients without neoadjuvant chemotherapy are not suitable. The goal of our study, therefore, is to assess whether the spectrum of various tissue types, for example, pure “fat” or a combination of fat and “connective” tissue, are significantly different in patients with and without neoadjuvant chemotherapy. In other words, we evaluate whether neoadjuvant chemotherapy induces changes in the breast tissue which may affect the use of DRS for margin detection.

We evaluate the optical measurements of surgical breast cancer specimens in a relatively large dataset of patients with and without neoadjuvant chemotherapy, when compared with other studies. First, we investigate the difference in the tissue composition between patients with and without neoadjuvant chemotherapy. For this purpose, we compare the histopathology of both the whole tissue slice and the measurement locations. Subsequently, two analyses were performed. In the first analysis, we compared if the measured diffuse reflectance at any of the wavelengths was significantly different in patients with or without neoadjuvant chemotherapy (spectral analysis). For the second analysis, fit parameters were derived from the measured spectra using an analytical fit model to quantify the measured spectra. These fit parameters were then compared to assess whether these were significantly different for the different tissue types in patients with and without neoadjuvant chemotherapy (fit parameter analysis).

2 Materials and Methods

2.1 DRS Measurements

2.1.1 Specimens

All measurements were performed on \textit{ex-vivo} breast samples from either breast-conserving surgeries or mastectomy procedures. No written informed consent from patients was required according to Dutch law (WMO) for the measurements. There was no selection of specimens based on tumor grade or type of breast cancer. Fresh specimens were brought to the pathology department where, after inking of the margins and slicing the specimen in a bread-loafed manner, one slice was provided for the optical measurements. Before measuring, the tissue was placed on top of a piece of black rubber in a macrocassette. All tissue slices were at least 2-mm thick.

2.1.2 Optical measurements

DRS measurements were obtained between 400 and 1600 nm. Details on the measurement setup were reported previously.\(^{26,29}\) In short, the light of a halogen light source was transferred to the tissue by means of the illuminating fiber that is integrated into a fiber-optic probe. Two other integrated fibers were attached to two spectrometers, one covering the visual wavelength range (Andor Technology, DU420A-BRDD, 400 to 1000 nm) and one covering the near-infrared wavelength range (Andor Technology, DU492A-1.7, 900 to 1600 nm), both of which collected the photons after interacting with the tissue. The setup was controlled with LabVIEW\(^{9}\) (Austin, Texas) software that performed a calibration from photon counts to diffuse reflectance and combined the output of both spectrographs to form one continuous spectrum from 400 to 1600 nm. For the calibration, a cap with Spectralon\(^{10}\) (SKR-99-100, Labsphere, Inc., Northern Sutton, New Hampshire) at the bottom was used. The fiber distance between the emitting and collecting fiber was 1 mm.

2.1.3 Grid

During the measurements, a custom-made grid was used to ensure a robust correlation between the locations of the optical measurements and the corresponding histopathology. Before the fiber-optic measurements, two white light images were acquired of the tissue in the cassette (1) with the grid and (2) without the grid. After the optical measurements, the grid was removed and tissue was processed according to standard protocol resulting in a hematoxylin and eosin (H&E) stained section of the whole tissue slice.

2.2 Correlating DRS Measurement to Histopathology

The whole H&E section [Fig. 1(a)] was evaluated by a pathologist who annotated areas with invasive carcinoma, ductal carcinoma \textit{in situ} (DCIS), and glandular ducts. The section and annotations of the pathologist were digitalized and, with thresholding, all other pixels in the H&E image were labeled as fat or connective in MATLAB R2018a, based on the fact that fat areas appear white in the H&E section, whereas connective tissue will stain pink. Glandular tissue was included in the connective tissue class. The result was an image in which each pixel had a label corresponding to a tissue type (fat, connective, or invasive carcinoma) [Fig. 1(b)]. The histopathology was registered with the white light image without the grid [Fig. 1(c)] to adjust for tissue deformation, which was introduced during histopathological processing. Another registration was made between this white light image and another white light image including the grid [Fig. 1(d)] to retrieve the actual measurement locations in the white light image [Fig. 1(e)]. Combining both registrations then allowed projecting the measurement locations in the annotated H&E section, which was corrected for tissue deformation [Fig. 1(f)]. This registration method is described in more detail in a previous paper.\(^{26}\) To calculate the mean percentage of tumor cells, an estimation was made of the ratio between tumor cells and connective tissue in all areas that were considered invasive carcinoma. As there were few measurement locations with DCIS \((n = 4)\) in the chemotheraphy patient group and the percentage of DCIS in those locations was less than 20%, this tissue type was not included in the analysis.

2.3 Composition of Measurement Locations

To investigate if the locations that were measured with the fiber-optic probe were a good representation of the tissue composition of the specimens, the composition of the tissue in the whole H&E sections of the specimens (from patients with and
without neoadjuvant chemotherapy) was compared with the composition of the measurement locations (from patients with and without neoadjuvant chemotherapy). A two-sided $t$-test was used to evaluate if the percentages of fat, connective, and tumor cells in the locations measured with the fiber-optic probe were significantly different from the percentages of those tissue types present in the whole section of the specimen. A $p$-value of 0.05 or smaller was considered significant.

### 2.4 Datasets and Analysis

In the analysis, we used four datasets comprising measurements with the same histopathology (Fig. 2). Datasets 1 and 2 consisted of locations that were >95% fat tissue or >95% connective tissue, respectively. Dataset 3 consisted of measurement locations that were a mixture of fat and connective tissues. The last dataset, dataset 4, comprised all locations that contained...
tumor cells in combination with some connective tissues. To select the measurements for each dataset, the estimated percentages of fat, connective, and tumor cells were used, as described in Sec. 2.2. In each of the four datasets, the difference between measurements with and without neoadjuvant chemotherapy was assessed by performing two analyses.

2.4.1 Spectral analysis

For the first analysis, only the intensities measured between 850 and 1600 nm were used. Although the measurements were acquired over the wavelengths between 400 and 1600 nm, the part of the spectrum between 400 and 850 nm was excluded in the analysis as pathology ink, present on the specimens, strongly influenced the measured intensities of the wavelengths below 850 nm. The spectra were not normalized before the analysis.

2.4.2 Fit parameter analysis

For the second analysis, the fit parameter analysis, the spectra were fitted with an analytical fit model, which was described in previous publications. This model estimated the composition of the measurement volumes based on diffusion theory using the measured spectrum and the absorption spectra of substances known to be present in the tissue. Included in the model were oxygenated and deoxygenated hemoglobin, fat, water, and collagen. The fit parameters, derived from the spectra with the analytical fit model, which are used in the analysis were: ratio between fat and water (F/W-ratio), fraction Mie scattering, Collagen, \( \alpha \), and \( b \). The latter two define the amplitude (\( \alpha \)) and slope (\( b \)) in the function that describes the reduced scattering.

To account for the influence of the pathology ink, spectra of the pathology inks were also included as fit parameters.

2.5 Statistics

In both analyses, the datasets were evaluated one by one via generalized estimating equation (GEE) models. This statistical approach is specifically suitable for modeling correlated data, such as repeated measurements in one patient or repeated measurements of tissue types in multiple patients. In such a case, incorporating within-subject and between-subject variations in the statistical analysis is important. A GEE model evaluates the association between measurements and covariates while taking into account the possible relations between measurements that are obtained in one patient. In the GEE models, an equicorrelated structure was used to describe the variance and covariance between the repeated measurements.

In the first analysis, a GEE model was generated for each wavelength, whereas in the second analysis a GEE model was generated for each fit parameter. Covariates included in the models were neoadjuvant chemotherapy ("yes"/"no") and "menopausal status" ("premenopausal or perimenopausal"/"postmenopausal"). Menopausal status was incorporated in the models, as this factor is known to be related to breast density.

Datasets 3 and 4 contained measurements that were a mixture of connective tissues in combination with either fat tissues or tumor cells. Since the ratio between two tissue types can also affect the spectrum, the percentage connective tissue estimated from the annotated H&E section was added as an extra covariate.
in the GEE models of these datasets to take into account this effect.

The analyses were performed in IBM SPSS Statistics 25 with help of the SPSS Python plugin module that provided the possibility to iterate over either the wavelengths or the fit parameters, making a GEE model for each one of them. The α-level was set to 0.05 for all the GEE models.

3 Results

In this study, measurements from 92 patients were used, of which 30 patients were treated with neoadjuvant chemotherapy. From each patient, one specimen was used for the optical measurements. The patient characteristics are listed in Table 1. The mean age of the group of pretreated patients was slightly younger, compared to the mean age of the group of patients without neoadjuvant chemotherapy. Furthermore, the majority of patients who were treated with neoadjuvant chemotherapy received a combination of different types of chemotherapeutics (anthracycline, alkylating agents, taxanes, and antimetabolites).

The response to neoadjuvant chemotherapy was evaluated by (1) comparing the tumor size on magnetic resonance imaging pretreatment and post-treatment (radiological response) and (2) histopathological assessment of the pathologist (pathological response). The combination of the radiological and pathological responses is also listed in Table 1. In 12 patients (40%), the response to neoadjuvant chemotherapy was complete, according to both the radiological and the pathological assessments. In another six patients (20%), both evaluation methods found the response to be “partial.” If a favorable or minimal radiological response is also considered as partial response, in five more patients (16.7%) the response in both evaluation methods is partial. In one patient, the radiological evaluation reported a progressive disease and the pathologist noted no response in the pathology report.

In five patients, either the radiological or pathological response was complete, whereas the other evaluation method asserted a partial response. In one patient, the radiological response was considered partial, whereas the pathologist stated that there was no response. Thus, in six patients (20%), the radiological response was not in accordance with the pathological response.

3.1 Composition of the Tissue Section

Figure 3 shows the mean percentages and standard deviations of the percentage of fat, connective tissue, and tumor cells in the whole H&E section and the measurement locations measured with the fiber-optic probe, from patients without neoadjuvant chemotherapy [Fig. 3(a)] and with neoadjuvant chemotherapy [Fig. 3(b)]. In the data from patients without neoadjuvant chemotherapy, the mean percentages of fat, connective tissue, and tumor cells calculated in the whole section are 63%, 40%, and 5%, respectively. For these tissue types in the same order, the mean percentages in the measured locations were 58%, 33%, and 6%, respectively. With a t-test, we did not find evidence of differences in the percentages of fat, connective tissue, and tumor cells between the whole sections and the measurement locations. None of the p-values were significant (fat: 0.30, connective tissue: 0.10, and tumor cells: 0.63).

The calculated mean percentages of fat, connective tissue, and tumor cells in the whole sections from patients with neoadjuvant chemotherapy were 61%, 39%, and 2%, whereas in the measured locations these mean percentages were 58%, 41%, and 2%, respectively [Fig. 3(b)]. Similar to the data from...
the patients without chemotherapy, a $t$-test was used to assess if the percentages were significantly different in the whole sections compared with the measured locations. The $p$-values for fat, connective tissue, and tumor cells were 0.64, 0.81 and 0.95, respectively, and thus not significant.

It was concluded that there was no bias in the selection of the fiber-optic probe measurement locations and that this dataset can be seen as a good representation of the tissue that is present in the whole section.

Table 2 lists the number of measurements with and without neoadjuvant chemotherapy in each of the four datasets, and the number of patients the measurements originated from, as well as the mean number of measurements per specimen with the standard deviation. The datasets including measurements of pure connective tissue (dataset 2) and measurements of a mixture of connective tissue and tumor cells (dataset 4) are relatively small and originate from a limited number of patients. Although the mean percentage of connective tissue is rather high (Fig. 3), locations of >95% connective tissue are quite rare. More often connective tissue is present in combination with fat tissue (dataset 3).

### 3.2 Intensity Measured between 850 and 1600 nm (Analysis 1)

With the GEE models, for each wavelength, a $p$-value was calculated to assess if the difference between the measured intensities in two tissue types was significant. The results of the GEE models for the pure datasets (pure fat and pure connective tissue) are shown in Fig. 4. As shown in Figs. 4(a) and 4(b), none of the wavelengths reach a $p$-value of 0.05 or lower. Thus, for pure fat or pure connective tissue, there are no wavelengths that have a significantly different intensity due to chemotherapy. Thus, no evidence was found for rejecting our null hypothesis that chemotherapy had no effect on the measurement. Furthermore, between premenopausal/perimenopausal and postmenopausal women no significant difference is seen. It should be noted, however, that for the pure connective

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**Table 2** Datasets used in the two analyses.

|                     | No neoadjuvant chemotherapy | With neoadjuvant chemotherapy |
|---------------------|-----------------------------|-------------------------------|
|                     | Total measurement locations (# of patients) | Average of measurement locations/patient (std) | Total measurement locations (# of patients) | Average of measurement locations/patient (std) |
| Dataset 1: Fat      | 222 (49)                    | 4.5 (4.0)                     | 144 (24)                       | 6 (4.7)                                |
| Dataset 2: Connective tissue | 19 (13)                 | 1.5 (0.8)                     | 32 (9)                         | 3.6 (2.1)                              |
| Dataset 3: Fat/connective tissue | 316 (56)               | 5.6 (4.5)                     | 236 (30)                       | 7.9 (4.7)                              |
| Dataset 4: Tumor cells/connective tissue | 43 (21)                | 2.0 (1.3)                     | 13 (6)                         | 2.2 (1.5)                              |
| Total # measurements | 600                         |                               | 425                            |                                       |
measurements a limited number of data points were available, which cause no detection of an existing effect.

In Fig. 5, the results of the GEE models for the mixture datasets are displayed. Figure 5(a) shows the results of the GEE models for the measurements of connective tissue in combination with fat tissue. There was no evidence found for rejection of our null hypotheses since a significant difference in intensity measured at any of the wavelengths in the group with neoadjuvant chemotherapy and menopausal status were not significantly different for any of the wavelengths. The percentage connective tissue in the measurement location was significant for wavelengths above 950 nm. The results of the dataset that consisted of measurement locations with tumor cells and connective tissue (dataset 4) are displayed in (b). Here, neither chemotherapy, nor menopausal status, nor the percentage connective tissue in the measurement location resulted in a significantly different measured intensity.

**Fig. 4** Result of GEE models: (a) p-values for pure fat (dataset 1) and (b) pure connective tissue (dataset 2). None of the wavelengths are significant for either two datasets comparing the intensities measured in patients with and without chemotherapy or comparing postmenopausal patients with premenopausal/perimenopausal patients.

**Fig. 5** Result of GEE models in (a) p-values for the two mixture datasets, with connective tissue in combination with fat tissue (dataset 3) and (b) tumor cells (dataset 4). None of the wavelengths are significant for either two datasets comparing the intensities measured in patients with and without chemotherapy or comparing postmenopausal patients with premenopausal/perimenopausal patients.
neoadjuvant chemotherapy compared with the group without neoadjuvant chemotherapy was detected. Neither did the menopausal status of the patient result in a difference in intensity measured for any of the wavelengths. As explained in Sec. 2, in the GEE models of the mixture datasets, the percentage of connective tissue was added as an extra covariate. In the dataset including a mixture of fat and connective tissue, covariate describing the percentage of connective tissue in the measurement location was significant for the wavelengths above 950 nm. In other words, the intensity measured at these wavelengths is dependent on the amount of connective tissue in the measurement.

In the other mixture dataset, with measurement locations that included tumor cells in combination with connective tissue (dataset 4), neither neoadjuvant chemotherapy nor the menopausal status showed significant effect on the intensity at any of the wavelengths [Fig. 5(b)]. In contrast to the other mixed tissue data set (dataset 3), the percentage connective tissue, or vice versa, in this case, the percentage of tumor cells, did not result in a significantly different intensity at any of the wavelengths.

3.3 Fit Parameters (Analysis 2)

For each of the fit parameters that were deduced from the DRS spectrum, a GEE model was generated for each dataset. The

| Table 3 | Result in p-values of the GEE models of fit parameters for the datasets of fat tissue (dataset 1) and connective tissue (dataset 2). |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Fit parameter | Fat dataset (dataset 1) | Connective tissue dataset (dataset 2) |
|----------|--------------------------|-------------------------------|
| F/W ratio | Neoadjuvant chemo 0.51 | Menopausal status 0.43 |
|          | Neoadjuvant chemo 0.19 | Menopausal status 0.77 |
| Fraction Mie scattering | Neoadjuvant chemo 0.52 | Menopausal status 0.99 |
|          | Neoadjuvant chemo 0.19 | Menopausal status 0.82 |
| Collagen (μM) | Neoadjuvant chemo 0.07 | Menopausal status 0.73 |
|            | Neoadjuvant chemo 0.48 | Menopausal status 0.24 |
| α         | Neoadjuvant chemo 0.49 | Menopausal status 0.19 |
|          | Neoadjuvant chemo 0.12 | Menopausal status 0.94 |
| b         | Neoadjuvant chemo 0.88 | Menopausal status 0.63 |
|          | Neoadjuvant chemo 1.00 | Menopausal status 0.66 |

3.4 Discussion

This research was focused on investigating whether DRS measurements of breast tissue are affected by neoadjuvant chemotherapy. First, the composition of tissue types of the whole H&E sections from patients with and without neoadjuvant chemotherapy was analyzed, as well as the composition of the

| Table 4 | Result in p-values of the analysis of fit parameters for the mixture datasets of a combination of connective tissue with fat or tumor cells (datasets 3 and 4). |
|---------|--------------------------------------------------------------------------------------------------|
| Fit parameter | Fat and connective tissue (dataset 3) | Tumor cells and connective tissue (dataset 4) |
|----------|--------------------------|--------------------------|
| F/W ratio | Neoadjuvant chemo 0.21 | Menopausal status 0.41 |
|          | % connective tissue 0.00' | |
| Fraction Mie scattering | Neoadjuvant chemo 0.13 | Menopausal status 0.22 |
|            | % connective tissue 0.05' | |
| Collagen (μM) | Neoadjuvant chemo 0.26 | Menopausal status 0.48 |
|            | % connective tissue 0.00' | |
| α         | Neoadjuvant chemo 0.56 | Menopausal status 0.10 |
|            | % connective tissue 0.00' | |
| b         | Neoadjuvant chemo 0.44 | Menopausal status 0.08 |
|          | % connective tissue 0.00' | |

*= Significant.
locations that were measured with the fiber-optic probe. The compositions were compared with investigate if the measured locations were representative of the tissue types in the specimen as seen in the whole H&E section. Next, the DRS measurements of the ex-vivo specimens of patients with and without neoadjuvant chemotherapy were compared by analyzing both the diffuse reflectance intensity data over the spectrum between 850 and 1600 nm (analysis 1) and the fit parameter data derived from the spectrum between 400 and 1600 nm (analysis 2). Both analyses were performed with GEE models that are suitable for assessing correlated data. In the GEE models, in addition to treatment with neoadjuvant chemotherapy, other possible confounding factors were included, such as the menopausal status, and for the mixture datasets, the amount of connective tissue, as these could also affect the DRS measurement.

Comparison of the percentages of fat, connective tissue, and tumor tissue present in the fiber-optic probe measurement locations with the percentages in the whole H&E section showed that these were not significantly different. Thus, showing that the probe measurements (which in essence are a subset of the whole sections) may be a representation of the tissue in both the patient groups, without and with neoadjuvant chemotherapy.

Subsequently, the intensity data (analysis 1: spectral analysis) was used to assess if there were wavelengths with significant different intensity in the patients treated with neoadjuvant chemotherapy and the patients that received treatment without neoadjuvant chemotherapy. This was done in four separate datasets, in which all measurements in one dataset had similar histopathology. Significance was not detected in all four datasets based on the measured intensity, indicating no intensity differences related to neoadjuvant chemotherapy at any of the wavelengths from 850 to 1600 nm. In the analysis, the menopausal status also did not show a significant result for any of the wavelengths in any of the datasets.

In the mixture datasets, consisting of connective tissue in combination with fat (dataset 3) or tumor cells (dataset 4), the percentage of connective tissue in the measurement was included as a covariate. In the dataset that included connective tissue with fat (dataset 3), this covariate was significant for wavelengths above 950 nm, indicating that the measured intensity at these wavelengths was significantly different in these two tissue types [Fig. 5(a)]. This is in line with the expectations, as there are clear biological differences between fat tissue and connective tissue that are displayed in the higher wavelength range. However, in the other dataset in which connective tissue was mixed with tumor cells, none of the wavelengths were significant. This finding may suggest that discriminating connective tissue from tumor cells is more difficult than discriminating connective tissue from fat tissue. For discriminating fat from connective tissue, the observed wavelengths can be used. For discriminating connective tissue from tumor cells, no such clear individual wavelengths could be identified by this approach, probably due to the fact that connective/glandular tissue can resemble tumor tissue. Potentially, a combination of wavelengths that are individually not significant could discriminate connective tissue from tumor cells, but this is not investigated in the current analysis.

The fit parameters that were derived from the measured spectra by using an analytical fit model were also evaluated with GEE models (analysis 2). As for the datasets of pure fat tissue and pure connective tissue (datasets 1 and 2), none of the fit parameters were significantly different in the groups with or without neoadjuvant chemotherapy and the groups of premenopausal/perimenopausal or postmenopausal patients. For these datasets, the results of analyses 1 and 2 are the same.

In the analysis of the mixture dataset that consisted of a combination of fat tissue and connective tissue (dataset 3), the fit parameters were also not significantly different in the measurements of patients who did or did not have neoadjuvant chemotherapy treatment or patients who were premenopausal/perimenopausal or postmenopausal. The percentage of connective tissue was significant for all fit parameters, indicating that all fit parameters changed significantly if the percentage of connective tissue changed and can thus contribute to discriminating fat tissue from connective tissue. For this dataset, the outcomes of analyses 1 and 2 are again the same. This is also in line with the expectations as the absorption coefficient of collagen, the predominant tissue constituent present in connective tissue, is different from the absorption coefficient of fat.

As for the last dataset, with measurements of tumor cells and connective tissue, four of the five fit parameters were not significant for any of the covariates, confirming the results of the first analysis performed with the measured intensities. These results are in line with the results of the spectral analysis, in which no significance was found for neoadjuvant chemotherapy, menopausal status, and connective tissue content. However, the fit parameter representing the amount of collagen in a measurement location appeared significant for all three covariates (neoadjuvant chemotherapy, menopausal status, and percentage of connective tissue). The fact that the fit parameter that describes the amount of collagen was related to the treatment with neoadjuvant chemotherapy, as well as menopausal status, as well as the percentage of connective tissue, may suggest that this fit parameter is significantly different in measurements of patients with and without chemotherapy, in patients with different menopausal status, as well as in measurements with different percentage of connective tissue. It was, therefore, concluded that this fit parameter should be used with caution when used for discriminating healthy tissue from tumor tissue in a dataset that includes patients with and without chemotherapy and with different menopausal statuses.

To our knowledge, this is the first research that assessed the effect of neoadjuvant chemotherapy on the optical spectra measured with DRS using a relatively large number of specimens. Nevertheless, the datasets of measurement locations of pure connective tissue and connective tissue in combination with tumor cells are small and, thus, conclusions should be drawn with reservation. The absence of evidence for significant differences in these datasets might be related to the small number of measurements.

There are a number of publications that describe the microscopic changes that were observed by the pathologist under the microscope or on magnetic resonance imaging. Commonly seen phenomena in nontumor-bearing breast tissue include lobular atrophy and atypia, as well as lobular sclerosis. We did not find any difference in the healthy measurements, which might be explained by the fact that the described structures are relatively small in comparison to our measurement volume. Some studies also report on a decrease in breast density. However, this effect was more profound in younger patients (<40 years). The mean ages of the patients to whom the specimens in this study belonged were 58 (without chemotherapy) and 51 (with chemotherapy) years. Potentially the involution of breast tissue was already present in these patients and, therefore, little difference
is seen between the ratio of fat tissue and connective tissue (Fig. 3). Another explanation could be that the morphological changes that are seen as a decrease in breast tissue are present on a larger scale (centimeters) than the fiber distance used in this study (1 mm) and were, therefore, not detected by the DRS measurements. In tumor tissue, the decrease in cellularity and formation of fibrous areas are often described as a marked feature of chemotherapy treatment. The reduction in the number of tumor cells and the increase in fibrosis could be an explanation for the difference found in the fit parameter that expresses the amount of collagen. However, it should be noted that including the percentage of tumor cells in the GEE model as a covariate should account for this, unless the estimations of the percentage tumor cells based on the H&E sections are not representative of the true percentages of tumor cells in the actual measurement volume.

Diffuse optical spectroscopy has been researched as a clinical tool for monitoring chemotherapy treatment and even for predicting therapy response. However, the main focus of these published papers is on comparing pre- and post-chemotherapy measurements of individual patients, whereas we are interested in comparing groups of patients with and without neoadjuvant chemotherapy. The results presented in this paper show no evidence for a profound effect of chemotherapy on the optical measurements. This does certainly not imply that chemotherapy response monitoring is not possible using DRS. It merely means that the contrast between healthy tissue and tumor tissue is not altered as a consequence of the neoadjuvant chemotherapy. In other words, the composition of the tissue might change due to treatment with neoadjuvant chemotherapy, but the optical response of the tissue type in itself is not significantly different in patients with and without neoadjuvant chemotherapy.

A previous study by Laughney et al. designed to investigate the use of spatial frequency domain imaging for margin assessment of breast specimens, also included patients who were treated with neoadjuvant chemotherapy and assessed the influence of chemotherapy on the optical contrast between tumor and healthy tissues. It was reported that measurements of tumor tissue that had been treated with neoadjuvant chemotherapy had an increased scattering slope, compared with the tumor measurements of patients without neoadjuvant chemotherapy, which was suggested to be related to cell shrinkage. This is different from our results as no difference was found for the scattering parameters. In another study by Keller et al. diffuse reflectance spectra of patients with and without neoadjuvant chemotherapy were measured and compared. They were able to discriminate normal measurements of patients who received neoadjuvant chemotherapy from normal measurements of patients without treatment with neoadjuvant chemotherapy, suggesting that there might be an influence of chemotherapy. However, in both studies, the analysis was based on a very limited number of patients ($n = 5$ and $n = 3$, respectively).

The response according to pathology and/or radiology was not included as a covariate in the GEE models, as the definition of response can be difficult and subjective. This can also be deduced from Table 1, which shows that in six patients (20%) the radiological response is different from the pathological response. Furthermore, patients were not subdivided based on neoadjuvant chemotherapy regime since patients are routinely treated with a combination of chemotherapeutics that have different mechanisms of action and thus all types of reactions are potentially present in each patient. In this study, we did not consider patients who were treated with neoadjuvant hormonal therapy. From the literature, it is known that this type of therapy can also induce tissue changes. However, the number of patients who were treated with neoadjuvant hormonal therapy during the time of this study was too small to include this group of patients in the analyses.

Since measurements were taken from ex-vivo samples after some processing at the pathology department, there was contamination of pathology ink in the measurements. The ink was deposited on the tissue slices by the knife when the tissue was sliced and by the gloves of the pathologist when palpating the tissue slices. By excluding all wavelengths below 850 nm and fit parameters of this wavelength region, influence of pathology ink was reduced as much as possible. However, as a consequence, the impact of chemotherapy on the visual wavelength range could not be investigated. In addition, with the current setup, we did not investigate if there are differences in these tissue types if measurements were acquired in vivo. However, it is not expected that the wavelength range that was evaluated in this study is different in the in-vivo setting compared with the ex-vivo setting. The ultimate goal is the intraoperative application of the technology, allowing the surgeon to assess the tissue when performing the resection. Future research, therefore, is directed toward in-vivo studies, which allow acquiring data from this visual part of the spectrum without the contamination of the pathology ink. In-vivo application of the technology also allows acquiring DRS measurements with larger fiber distances (~3 mm) to increase the depth sensitivity of the system. In this study, this was not possible as the thickness of the tissue was a constraint in the maximum fiber distance that could be used.

In conclusion, we used two different analyses (diffuse reflectance intensity at a specific wavelength and fit parameter data) to assess the influence of chemotherapy on optical characteristics of breast tissue by assessing four different datasets in which all measurements in one dataset had similar histopathology. In addition, the influence of menopausal status on the measurements was assessed, and for the datasets with a mix of tissue types, the percentage of connective tissue was included in the analysis. The measured intensity for the different tissue types was not significantly different in patients with or without neoadjuvant chemotherapy in any of the four datasets. Also, comparing the intensity in the measurements of the group of patients who were postmenopausal to those who were premenopausal/perimenopausal, no significant differences were found in any of the wavelengths. These data were confirmed by the analysis of the fit parameters. None of the fit parameters in the different datasets were affected by neoadjuvant chemotherapy, except for the fit parameter that reflects the amount of collagen, which showed a significant difference in only one dataset that comprised the measurements of tumor cells in combination with connective tissue. These findings seem to indicate that the impact of neoadjuvant chemotherapy on the optical characteristics of breast cancer tissue and healthy breast tissue types is limited, although some caution is warranted for drawing hard conclusions in the small numbered datasets. This means that also after neoadjuvant chemotherapy, margin assessment by DRS during breast-conserving surgery seems feasible.

Disclosures
One of the authors is affiliated with Philips Research; B. H. is an employee of Philips Research; however, B. H. has no financial

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