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Using DRS during breast conserving surgery: identifying robust optical parameters and influence of inter-patient variation

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Abstract: Successful breast conserving surgery consists of complete removal of the tumor while sparing healthy surrounding tissue. Despite currently available imaging and margin assessment tools, recognizing tumor tissue at a resection margin during surgery is challenging. Diffuse reflectance spectroscopy (DRS), which uses light for tissue characterization, can potentially guide surgeons to prevent tumor positive margins. However, inter-patient variation and changes in tissue physiology occurring during the resection might hamper this light-based technology. Here we investigate how inter-patient variation and tissue status (*in vivo* vs *ex vivo*) affect the performance of the DRS optical parameters. *In vivo* and *ex vivo* measurements of 45 breast cancer patients were obtained and quantified with an analytical model to acquire the optical parameters. The optical parameter representing the ratio between fat and water provided the best discrimination between normal and tumor tissue, with an area under the receiver operating characteristic curve of 0.94. There was no substantial influence of other patient factors such as menopausal status on optical measurements. Contrary to expectations, normalization of the optical parameters did not improve the discriminative power. Furthermore, measurements taken *in vivo* were not significantly different from the measurements taken *ex vivo*. These findings indicate that DRS is a robust technology for the detection of tumor tissue during breast conserving surgery.

OCIS codes: (300.0300) Spectroscopy; (060.2310) Fiber optics; (170.6935) Tissue characterization.

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1. Introduction

There is an unmet clinical need for intra-operative detection of tumor deposits on resection margins during breast conserving surgery. Presently, surgeons still have to rely on their visual and tactile abilities when determining the optimal resection plane. Since tumor tissue in the breast is difficult to feel or see, the surgeon's capability to distinguish tumor tissue from normal tissue during surgery is often compromised. Leaving tumor tissue behind during surgery will increase the chance of developing local recurrence, and for this reason, in patients with tumor positive resection margins additional measures such as re-excision or boost radiation therapy may be indicated. On the other hand, removing too much normal breast tissue will impair cosmetic outcome. To guide the surgeon in this delicate balance, several margin assessment techniques are currently available. Traditionally used microscopic techniques, such as frozen section analysis and imprint cytology however, proved to have limited accuracy [1]. Imaging methods, like intra-operative ultrasound, decrease the number of positive resection margins but still lack sensitivity for small tumor deposits [2]. A recently introduced margin evaluation tool is the MarginProbe (DUNE Medical Devices, Paoli, PA, United States), which uses radiofrequency spectroscopy for positive resection margin detection. Currently this technology is under evaluation for more widespread intraoperative use during breast conserving surgery (BCS). First results indicate that with this device the number of re-excisions decreases although also larger volumes of tissue are resected [3, 4].

In an attempt to characterize tissue and perform margin assessment, optical technologies such as Raman spectroscopy [5, 6], fluorescence spectroscopy [7], optical coherence spectroscopy [8], and diffuse reflectance spectroscopy (DRS) [9–12], have been investigated. These optical technologies build on the principle of measuring optical responses, which are influenced by the structure and composition of the underlying tissue. Specifically, DRS with its ability to perform near real-time tissue characterization of tissue volumes of several mm^3,

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without the need for exogenous contrast agents, seems a suitable candidate for margin assessment during breast conserving surgery.

Much of the previously published data demonstrates the potential of DRS to discriminate normal tissue from tumor tissue with high sensitivity (74-79%) and specificity (78-93%). However, many of these studies have been conducted in a well-controlled *ex vivo* setting which does not necessarily resemble intra-operative circumstances when trying to discriminate normal tissue from tumor tissue in an individual patient.

One of the challenges that need to be addressed is the non-uniformity of breast tissue both in an individual patient and between patients. In previous publications, the heterogeneity of normal breast tissue is reported to range between 20%-40% for absorption (μ_a) and 5%-20% for scattering (μ_s) [13, 14]. Besides this intra-subject variation, also inter-subject variation is present in optical measurements of breast tissue [15, 16]. Various publications report on the influence of menopausal status on breast tissue, many of them indicating that breast tissue of pre-menopausal women has higher scattering values, water content, and concentrations of hemoglobin in comparison to post-menopausal women, whereas in the post-menopausal group lipid content is reduced [13, 14, 17, 18]. Furthermore, large inter-subject variation is found [19]. Such inter-patient variation could negatively affect the potential of optical DRS parameters to discriminate normal tissue from tumor tissue.

Another challenge may be the physiological differences between *in vivo* and *ex vivo* measurement. Due to the loss of blood flow and exposure to air, tissue physiology will be different in resected tissue. Such phenomena could impair optical parameters, especially those that are related to blood, thereby hindering one-on-one translation of *ex vivo* results to an *in vivo* setting [20, 21].

Both aspects, the inter-patient variation and changing physiology during the resection of the tissue, can hamper the use of optical spectroscopy for the detection of tumor tissue at a resection margin during breast conserving surgery. To further investigate whether these aspects could hamper future clinical implementation of DRS in BCS we evaluated the optical parameters derived from the DRS measurements for the discrimination of normal tissue from tumor tissue and assessed the influence of inter-patient variation. Furthermore, we explore the potential of data normalization to diminish this phenomenon. Finally, we examined the influence of tissue status (*in vivo* vs *ex vivo*) on the optical measurements to investigate if the physiological changes occurring in tissue during surgery impede the use of DRS as an intra-operative tool.

2. Materials & methods

Spectroscopy system

The optical system used for the spectroscopic measurements consisted of a broadband light source and two spectrometers for 1) the visual wavelength region (Andor Technology, DU420A-BRDD), and 2) the near infrared wavelength region (Andor Technology, DU492A-1.7). The spectrometers and light source were controlled on an attached laptop with LabView and allowed measuring diffuse reflectance spectra between 400 and 1600nm [22]. The measurement set-up was approved for use in the operating theater. The measurements were obtained through a beveled optical needle (18°) with a fiber distance of approximately 0.17cm between the illuminating and collecting fiber at the tip of the needle (Fig. 1). This needle allows to obtain optical data of tissue volumes of a few mm^3.

Data acquisition

Female patients diagnosed with an invasive carcinoma or *in situ* carcinoma, ductal or lobular, were included. The Medical Ethics Committee of the Netherlands Cancer Institute – Antoni van Leeuwenhoek approved the study and all patients signed informed consent.

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Under ultrasound guidance, a coaxial 15/14G needle cannula (Invivo, Schwerin, Germany) was placed at the first measurement location. Through the cannula the optical needle was inserted to collect *in vivo* measurements of non-malignant tissue at a distance of a few centimeters from the tumor. Once data acquisition at the first location had finished the cannula and the optical needle were moved forward to the second measurement location in normal tissue. After the spectra of the second measurement location had been acquired, the optical needle was retracted and through the same cannula a twistmarker (BARD GmbH, Karlsruhe, Germany) was inserted to mark the second measurement location. Next, the optical needle was introduced a second time and the tip of the cannula and optical needle were positioned in the tumor. Similar to the measurements in normal tissue, two tumor locations were measured followed by the insertion of a second twistmarker (Fig. 1). During histopathologic evaluation, the pathologist, who was blinded for the outcome of the spectroscopic measurements, sampled tissue around the twistmarkers to confirm histopathology at the measurement locations (twistmarker verification). However, to facilitate straightforward correlation between histopathology and the measurement locations the study protocol was amended halfway allowing biopsies to be taken from the measurement locations (biopsy verification). Needle biopsies were obtained of the second normal measurement location and the second tumor measurement location.

Once the specimen was resected, the *ex vivo* measurements were obtained, using ultrasound to guide needle placement in normal and tumor measurement locations.

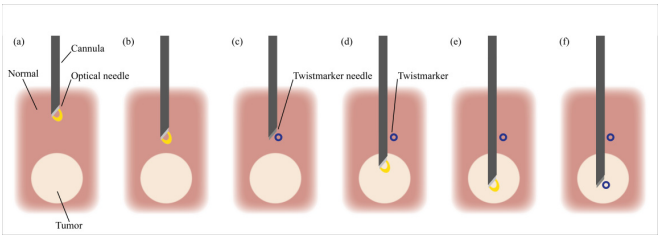

Fig. 1. Schematic image of data acquisition. A cannula with the optical needle are placed in normal tissue to acquire spectra at the first measurement location (a) and subsequently at the second measurement location (b). Afterwards the second measurement location is marked with a twistmarker (c). The optical needle is than again introduced to obtain measurements of tumor tissue (d & e) and similarly the second tumor measurement is marked with a twistmarker (f).

Data processing

The obtained spectra were quantified with an analytical model that was derived from the diffusion theory and has been described extensively in previous publications [22, 23]. In short, the acquired optical spectra of tissue and absorption spectra of known absorbers in the breast are provided as input to the model which uses a Trust Region algorithm to translate the obtained spectra into estimations of optical parameters (Matlab 2015a). Optical parameters evaluated by the model were; blood (%), oxygen saturation (%), scattering at 800nm (cm-1), fraction Mie scattering, β-carotene concentration, collagen fraction, fraction of fat ([fat]/([water] + [fat])), and total volume of water and fat ([water] + [fat]). The latter two were used to calculate the ratio between fat and water (F/W-ratio).

The spectra with more than 20% blood or more than 10% patent blue according to the fit results were excluded. More than 20% blood indicates pooling of blood around the tip of the optical needle whereas patent blue, a dye injected peritumoral for sentinel node detection, may confound data analysis even when included in the fitting model.

Analysis

Each optical parameter was modelled using the Generalized Estimating Equation (GEE) with clustering of all measurements obtained in the same individual subject and assuming

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equicorrelated structure of the covariance matrix for correlated measurements. This GEE method allows taking into account relations within individual measurements when assessing association between optical parameters and covariates [24]. The GEE model evaluates a certain optical parameter by comparing two instances (e.g. normal vs tumor or *in vivo* vs *ex vivo*) while fixing all other covariates. For each optical parameter, the model returns a β-value and a p-value indicating significance. The β-value reflects the change of the optical parameter comparing the two instances. In example, a β-value of 10 of the optical parameter blood in the comparison of normal tissue with tumor tissue means that the average percentage blood increases with 10% between the measurements in normal tissue and the measurements in tumor tissue. Visa versa a negative β-value indicates a decrease. For each optical parameter the β-values for the covariates can be compared.

Covariates included in these models were; 'Tissue Type' (normal or tumor tissue); 'Histological verification method' (twistmarker or biopsy); 'Tissue Status' (*in vivo* or *ex vivo*); 'Menopausal Status' (pre- or post-menopausal); 'Chemotherapy' (neo-adjuvant chemotherapy or not); and 'Hormonal Therapy' (neo-adjuvant hormonal therapy or not). Both neo-adjuvant chemotherapy and hormonal therapy were provided as covariates to the model because in literature effects of these treatments are described for both tumor tissue as well as non-tumor bearing breast tissue [25–30]. Patients who were peri-menopausal (n = 7) were included in the pre-menopausal group. A p-value of 0.05 or smaller was considered significant; all analyses were performed using Matlab R2015a software.

For all optical parameters that were significant in the GEE analysis, median values were calculated at each measurement location and used to evaluate ROC curves and associated 95% confidence intervals in MedCalc. For calculation and comparison of the ROC the methodology as described by DeLong et al. was used [31].

To nullify the potential influence of inter-patient variation and thereby improve the discriminative power of the optical parameters, the optical measurements were normalized by using the patient as her own reference. Normalized data was computed by dividing the measurements from a certain location with the median of all the normal measurement of the same patient.

3. Results

Table 1 lists characteristic of all patients (n = 45) used for analysis. Across these patients 1394 DRS spectra were obtained from 263 locations. The coefficient of variation of the spectra at a measurement locations was <25% for 82% of the measurement sites.

Measurement were obtained of normal tissue *in vivo* (n = 85 locations), normal tissue *ex vivo* (n = 73 locations), tumor tissue *in vivo* (n = 60 locations) and tumor tissue *ex vivo* (n = 45 locations). In incidental cases the tumor could not be clearly identified in the ex vivo setting and in some cases less measurements were obtained due to time restrains in the OR-schedule.

Table 1. Patient characteristics

		Twistmarker verification (n = 25)	Biopsy verification (n = 20)
Mean age (SD)		55.9 year (8.5)	58.3 year (12.1)
Menopausal status	Pre	13	6
	Post	12	14
Previous treatment	Chemotherapy	4	5
	Hormonal therapy	1	2
	None	21	17
Invasive carcinoma	Ductal	23*	17
	Lobular	3*	3

*in one patient histopathological evaluation showed a mixture of ductal and lobular carcinoma *in situ.*

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The β-values calculated in the GEE analysis are displayed in Table 2, in which the scorings of each covariate can be compared with each other for each optical parameter.

It is shown that the parameters F/W-ratio, β-carotene, blood, scattering at 800nm, and fraction Mie scattering, significantly varied between tissue types (first column of Table 2). The optical parameters blood and scattering at 800nm were higher for tumor tissue measurements than for normal tissue measurements. Conversely, F/W-ratio, β-carotene, and fraction Mie scattering were higher for normal tissue than for tumor tissue. Other optical parameters, StO2 and collagen, were not significantly different between normal tissue and tumor tissue.

There were no significant differences between the optical measurements obtained in pre-menopausal and post-menopausal patients (second column Table 2).

F/W-ratio and Mie scattering fraction were significantly different comparing patients with hormonal therapy to those without hormonal therapy. However, the groups of patients who received neo-adjuvant chemotherapy (n = 9) or hormonal therapy (n = 3) are small.

The comparison between the two verification methods (twistmarker or biopsy) was only significant for the F/W-ratio (fifth column Table 2), suggesting that measurements in the biopsy study were less fatty and/or contained more water. A more detailed analysis of the data revealed that the difference in F/W-ratio between the studies was mainly induced by the measurements of normal tissue. No specific reason for this difference could be identified.

Table 2. β-values of the covariates in the GEE analysis. An asterisk indicates significance.

Parameter	Tissue Type[a]	Menopausal Status[b]	Chemo-therapy[c]	Hormonal Therapy[c]	Verification method[d]	Tissue Status[e]
F/W-ratio	–9.11*	1.56	–1.25	–3.17*	–2.40*	0.86
β-carotene (μM)	–4.37*	–0.93	0.69	1.07	–1.36	0.90
Blood (%)	3.06*	0.36	–0.33	–0.76	–0.21	0.14
Scat. 800nm (cm^{-1})	9.19*	–3.85	0.43	–1.37	3.07	–0.31
Mie scat. fraction	–0.08*	–0.01	0.01	–0.09*	0.03	–0.01
Collagen (μM)	0.04	–0.02	–0.02	0.03	0.03	–0.03
StO_2 (%)	–2.12	–6.21	1.79	4.69	6.55	–5.92

[a]Comparing tumor tissue to normal tissue, [b]Comparing postmenopausal measurements to premenopausal measurements, [c]Comparing neo-adjuvant therapy to no neo-adjuvant therapy, [d]Comparing biopsy verification to twistmarker verification, [e]Comparing *ex vivo* measurements to *in vivo* measurements.

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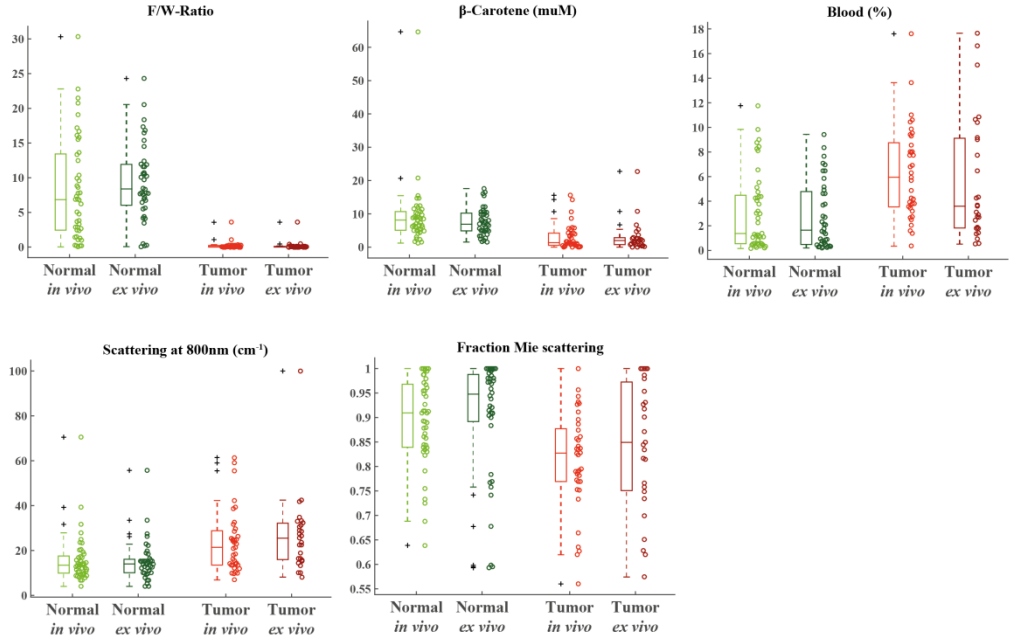

Fig. 2. Boxplot and scatter plot of the five parameters that were significant in the GEE analysis (F/W-ratio, β-carotene, blood, scattering at 800nm, and fraction Mie scattering). The figures comprise the medians from all measurement locations categorized based on the type of tissue (normal or tumor) and tissue status (*in vivo* or *ex vivo*).

Figure 2 illustrates the medians of all measurement locations obtained in an *in vivo* and *ex vivo* setting, represented as a boxplot as well as a scatter plot and obtained for the optical parameters that were significant in the GEE analysis. These graphs capture differences between the normal and tumor measurements but also demonstrate that there is variation in the measured values.

To evaluate the true potential of the optical parameters in this data set to discriminate normal tissue from tumor tissue the Receiver Operator Characteristic (ROC) curves and corresponding Area Under Curve (AUC) values were calculated for the optical parameters that were significant in the GEE analysis (Fig. 3). The F/W-ratio was able to discriminate normal tissue from tumor tissue with highest value of the AUC of 0.943. The AUC values for β-carotene, blood, scattering at 800nm and fraction Mie scattering were 0.824; 0.746; 0.715; and 0.679 respectively.

The data was normalized in an attempt to limit the influence of inter-patient variation. The ROC curves of the normalized data are depicted in Fig. 4. Similar to the absolute data the F/W-ratio was the best discriminator in the normalized data with an AUC of 0.951. All other optical parameters performed slightly worse with AUCs of; 0.826 (β-carotene); 0.751 (blood); 0.752 (scattering at 800nm); and 0.647 (fraction Mie scattering).

The AUCs of the absolute data and the normalized data were compared in MedCalc with a ROC comparison method [31], to investigate the benefit of normalization. The p-values for the comparison between the absolute data and the normalized data were; 0.68 (F/W-ratio), 0.96 (β-carotene), 0.91 (blood), 0.43 (scattering at 800nm) and, 0.53 (fraction Mie scattering); indicating that there were no significant differences between the AUC of the absolute data and the normalized data.

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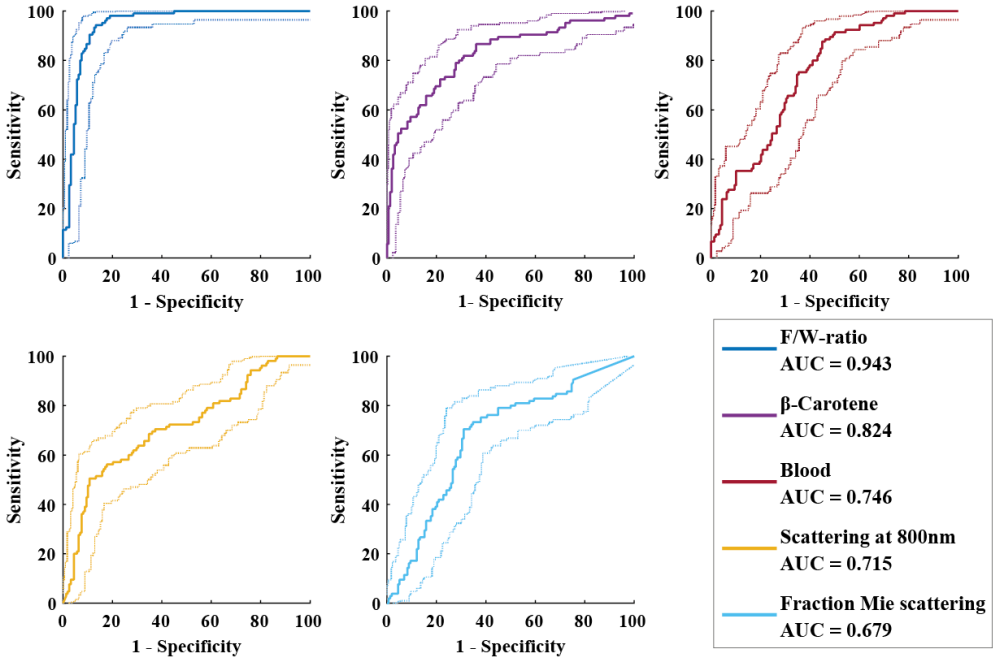

Fig. 3. ROC curves of the optical parameters that were significant in the GEE analysis. The dotted lines around the ROC represent the 95% confidence intervals.

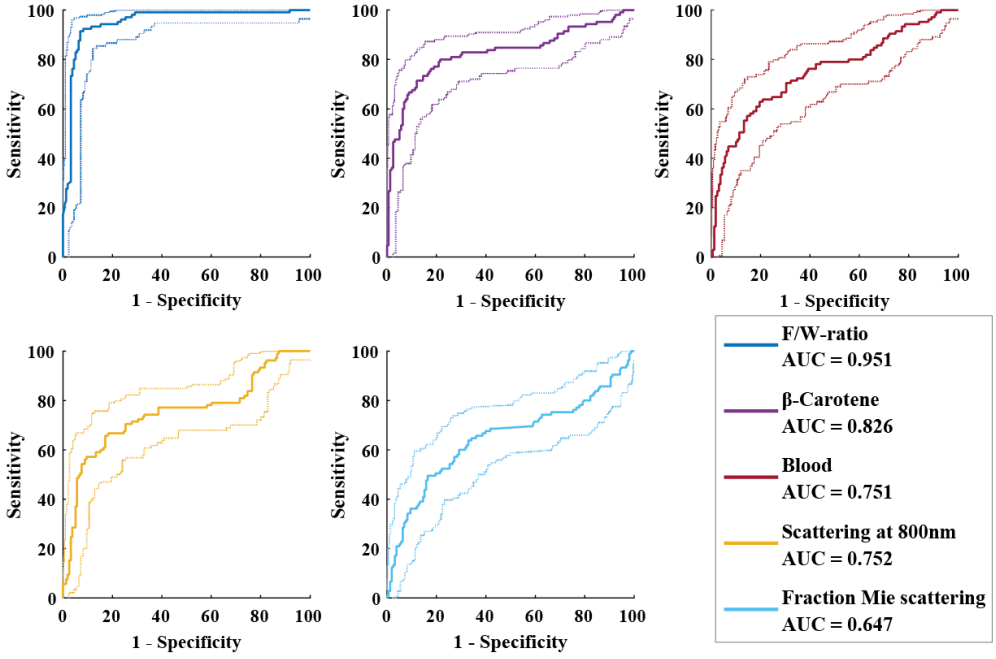

Fig. 4. The ROC curves of the normalized data of the optical parameters. The dotted lines represent the 95% confidence intervals.

Since tissue status (*in vivo* or *ex vivo*) could potentially be a confounding factor in the discrimination between normal tissue and tumor tissue this variable was also included in the GEE analysis. The sixth column of Table 2 comprises the results for the comparison between the *in vivo* and *ex vivo* data and presents no significant differences in all the optical

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parameters. As can be seen in Fig. 5 the ROC curves and the 95% confidence intervals of the *in vivo* and *ex vivo* data of the F/W-ratio almost entirely overlap (p-value: 0.20) which confirms that discriminative power is not significantly different between the *in vivo* and *ex vivo* data.

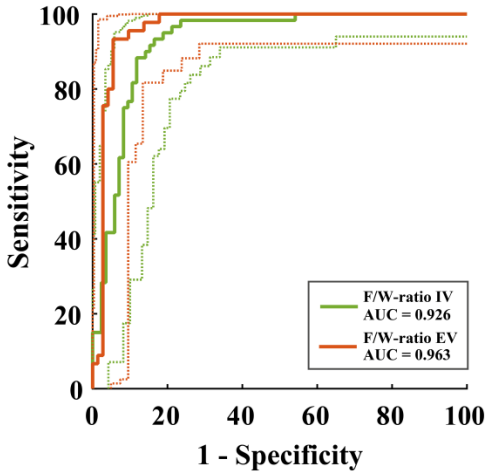

Fig. 5. The F/W-ratio ROC curves of the *in vivo* measurements (green) and the *ex vivo* measurements (red). The dotted lines represent the 95% confidence interval.

4. Discussion

In this study, we investigated the usability of optical spectroscopy for margin assessment during breast conserving surgery by evaluating the discriminative power of optical parameters for tissue differentiation between normal breast tissue and tumor tissue. In this evaluation, we took in consideration confounding factors such as menopausal status (pre- or post-menopausal), previous treatments (neo-adjuvant chemotherapy/hormonal therapy or not), verification method (twistmarker or biopsy) and tissue status (*in vivo* or *ex vivo*) that might have an influence on the optical parameters. ROC-curves were calculated of those optical parameters that were significant in the GEE analysis to consider the true potential to discriminate normal tissue from tumor tissue in this data set. Additionally, focus was on the effect of data normalization to improve discriminative power of the optical parameters, as well as on the effect of change in tissue status (from *in vivo* to *ex vivo*).

The optical parameter F/W-ratio provided the best discrimination between normal tissue and tumor tissue in this study, which is in line with previous published results [32, 33]. Other optical parameters, i.e. β-carotene, blood, scattering at 800nm and fraction Mie scattering displayed significance in the GEE analysis and revealed AUC values in ROC curves between 0.647 and 0.826. This demonstrates that these optical parameters have some potential to discriminate measurements of normal tissue from measurements of tumor tissue but are less distinctive than the F/W-ratio.

Experience learned that sometimes the twistmarker was difficult to find for the pathologist. To avoid problems, the measurement protocol was amended and thereafter biopsies were taken from measurement sites. The influence of this alteration in protocol was assessed by incorporating verification method as a covariate in the GEE analysis. Except for F/W-ratio, all optical parameters were comparable between data acquired in the two protocols. Since the significant difference between the twistmarker verification and the biopsy verification was only present in the measurements of normal tissue, we hypothesize that the density of the breast tissue of the women in the biopsy study might have been higher compared to the density of those women in the twistmarker study despite their comparability in age (Table 1).

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Menopausal status, although highly correlated with involution of glandular tissue in the breast, did not significantly influence our results, similar to the results of Pogue et al [19]. Potentially this is caused by the fact that, although involution of glandular tissue is correlated to menopausal status this does not necessarily imply that post-menopausal women cannot have dense breast tissue. Stomper et al. reported a parenchymal breast density of >50% in 26% of cases in a group of women aged 55 years and above [34].

We included both neo-adjuvant chemo- and hormonal therapy in the analysis since previous research found an influence of these therapies on the optical characteristics of both the normal breast tissue and tumor tissue. Whereas F/W-ratio and fraction Mie scattering were correlated with previous hormonal therapy, previous chemotherapy did not affect any of the optical parameters. The number of patients in both groups was small and any definitive conclusions on the influence of neo-adjuvant therapy on optical measurements cannot be drawn from these numbers. It should be noted that the primary goal of this study was not to assess the influence of menopausal status or neo-adjuvant therapy on the optical parameters as these factors were only included to control the differences in optical parameters between normal and tumor tissue. In other words, incorporating them as covariates in the GEE analysis ensured potential influence of these factors was not falsely attributed to optical differences between normal and tumor tissue. In other studies, also BMI and breast density information were used in covariate adjustment [35], however for the patients included in this study this information was not available. In future studies, we will record both BMI and breast density for all patients.

To improve the discriminative power of the optical parameters and nullify the influence of inter-patient variation we normalized the individual patient data. Previous work by our group used a similar approach and found an increase in both sensitivity and specificity in the individual patient data analysis compared to the cohort data analysis [33]. However, in this research a Classification and Regression tree (CART) algorithm with Leave One Out (LOO) cross validation was used which can be prone to biased parameter selection resulting in improved sensitivity and specificity that is not necessarily associated with differences in optical characteristics of normal tissue and tumor tissue. In the current study, the measurements were normalized by dividing them with the average value of normal tissue of the patient, the CART classification was omitted, and instead ROC curves were calculated and compared. In contrast to what was expected, the normalization step did not improve or deteriorate the ability of any of the optical parameters to discriminate normal tissue from tumor tissue. This could suggest that the influence of inter-patient variation is negligible in this data set, since otherwise the normalization step would have improved the discriminative power. However, potentially not only inter-patient variation but also intra-patient variation influences the optical measurements since the composition and structure of breast tissue types in each individual may vary as well. The performed normalization step did contribute in diminishing the influence of inter-patient variation, but did not fully address the heterogeneity observed in tissue types of an individual patient, which might be a more important factor. Although research by Taroni et al. concludes that the optically derived parameters of a single point time-resolved measurement at a central position in the breast are comparable to a full scan of the breast [35], this probably did not apply to the measurements in this research. Presumably the fact that the measurement volumes of a single measurement in the study of Taroni et al. were much larger compared to the probed volume in this study can explain why in the current study using a single measurement as reference could not account for all intra-patient variation. Obtaining measurements on a number of sites might be necessary to address the intra-patient heterogeneity with our optical measurement set-up.

As for tissue status, measuring *in vivo* or *ex vivo* did not result in significant different measurements for any of the optical parameters. The ROC curve and confidence intervals of the F/W-ratio of the *in vivo* measurements overlap with those of the *ex vivo* measurements indicating that this optical parameter had the same outstanding performance in both settings.

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A direct one-on-one comparison of the *in vivo* with *ex vivo* measurements could, however, not be performed since the sites measured *ex vivo* on the tissue sample were not exactly the same as the locations of the *in vivo* measurements. This is a clear limitation of the study.

Comparisons between *in vivo* and *ex vivo* DRS measurements of normal and tumor tissue are sparse in literature. Many studies are performed in animals comprising only measurements of normal tissue [20, 21], or data is obtained with different optical technology such as Raman [36], fluorescence [21] or electrical and impedance spectroscopy [37]. Optical differences between *in vivo* and *ex vivo* measurements that are described in these studies are mainly attributed to the change of oxygenation state of hemoglobin and blood loss of the tissue. In a study comparing site-matched *in vivo* and *ex vivo* DRS measurements of adenocarcinoma and normal tissue in pancreatic cancer patients the measurements of the two tissue statuses were found to be qualitatively and quantitatively in agreement [38]. To our knowledge the difference between *in vivo* and *ex vivo* measurements of breast tissue, both normal tissue and tumor tissue has not been investigated before. Bydlon et al. did comment on the use of hemoglobin saturation as a parameter for margin assessment. Since excessive changes in oxygenated and deoxygenated hemoglobin were found post-excision, due to oxygen being consumed by metabolic active tissue immediately after excision, they concluded that hemoglobin saturation is an unreliable parameter for this purpose [39]. In this study, measurements were obtained directly after resection, which might be different to other studies where time between resection and measurement is longer. It should also be noted that it is difficult to determine whether a measurement should be considered as *in vivo* or *ex vivo* since tissue characteristics will alter immediately when tissue is manipulated during the resection (e.g. changes in blood flow, secretion of cytokines).

Besides the previous limitations regarding the changes in protocol and the fact that the *in vivo* and *ex vivo* measurements were not acquired at the exact same measurement spot another important limitation is the correlation with the histopathology. In the twistmarker study tissue for histopathological evaluation was obtained close to the twistmaker, however this might not have been the exact same location. Something similar concerns the biopsy study when two different needles were used for measuring and taking the biopsy. Tissue can be classified reliably as ‘normal’ or ‘tumor’ since ultrasound imaging was used to guide the needle and confirm placement in the targeted tissue. Direct correlation between the optical measurements and the actual histopathology at a measurement site however was not possible. This was an important reason why the measurements were not subdivided into more detailed groups than normal and tumor. Since normal tissue consists of both fatty tissue and fibrous/glandular tissue in varying ratios, classifying all these measurement locations as normal might be considered as somewhat limited. To be able to claim that DRS can also be used for discriminating all types of tissue present in the breast a more refined classification of the measurement locations based on direct correlation between optical measurements and histopathology is necessary. The fact that normal measurements can have different underlying tissue types may also explain the heterogeneity seen in the normal tissues as described previously in the discussion.

The results of the current study indicate that the F/W-ratio outperformed all other optical parameters in discriminating between normal tissue and tumor tissue with and AUC of 0.94. Furthermore, it was concluded that this parameter was not affected by tissue status because the performance in the *in vivo* and *ex vivo* setting was similar. For the application of DRS as a margin assessment tool, these results imply that DRS technology based on F/W-ratios is a suitable candidate for intra-operative use, even if during the resection tissue physiology will change. The data was normalized in an attempt to overcome inter-patient variation. However, the normalization of data did not result in the hypostasized enhanced performance of the optical parameters. Potentially increasing the number of normal measurement that are used for determining a reference value can help in establishing a more robust baseline

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measurement of normal tissue. The results of this study support the potential use of DRS during breast conserving surgery for the detection of tumor on resection margins.

Conflict of interest. This study was supported by Philips Research, Eindhoven, Netherlands. The author who is affiliated with Philips Research (BH) only has financial interests in the subject matter, materials, and equipment, in the sense that he is an employee of Philips. None of the other authors have any financial relationship with Philips Research or conflict of interests.

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