Introduction

Optoacoustic (OA) imaging is an emerging low-cost hybrid imaging modality currently in clinical feasibility studies for breast cancer diagnosis and staging (1–7). The technique applies pulsed light to the tissue of interest, where molecules absorb the light photons and generate acoustic pressure waves. The resulting acoustic responses are detected using ultrasound transducers and converted into images. Image contrast within a pixel is dependent on the relative concentration and absorption characteristics (i.e. spectrum) of the chromophores within the illuminated tissue. Thus, tissue responses from illumination using multiple wavelengths, chosen to reflect the differential absorption of oxy, deoxy and total haemoglobin (Hb), can be measured. In turn, these signals can be regarded as surrogate measures of tissue hypoxia and neoangiogenesis, hallmarks of cancer associated with adverse outcomes in cancer patients (8–11).

Normal breast tissue undergoes cyclical physiological changes (12) during the second half of the menstrual cycle (days 15-27 – luteal/secretory phase), leading to increased parenchymal vascularity and proliferation of the ducts and acini (13) (14). In addition, steroid hormones (such as estrogen) cause vasodilation and increased vascular permeability, effects that peak during the secretory phase (14). Optoacoustic imaging in pre-menopausal women may therefore be influenced by the rise in vascularity and vascular permeability of the normal breast parenchyma between the proliferative and secretory phases of the menstrual cycle.

We sought to establish the range of qualitative appearances and quantitative measures of OA imaging in the normal physiological range of the healthy breast tissue. Characterising the expected normal ranges and determining the effect of physiological variations on OA imaging are critical steps in validating a new imaging modality for clinical applications, as this allows to differentiate diseased states from physiological
change. Previous studies have shown that changes in breast vascularity and oedema during the menstrual cycle in healthy volunteers and women with benign lesions affect dynamic contrast enhanced (DCE) magnetic resonance imaging (MRI) (14–16), diffusion weighted MRI (17), elastography (18) and near-infrared spectroscopy measurements (19). Such variations are linked to an increase in parenchymal contrast during the secretory phase of the menstrual cycle (14–16) resulting in an increased Apparent Diffusion Coefficient (ADC) (17), increased stiffness (18) and increased measured haemoglobin content, respectively (19).

The aim of our study was to assess the ability of optoacoustic imaging with ultrasound (OPUS) to detect physiological changes in the breast during the menstrual cycle and to determine qualitative and quantitative metrics of normal parenchymal tissue in pre- and post-menopausal women. The secondary aim was to assess intra-observer OPUS repeatability.
Materials and Methods

Following institutional review board approval this prospective study was performed between Jan 2016 and July 2016. The study was conducted according to the protocol of the latest World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects.

Healthy female volunteers were recruited from our institution’s breast clinic with the following inclusion criteria: ability to give informed consent; age ≥ 18 years; pre-menopausal with a regular menstrual cycle or post-menopausal (1 year without a period). Exclusion criteria were: pregnancy; lactation; current use of the oral contraceptive pill or hormone replacement therapy (HRT); history of breast cancer; known high risk of breast cancer; breast implants; tattoos; or skin disease affecting the breast. Pre-menopausal volunteers were imaged in the proliferative/follicular phase (day 5-14; referred to as ‘proliferative’ for the remainder of the paper) and secretory phase (day 21-28) of the menstrual cycle. These times points were chosen based on the well-established menstrual cycle classification by Vogel et al (12).

We used an OPUS system with a 2D handheld detector (cylindrically focused 256-element detector array at a center frequency of 4MHz, a send/receive bandwidth of >50% and 160° coverage) with ~ 200 µm resolution to acquire 2D cross-sectional images with a field of view of 30 mm × 30 mm (MSOT Experimental Imaging Platform, iThera Medical GmbH, Germany). The same detector was used to generate both optoacoustic images and pulse-echo ultrasound images; as a result, ultrasound and optoacoustic images remained intrinsically spatially co-registered. For the optoacoustic images, diffuse illumination was created by an optical fiber bundle with a diffusor mounted at a small angle from the transducer, the light being provided by a
nanosecond pulsed optical parametric oscillator (OPO) laser at 25 Hz. The illumination wavelength was tunable between 680 nm and 1300 nm. For pulse-echo ultrasound, transmitted pulses had a peak-to-peak voltage of 20 V at 6 MHz and were reconstructed using synthetic transmit aperture technique at a field of view of 30 mm × 30 mm with a pixel size of about 200 µm.

OPUS was performed using illumination wavelengths of 700 nm, 800 nm, and 850 nm. These wavelengths were chosen based on the spectral features of oxy, deoxy haemoglobin within the operating range of the laser in the near-infrared optical window (Fig. 1) (20) (21) as follows: at 700 nm deoxy-Hb is most absorbing; 800 nm is the isosbestic point and considered proportional to the sum of deoxy-Hb and oxy-Hb (thus proportional to total Hb); 850 nm is the wavelength at which oxy-Hb is most absorbing.

During the examination, the volunteer laid supine on an ultrasound examination couch (identical to when performing a routine breast ultrasound). Ultrasound gel was applied on the skin for acoustic coupling. The OPUS probe was positioned consistently 4 cm from the nipple in the upper outer quadrant of the left breast to achieve: (1) comparable measurements between the different time points in pre-menopausal volunteers; (2) standardization between pre- and post-menopausal volunteers; and (3) maximal repeatability. The left breast upper outer quadrant was chosen as the volume of glandular tissue is highest in the upper outer quadrant of the breast and the operator could position her forearm across the volunteer and stabilize the large probe of the prototype clinical OPUS system. Repeatability scans were performed within the same day by the same operator. i.e. all scans were performed by a radiologist with five years of experience in breast ultrasound imaging.
Region of interest (ROI) analysis was performed using dedicated software (cLabs 2.23, iThera Medical GmbH, Germany). ROIs were drawn on the reflection ultrasound computed tomography (RUCT) image, which provided normal sonographic anatomical contrast to distinguish fat from parenchymal tissue. Two experienced radiologists, with five years of experience and over 20 years of experience in breast imaging drew all ROIs in consensus. An elliptical ROI with a fixed area of 25 mm$^2$ was placed over a region of fibroglandular tissue. In the same volunteer between the proliferative and secretory phase we attempted to draw the roi at the same depth with a maximum of variation on 0.5cm comparison to the previous placing. Depth was calculated from the skin surface to the most superficial point on the ROI.

(Fig. 2). The ROI was then used to derive mean optoacoustic (OA) image intensity at each wavelength in the intrinsically co-registered OA image.

Statistical analysis was performed using GraphPad Prism (version 7, GraphPad Software Inc, La-Jolla, CA) and R (version 3.2.1). After confirming normality using the Shapiro-Wilk’s test, paired Student’s t-tests were used to analyse the differences in signal intensity between the proliferative and secretory phase of the menstrual cycle at 700 nm, 800 nm and 850 nm. Unpaired Student’s t-tests were used to analyse the difference between the pre-menopausal and post-menopausal group. Repeatability data was assessed using the interclass correlation coefficient and Bland-Altman plots. A p-value <0.05 was defined as statistically significant.

**Results**

Overall, 22 pre-menopausal and 8 post-menopausal volunteers were recruited (age range 22 – 61 years). Six pre-menopausal volunteers were excluded from comparative assessments between the proliferative and secretory phases of the menstrual cycle due to an inability to complete both scans (n = 1), use of oral contraceptives (n = 3), change in hormonal status (n = 1) and technical errors during acquisition (n = 1). Repeatability studies were available in 21 volunteers.
The technical error was operator dependent. It was the first volunteer scanned and there was significant movement artefact. However, it served as a learning opportunity and the scanning technique was modified accordingly.

The optoacoustic image alone appeared relatively homogenous and featureless in the proliferative phase and provided no delineation of the parenchyma or fat. Optoacoustic features remained unchanged in the secretory phase and similar findings were observed in the post-menopausal group. A representative set of images from the breast of a healthy volunteer in the proliferative and secretory phases of the menstrual cycle is shown in Fig. 2. As expected, a gradual loss of image intensity from the skin to the chest wall was observed due to the absorption of light as it travelled through the tissue. No light fluence compensation (i.e. correction algorithm for depth) was applied to the images. The high optical absorption of haemoglobin allowed blood vessels to be visualized as bright (cyan, yellow and red in the colour scheme used) linear or circular structures, depending on the position of the cross sectional plane. The skin line usually appeared as a bright structure, likely due to the high density of the capillary network in the skin and the absorption of light by cutaneous melanin.

A statistically significant increase was observed in measurements at all three wavelengths between the proliferative and secretory phase of the menstrual cycle $p < 0.001$ (Fig. 3) and Table 1. Linear regression and correlation analyses showed that the changes in OA-intensity at 700 nm (Supplemental Fig. 1, $r = 0.81$) and 850 nm ($r = 0.86$) correlated well with those established at 800 nm ($r = 0.81$), suggesting that changes in oxy and deoxy-haemoglobin parallel the overall change in total haemoglobin expected based on higher perfusion or vascularization of the fibroglandular tissue during the secretory phase of the menstrual cycle.
Table 1. Mean ± SD values for each wavelength in the proliferative and secretory phases.

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<th>Proliferative Phase Mean ± SD</th>
<th>Secretory Phase Mean ± SD</th>
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<tbody>
<tr>
<td>800nm (surrogate for total Hb)</td>
<td>14.23 ± 2.56</td>
<td>17.85 ± 2.67</td>
</tr>
<tr>
<td>700nm (surrogate for deoxy weighted Hb)</td>
<td>14.07 ± 2.61</td>
<td>18.02 ± 3.00</td>
</tr>
<tr>
<td>850nm (surrogate for oxy weighted Hb)</td>
<td>12.78 ± 2.81</td>
<td>17.06 ± 3.09</td>
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Comparison of post-menopausal to pre-menopausal measurements with OPUS showed a statistically significant difference between the secretory phase of the pre-menopausal group and post-menopausal group at all three wavelengths p < 0.05 (Fig. 4). We observed no significant differences when comparing measures from the post-menopausal group to measures from the proliferative phase of the pre-menopausal group.

Repeatability of OA-image intensities was assessed by correlating the mean intensity of ROIs at 700 nm, 800 nm and 850 nm between repeatability scans in the same volunteer (intra-observer variability). ICC at 700 nm, 800 nm and 850 nm was 0.669, 0.755 and 0.792 respectively, indicating good to excellent agreement between scans (22). The variability between repeated scans was visualised using Bland-Altman plots (Supplemental Fig. 2). In agreement with the ICC values, the Bland-
Altman plots show a greater spread of values for the 700 nm wavelength but with good reproducibility at the 800 nm and 850 nm wavelengths.

**Discussion**

The ability to demonstrate small differences in tissue function is a critical step in the clinical translation of this new imaging tool for breast cancer assessment. At present, mammography and ultrasound provide anatomical delineation of cancerous lesions and healthy tissue, but these techniques lack the ability to characterize tissue function, limiting the potential for early detection, as well as lesion heterogeneity and aggressiveness assessment. DCE-MRI addresses some of these limitations, but it remains hampered by higher costs, the need for dedicated image processing capabilities and potential long-term effects from gadolinium exposure. Hence, there is an unmet clinical need for low-cost imaging approaches that can provide functional information and assess molecular changes non-invasively. Optoacoustic imaging has the potential to meet these needs in breast imaging and early clinical studies using optoacoustic imaging in the human breast cancer have already shown promise in visualizing tumours (1,5,4,6). To our knowledge, the sensitivity of OPUS in characterising the normal physiology of breast parenchyma has not been reported.

In our study, we assessed changes in healthy breast parenchyma from pre- and post-menopausal volunteers and at different phases of the menstrual cycle. This was made possible by the recent development of a novel clinical ultrasound-integrated optoacoustic imaging system (OPUS) to characterise functional variations in healthy and diseased parenchymal breast tissue. In this setup RUCT ultrasound provided delineation of the parenchymal tissue and facilitated ROI analysis while measured OA-image intensity values provided quantitative metrics for functional assessment of breast tissues. Using this methodology, we observed an increase in OA-image
intensity in the breast parenchyma in the secretory phase of the menstrual cycle at each chosen wavelength. These imaging wavelengths (700 nm, 800 nm, and 850 nm) were specifically chosen to reflect the spectral features of oxy, deoxy and total haemoglobin, respectively. The increases in OA-image intensity at 700 nm and 850 nm in the secretory phase of the menstrual cycle correspond to increases in oxy and deoxy haemoglobin content. These findings are strengthened by the observed rise in OA-intensity at 800 nm, suggesting an increase in total haemoglobin in the secretory phase of the menstrual cycle. Collectively, our results confirm the increase in overall perfusion and vascularity of the breast parenchyma during the secretory phase of the menstrual cycle and they are in agreement with published results from other existing clinically-approved imaging modalities (14–16,19). Furthermore, the breast parenchyma of post-menopausal volunteers showed similar OA-intensity to that of pre-menopausal volunteers in the proliferative phase of the menstrual cycle. Appreciation of the quantitative metrics within healthy breast parenchyma will assist future research when evaluating quantitative metrics of benign and malignant lesions and assist in defining optimal cut offs. It must also be appreciated that every optoacoustic scanner may generate numerical values and thresholds between health, benign and malignant disease should be refined accordingly.

Results from the repeatability study provide correlation coefficients that support confidence in the sensitivity of OPUS to physiological changes in breast parenchyma. OPUS repeatability was good at 700nm, very good at 800 and 850nm (22) and within the range of other handheld techniques at 0.6 - 0.9 (23) (24) (25). Comparison of OPUS to other standard techniques like CT or MRI is not as valid due to mobility of the breast and handheld nature intrinsic to this scanning technique.

Our study's strengths include its prospective nature and longitudinal design that enabled us to assess changes with respect to hormonal status. Furthermore, acquiring
all OPUS measurements using the same operator eliminated inter-operator variability. In addition, further hormonal variations were removed by excluding patients taking oral-contraception or hormone replacement therapy.

Our study’s main limitation was the small sample size, but this was similar to other prospective studies (14) (17) which evaluated imaging appearances of hormone-related changes in the breast. We were unable to correlate our results with breast density, as we assessed healthy volunteers that could not be exposed to unnecessary radiation from mammography. Some other limitations related to technical challenges arising from the prototype OPUS system. Optoacoustic assessment becomes more difficult with increasing tissue depth due to variable light fluence at depth. Furthermore, incident light to the skin surface is scattered and absorbed as it passes through tissues, resulting in reduced OA absorption and reduced image contrast with increasing depth. In this study, we did not apply fluence correction to compensate for light attenuation as a function of depth but instead we controlled for the potential impact of depth on our results by drawing ROIs at approximately the same level when comparing different phases of the menstrual cycle. However, in a clinical setting when patient management decisions need to be made, the quantitative measurements from OPUS imaging of breast lesions could vary depending on their location (depth). To overcome this limitation, future studies should evaluate the performance of fluence-correction algorithms (26) (27) on a per-patient basis in comparable analyses at variable depths. In addition, future studies should be performed to evaluate the usefulness of spectral unmixing algorithms for breast data acquired at a larger number of wavelengths that may allow the specific separation of the different endogenous chromophores that contribute to OA contrast (including oxy and deoxy haemoglobin, melanin, water and lipids) (20). Signal unmixing approaches may not only provide semi-quantitative assessment of endogenous chromophores, but also allow the development of approaches to mitigate against the strong optical absorption of melanin, an important factor when applying this technology to a larger population with various skin tones. In
the present study, our goal was to characterize haemoglobin content in breast parenchyma and thus we focused on single wavelength image analysis that is weighted by oxy, deoxy and total-haemoglobin. In our study, the recruited volunteers were of fair complexion where single wavelength images could potentially provide robust analyses by minimising the impact of variable melanin content in the skin.

Conclusion
To our knowledge, this is the first report to demonstrate menstrual cycle-dependent changes in breast parenchyma using optoacoustic imaging. Our observations correlate well with known changes in vascularity during the menstrual cycle and showed an increase in intensity in the secretory phase compared to the proliferative phase. suggesting that OPUS has the potential to be applied for functional and molecular characterization of breast tissue.
REFERENCES


