Clinical experiences with photoacoustic breast imaging

the appearance of suspicious lesions

Michelle Heijblom

**CLINICAL EXPERIENCES WITH
PHOTOACOUSTIC BREAST IMAGING**
THE APPEARANCE OF SUSPICIOUS LESIONS

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CLINICAL EXPERIENCES WITH PHOTOACOUSTIC BREAST IMAGING

THE APPEARANCE OF SUSPICIOUS LESIONS

DISSERTATION

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the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
Prof. dr. H. Brinksma,
on account of the decision of the graduation committee,
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1

Introduction

Breast cancer is the most common form of cancer among women. Conventional imaging modalities, both in screening and diagnosis, suffer from limitations. A technique with high theoretical potential for breast cancer visualization is photoacoustic mammography, which combines high intrinsic optical absorption contrast with ultrasound resolution in detection. Until 2009, clinical photoacoustic data remained limited. New clinical studies were designed using the Twente Photoacoustic Mammoscope (PAM) in order to provide information on the potential of photoacoustic mammography in breast imaging.

1.1 Introduction

Breast cancer is the most common form of cancer among women [1, 2], accounting for about 14000 new cancer cases (Figure 1.1) and more than 3000 deaths in the Netherlands a year [3]. Early detection of breast cancer can significantly improve survival chances [2], but conventional imaging techniques suffer from limitations in performance and comfort. Hence the continuous search for improved breast imaging techniques. This thesis describes clinical results obtained with a novel technology: photoacoustic (PA) mammography. In this chapter, an introduction on breast cancer, breast imaging and PA mammography is given.

Figure 1.1 The number of new cancer diagnoses per year in the Netherlands for the four most common types of cancer in women [3].

1.2 The breast and carcinoma of the breast

1.2.1 Normal breast

The three basic structures that compose the breast are the skin, subcutaneous fat and the breast tissue. The latter comprises the parenchyma (functional part of the gland) and stroma (the supporting tissue of the gland). The dorsal side of the breast is delineated by the pectoralis muscle [4]. The parenchyma is composed of 15-25 segments or lobes, corresponding to each of the major lactiferous ducts that terminate in the nipple (Figure 1.2a) [4, 5]. Each duct drains a lobe that further consists of 20-40 terminal duct lobular units (TDLU, Figure 1.2b) [4]. The TDLU is a 1-8 mm hormone sensitive gland, which has the potential of milk production and is believed to be a basic histopathologic unit of the breast from which many benign and malignant lesions arise [4]. Small branches of the major duct lead into terminal ducts that drain a single lobule. The lobule is further

composed of intralobular terminal ducts and blindly ending ductules. The lobules normally regress at menopause, leaving blunt terminal ducts [4].

The functional glandular elements are embedded in fibrofatty tissue, which forms most of the mammary gland. The relative proportions of fat and collagenous stroma vary greatly among individuals and age. The combination of stromal and epithelial components is responsible for the radiographic appearance of the breast structure in normal and pathologic states [5]: the greater the ratio of parenchyma to stromal tissue, the higher the so-called radiographic breast density. The number and size of the lobules increases from childhood to puberty, is maximum during lactation and the lobules involute during menopause. Therefore, the breast density is generally larger in premenopausal women [4].

Figure 1.2 Normal breast anatomy. **a)** The breast is composed of 15-25 lobes (segments), each with a major lactiferous duct, draining the terminal duct lobular unit (TDLU, b) and ending in the nipple. **b)** The TDLU is a hormone sensitive gland, consisting of ductules and lobules. The image of the TDLU is obtained, with permission, from [6].

1.2.2 Carcinoma of the breast

The majority of women with breast cancer present symptomatically, although the introduction of breast screening has led to an increasing proportion of asymptomatic cases being detected mammographically [7]. Breast cancer does not have specific signs and symptoms that allow reliable distinction from various forms of benign breast disease. However, benign conditions are more common in younger women, while breast cancer is the most common cause of symptoms in older women [7]. The focus of this thesis is primarily on malignant breast lesions. Therefore, a short description of the most common malignant breast lesions is given in this section. In addition, a short introduction on benign

cysts can be read in Chapter 5. More information on other common benign breast abnormalities such as fibroadenoma, adenosis, hemangioma and phyllodes tumors, can be found elsewhere [7].

Breast carcinoma arise from the mammary epithelium and most frequently from the epithelial cells of the TDLU. Between 40 and 50% of tumors occur in the upper outer quadrant of the breast and there is a decreasing order of frequency in the other quadrants from the central, upper inner, lower outer to the lower inner quadrant [7]. The epithelial cell lesions can further be subdivided in in situ and invasive tumors. The most common in situ cancer is the ductal carcinoma in situ (DCIS). DCIS is by definition an atypical proliferation of cells confined by an intact basement membrane to the ductulobular system of the breast. It cannot cause serious morbidity unless it becomes invasive. The major goal of any pathologic or radiologic evaluation of DCIS is thus to determine the level of risk of invasion so that optimal treatment can be given and under- or overdiagnosis can be avoided [8]. Lobular carcinoma in situ (LCIS) is by definition a microscopic process and is therefore almost always an incidental finding associated with other abnormalities in diagnostic images. In LCIS, the lobular acini are filled with poorly cohesive proliferations of specific appearing cells (round nuclei, scant cytoplasm and inconspicuous nucleoli) [8].

Invasive (infiltrating) carcinoma can grossly be divided in infiltrating ductal carcinoma (IDC) and infiltrating lobular carcinoma (ILC). Other less common carcinoma types are amongst others: tubular carcinoma, invasive cribriform carcinoma, medullary carcinoma, mucinous carcinoma and inflammatory carcinoma. IDC comprises the largest group, accounting for 50-75% of all invasive breast cancers [7]. The traditional concept was that these tumors are derived exclusively from mammary ductal epithelium, while lobular carcinoma were deemed to have arisen from within lobules, hence the nomenclature. However, there is no evidence for this concept and it has been shown that the TDLU should be regarded as a single entity from the point of view of the site of origin of most breast carcinoma [7]. Therefore, the distinction between IDC and ILC is based on the histopathological appearance, with ILC (accounting for about 5-15% of invasive breast tumors) composed of non-cohesive cells individually dispersed or arranged in a single-file linear pattern in fibrous stroma [8]. Invasive carcinoma that do not describe these features (or the features of one of the other types of carcinoma) are considered to be IDC [7, 8] which therefore forms a heterogeneous group of tumors. Macroscopically, the tumors do not have specific features [7], but classically, ductal carcinomas are firm or even hard on palpation. The surface is usually grey-white with yellow streaks [7]. In contrast to IDC, ILC presents as a less distinct tumor that is less apparent on physical examination and mammography [8].

Invasive carcinoma are histopathologically graded based on assessment of tubule/gland formation, nuclear pleomorphism and mitotic counts. In the Bloom Richardson classification scale [9], a numerical scoring system of 1-3 per category is used

to ensure that each factor is assessed individually. The three values are added together to produce scores of 3-9, to which the grade is assigned as [7]:

- Grade 1: well differentiated (3-5 points);
- Grade 2: moderately differentiated: (6-7 points);
- Grade 3: poorly differentiated (8-9 points);

Therefore, the grade reflects how well the tissue is differentiated and thus how much it resembles the original breast tissue. Many studies have demonstrated a significant relation between histological grade and survival in invasive breast cancer: the higher the grade of the carcinoma, the lower the survival chances [7].

1.3 Breast imaging

In the description of breast imaging techniques, a differentiation between the two major breast imaging disciplines should be made: screening and diagnosis.

Breast cancer screening is usually performed using x-ray mammography. In the Netherlands, women between 50 and 75 years of age are invited every two years to participate in the screening program. The incidence of breast cancer in this age group can be seen in Figure 1.3. The screening mammograms are judged by two radiologists and in case of suspiciousness, women are referred for further diagnostic imaging.

Figure 1.3 The number of breast cancer diagnoses (in women) in 2011 per age group [3]. The black dashed box indicates the screening age range.

Diagnostic imaging is usually performed at specialized breast departments of the hospital (mammapoli's in Dutch). In addition to women referred from the national screening program, also women with self-detected abnormalities or women with a history of breast cancer are investigated at such departments. Breast abnormalities should be evaluated by

triple assessment including clinical examination, imaging and tissue sampling by either fine needle aspiration cytology (FNA) or core needle biopsy (CNB) [7]. The biopsy is performed under ultrasound guidance; however, in case the lesion is occult in US, a biopsy can also be performed using stereotactic x-ray guidance. Following histopathological assessment, in some cases diagnostic imaging is extended with Magnetic Resonance Imaging (MRI). Reasons to opt for such an investigation are:

- A high percentage of glandular tissue (high breast density) making it impossible to exclude the presence of more malignancies elsewhere in the breasts;
- Inconclusive conventional diagnosis;
- The need for assessment of lesion extent and its position with respect to the nipple, skin or pectoralis muscle for proper surgical planning;
- The need for characterization of the lesion preceding neoadjuvant chemotherapy.

The suspiciousness of the lesion in conventional images is graded according to the BI-RADS (Breast Imaging Reporting And Data System) classification [10], see Table 1.1

Table 1.1 *BI-RADS classification scale [10]*

BI-RADS	Description
0	Incomplete - image cannot be judged because of technical limitations
1	Negative - no lesion found
2	Benign finding - no malignant features; e.g. cyst
3	Probably benign finding - malignancy is highly unlikely; e.g. fibroadenoma
4	Suspicious abnormality - low to moderate probability of cancer
5	Highly suggestive for malignancy - almost certainly cancer
6	Proven malignancy ¹ - biopsy proven malignancy

¹ This classification is used for imaging that is performed after biopsy has proven the presence malignancy. For example, if MRI is being performed to check for the presence of more abnormalities elsewhere in the breast, the malignancy that has already been diagnosed is indicated as a BI-RADS 6 lesion.

All information from x-ray mammography, ultrasonography, MRI and histopathology is used to define treatment or follow-up strategies. Figure 1.4 shows the most common procedures in a flow diagram.

The different purposes of screening and diagnosis lead to different requirements for the imaging techniques. Where screening methods should be fast and relatively cheap, allowing for high throughput, this is less of importance for diagnostic modalities. For screening, the general consensus is that the sensitivity should be high and that this might be obtained at the cost of specificity. For diagnostic modalities, both sensitivity and specificity should be high, hence the multimodal approach in this discipline.

Figure 1.4 Flow diagram of breast imaging in the Netherlands. The most common pathways are described here. Patients without malignancies return to the normal screening program, for which they are invited every two years. Screening and diagnosis have a different function; therefore the requirements for the technologies are different.

1.4 Conventional breast imaging modalities

In the following sections and in Table 1.2, the three conventional breast imaging techniques are further explained together with their major advantages and disadvantages.

1.4.1 X-ray mammography

X-ray is the method of choice for breast cancer screening and diagnostic purposes. It is a relatively cheap and easy method. Above all, it is the only method found sensitive enough to be applied for screening [11]. The strength of x-ray is that it can detect breast cancer at a small size and early stage [12]. However, its specificity is still insufficient: a large number of the suspicious lesions on screening mammograms turn out to be noncancerous after call-back. Of all people that were referred for further diagnosis following screening in the Netherlands in 2009, only 30% was diagnosed with breast cancer [13]. Besides, x-ray uses potentially hazardous ionizing radiation and the required compression of the breast is painful [14]. The maximum tumor to normal contrast is small, especially in dense breast tissue [15]. This causes that x-ray has a prominent false negative rate [11]. Sensitivities

around 90% are reported in the older age groups (> 40 years) [16, 17], but sensitivities are significantly lower in young women (<40 years), with values decreasing to 70% or lower [16, 18]. This is mainly due to the great density of the glandular parenchyma in those women and the resulting low radio-sensitivity [17, 18].

1.4.2 *Ultrasonography*

Ultrasound (US) is an excellent method for assessing palpable abnormalities, differentiating between cystic and solid lesions and classifying solid masses. Moreover, it is the method of choice to guide core needle biopsies [17] and to investigate young patients with high (hereditary) risk for developing breast cancer. The resolution of US is good, but the soft tissue contrast is poor. The spatial resolution of modern breast ultrasound is in the range of ductal and lobular anatomy, making detection of lesions smaller than 1 cm possible [17]. One of the largest advantages of ultrasound is that it can detect malignancies in women with dense breast tissue [11].

Sensitivity and specificity numbers for breast cancer diagnosis are highly varying. Mostly a sensitivity of 80 to 90% is reported, but exact numbers depend on the type of the lesion and on the location of the lesion in the breast [16, 18, 19]. As was the case with x-ray, also US breast imaging suffers from a high rate of false positives. In addition to the non-perfect sensitivity and specificity, it is difficult to verify that the entire breast has been covered with US breast imaging and US suffers from a major inter-user variability [17]. At last, US does not give sufficient functional information about the tissue under investigation.

1.4.3 *MRI*

When tumors grow beyond the size of a few millimeters, their metabolic demand cannot be fulfilled by diffusion of oxygen and nutrients from the normal breast vasculature. The lack of sufficient nutrients and oxygen causes hypoxic stress in the tumor cells, which in turn can promote the formation of new vessels via the release of pro-angiogenic growth factors. This process is called angiogenesis [20] and will be explained in more detail in **Chapter 3**. The angiogenic process causes a higher density of blood vessels around the tumor than in other breast areas. Furthermore, tumor angiogenesis results in chaotic, structurally abnormal and permeable vessels that are distinct from the normal vasculature [21, 22].

Angiogenesis constitutes the basis for Dynamic Contrast Enhanced MRI (DCE-MRI) breast cancer imaging. In DCE-MRI, a T1 shortening contrast agent (gadolinium) is injected in the blood stream causing contrast enhancement in areas with greater vessel density. Furthermore, because of the altered structure and permeability of the abnormal vessels, the kinetic behavior of the contrast enhancement is altered [23]. The advantage of DCE-MRI is that it does not make use of ionizing radiation. Besides, DCE-MRI exhibits an excellent soft tissue resolution, uses a tomographic imaging principle and enables one to obtain multiple planes [17, 24]. DCE-MRI has very good sensitivity compared to X-ray mammography and ultrasound imaging [24, 25]. This sensitivity is not impaired by the

density of the fibroglandular tissue, nor by scar tissue, radiation therapy or breast reconstruction surgery [23].

There are, however, numerous disadvantages associated with DCE-MRI breast cancer imaging. The method cannot be used in pregnant patients, patients with pacemakers, implanted non-titanium metallic clips or with claustrophobia [24]. Moreover, the technique is time-consuming, the results are dependent on the hormonal status of the patient and the intravenous application of a paramagnetic contrast agent is required [26]. One of the major disadvantages of DCE-MRI is that its specificity is low. Not only the number of false positives is high, but MRI also often overestimates the tumor size and stage [24, 26, 27]. DCE-MRI is further described in **Chapter 3**.

Table 1.2 *Performances of conventional breast imaging techniques*

	X-ray mammography	Ultrasonography	Magnetic Resonance Imaging
Sensitivity ¹	Diagnosis: 85% [28]	79% [29]	88% [30]
Specificity ¹	Diagnosis: 93% [28]	88% [29]	68% [30]
Tumor-normal contrast	Poor	Poor	Reasonable
Resolution	Very good ~100 μm [31, 32]	Good < 0.5 mm	Good <1 mm voxel size [23] ²
Ionizing radiation	Yes	No	No
Contrast agents	No	No	Yes
Comfort	Poor	Good	Good-reasonable
Performance in young women	Significantly lower sensitivity and specificity in premenopausal patients [16]	Significantly higher sensitivity and specificity numbers than x-ray for women <45 years of age [33]	Comparable sensitivity and specificity numbers for pre- and postmenopausal patients [30] if imaging is being performed in the 2 nd week of the menstrual cycle in the premenopausal group
Acquisition time	Very fast (seconds)	Real-time, reasonable procedure time	Slow (>15 minutes)
Specific limitations	Not applicable in pregnant patients Projection (2D) technique	Operator dependence	Not applicable in patients with metallic implants, claustrophobic patients and pregnant patients. Dependence on the week of menstrual cycle of the patient

¹ Sensitivity and specificity numbers are highly varying throughout literature and strongly depend on the study set-up. More numbers on sensitivity and specificity and a discussion on the use and meaning of such numbers can be found in Chapter 3.

² Resolution numbers for MRI strongly depend on the system and imaging protocol used. Higher spatial resolution occurs at the cost of temporal resolution, making tumors with prominent wash-out less visible. Mostly in-plane pixel sizes of 0.5-1 mm are used [23]

1.5 Visualizing tumor vascularization using light

The former sections indicate the need for improvements, both in diagnostic as well as screening breast imaging. The high sensitivity of MRI for the visualization of breast cancer indicates that tumor vascularization is a good imaging hallmark for malignancy.

Far-red and near infrared (NIR) light (650-1100 nm) can travel across several centimeters of breast tissue due to low absorption by soft tissue chromophores in this wavelength region [34] as can be seen in Figure 1.5.

Figure 1.5 Between 700 and 900 nm, the sum of the total optical absorption in tissue by oxygenated blood, deoxygenated blood, water and lipid reaches a minimum, making this so-called therapeutic window ideal for medical imaging. Between 1000 and 1100 nm, local dips in the water and lipid absorption curves provide other opportunities for deep tissue imaging. Green: water [39]; Purple: lipid (mammalian fat [40]); red: oxygenated whole blood (45 % hematocrit (HCT), oxygen saturation (SO₂) >98% [41]); blue: deoxygenated blood (45% HCT, SO₂ 0% [41]).

The diffuse transmittance or reflectance of NIR light can provide information about the local absorption and scattering coefficients of tissue [35, 36]. Hemoglobin is a strong intrinsic absorber of NIR light, which causes the absorption coefficient of blood vessels to be significantly higher than that of surrounding glandular and adipose breast tissue [37]. The locally increased concentration of hemoglobin at malignant sites in combination with

the strong optical absorption of hemoglobin makes it potentially possible to visualize breast cancer with safe, non-invasive light, using so-called ‘optical imaging techniques’ [34]. Optical mammography is further explained in **Chapter 3**, where a review is given on imaging techniques that aim at visualizing tumor vascularization for detection and diagnosis of breast cancer. Despite the high intrinsic optical absorption contrast between healthy and malignant tissue, optical mammography is not the ideal method. The high scattering of light in biological tissue causes the technique to have a poor resolution beyond several mm’s in depth [38]. At a few centimeters depth, the resolution lies in the range of 3 mm and it worsens with depth [15, 38].

1.6 Photoacoustic imaging

A technique that also exploits the high optical contrast of malignancies is photoacoustic (PA) mammography (Figure 1.6). In PA mammography, the breast is illuminated by short pulses of laser light, usually in the NIR regime. The light will be absorbed by the increased amounts of hemoglobin at malignant sites. The resulting temperature increase leads, via the process of thermo-elastic expansion, to a pressure wave that propagates through the tissue and can be detected by ultrasound detectors with the appropriate frequency and bandwidth. The detection of the low scattered ultrasound rather than the highly scattered light, causes that the resolution problems of purely optical techniques can largely be overcome [42].

Figure 1.6 Photoacoustic imaging. Upon illumination of a highly scattering medium (e.g. breast tissue), the light can reach the malignancy where it will be absorbed by the local abundance of hemoglobin. The resulting temperature increase leads, via the process of thermo-elastic expansion, to a pressure wave, with its frequency in the ultrasound regime. The bipolar waves, with peak-to-peak time τ_{pp} , are characteristic for spherical absorbers with radius r_0 (Equation 1.4)

In photoacoustic or optoacoustic imaging, for light pulses shorter than the thermal and stress relaxation times, the initial pressure generated at the absorption site can be described by:

$$p_0 = E_a \Gamma, \quad (1.1)$$

with p_0 the initial pressure distribution (Pa), Γ the (dimensionless) Grüneisen coefficient and E_a the local absorbed energy density (Jm^{-3}), described by

$$E_a = F \mu_a, \quad (1.2)$$

where F is the optical fluence (Jm^{-2}) and μ_a is the optical absorption coefficient (m^{-1}). Γ is given as:

$$\Gamma = \beta c^2 / C_p, \quad (1.3)$$

with β the isobaric volume expansion coefficient in K^{-1} , c the speed of sound in the medium (m s^{-1}) and C_p the specific heat capacity at constant pressure in $\text{J}/(\text{K kg})$.

The duration of the photoacoustic signal and therefore the frequency of the generated ultrasound wave is dependent on the size of the optical absorbers. For a spherical absorber with radius r_0 a bipolar pulse is generated (see Figure 1.6), for which the peak to peak time τ_{pp} is described by [43]:

$$\tau_{pp} = \sqrt{2} \frac{r_0}{c} \quad (1.4)$$

1.6.1 Clinically applied photoacoustic breast imaging

Table 1.3 provides an overview of the most important clinical studies that exploit the PA effect for visualization of breast cancer. Oraevsky et al. [44, 45] were the first to publish clinical results using near infrared light PA imaging (NIR PAI) to visualize breast cancer as a consequence of the increased hemoglobin concentration at malignant sites. The Laser Optoacoustic Imaging System (LOIS), which uses an arc-shaped 32-element piezoelectric polyvinylidene fluoride (PVDF) transducer array in combination with either 757 or 1064 nm light excitation, provided 2D slices through the breast. Already in 2001, initial clinical studies showed the possibility of NIR PAI for visualizing breast malignancies with the PA amplitude of tumors at least twice the amplitude of the background tissue [44, 45]. Moreover, the PA contrast correlated with the oxygen saturation of hemoglobin in the tissue vasculature [45]. Further modifications of the system led to LOIS-64 for which clinical results were published in 2009 [46]. In this study 18 of 20 malignancies could be visualized [46].

In the early work of Kruger et al. [47], the term photoacoustic imaging is used equivalent to optoacoustic and thermoacoustic imaging, all having in common that incident energy in the form of photons is used for excitation, where the detected signals are in the ultrasound regime. Using radio- waves at 434 MHz for ultrasound generation in combination with three planar detection arrays of 128 elements in total, the rotating thermoacoustic computed tomography (TCT) scanner provided a uniform detection over a hemispherical surface, giving a near complete coverage of the entire breast [47]. Hereby, 3D maps of ionic water concentration in the breast could be provided. It is expected that the ionic water concentration is increased at malignant sites. A feasibility study in 5 breast cancer patients has been performed, showing contrast enhancement in three patients prior to chemotherapy and no contrast enhancement in the two patients after chemotherapy [47]. The hemispherical configuration has further been exploited in later photoacoustic tomography (PAT) systems. Using 800 or 756 nm light excitation in combination with 5 or 2 MHz ultrasound detectors, the PAT systems can provide highly detailed maps of the breast vasculature in healthy human subjects [48, 49].

Recently, Kitai et al. [50] published results on their photoacoustic mammoscope (PAM) which images a small (30x46 mm) region of interest (ROI) on the breast in forward mode, using multi-wavelength excitation (1064, 825, 797, 756 nm) in combination with a 1 MHz ultrasound detector array for detection. In this study, 20 of 27 malignancies could be visualized. The use of multiple wavelengths allowed for first estimations of the total hemoglobin content (Hb_T) and tissue saturation (StO_2) of the lesions [50].

1.6.2 PAM 1

Researchers of the Biomedical Photonic Imaging Group (BMPI) implemented the technique in the first prototype of the Twente Photoacoustic Mammoscope (PAM) in 2005, of which a schematic can be seen in Figure 1.7 [51].

The patient lies in prone position on the bed, with her breast pendant through an aperture. In PAM, the breast is slightly compressed between a glass plate for laser illumination at the cranial side of the breast and the US detector array at the caudal side of the breast. The laser pulses at 1064 nm, with 10 ns pulses at a repetition rate of 10 Hz. The overall absorption by tissue chromophores is relatively low at 1064 nm compared to other wavelengths in the NIR regime (Figure 1.5) allowing for high penetration depth of the light and thus relatively high F at the depth of the malignancy. Meanwhile the ratio between hemoglobin absorption and the absorption by other chromophores is high at 1064 nm, causing the high optical absorption (μ_a) contrast that is required. Signals are detected by a 1 MHz (130% bandwidth) unfocused ultrasound detector array. Assuming a speed of sound of 1540 m/s in breast tissue, this detector is most sensitive to structures between approximately 1 to 5 mm in size [52, 53]). All signals are reconstructed off-line, using a 3D reconstruction algorithm.

Table 1.3 Overview of the clinical studies using the photoacoustic effect for breast imaging.

<i>System</i>	<i>Ref</i>	<i>System description</i>	# <i>healthy</i> <i>subjects</i>	# <i>patients</i>	<i>Most important results</i>
< 2009					
TCT <i>Indiana</i> <i>University</i> <i>OptoSonics</i>	[47]	434 MHz excitation (thermography), 3 planar detection arrays (128 elements in total) to provide ionic water maps.		5	• In the three patients that were imaged prior to chemotherapy, contrast enhancement was observed. • In the two patients that had received chemotherapy, no contrast enhancement was observed.
LOIS-02 <i>University of</i> <i>Texas</i>	[44]	Single breast slice using arc-shaped transducer (32 elements), 1064 nm light excitation		5	• Photoacoustic tomography provides enhanced contrast between normal tissues and cancerous tumors.
LOIS-02 <i>University of</i> <i>Texas</i> <i>LaserSonics</i> <i>Technologies</i>	[45]	Single breast slice using arc-shaped transducer (32 elements), 757 and 1064 nm light excitation		Not mentioned	• Photoacoustic tomography provides substantially enhanced contrast between normal tissues and tumors. • The photoacoustic contrast correlates with the oxygen saturation of hemoglobin in the tumor microcirculation.
PAM <i>University of</i> <i>Twente</i>	[54]	Forward mode, detector array (590 elements), 1 MHz (130% bandwidth), 1064 nm excitation, 4x4 cm ² Field Of View (FOV)	-	13	• 4 of 5 malignancies visible.
LOIS-64 <i>Fairway</i> <i>Medical</i> <i>Technologies</i>	[55]	Single breast slice using arc-shaped transducer (64 elements), 1 MHz (bandwidth up to 2.5 MHz), 757 nm light excitation	-	27	• 18 of 20 malignancies visible.

Table 1.3 Overview of clinical PA breast imaging studies - continuation

<i>System</i>	<i>Ref</i>	<i>System description</i>	<i># healthy subjects</i>	<i># patients</i>	<i>Most important results</i>
2009-2013					
PAT <i>OptoSonics</i>	[49]	Hemispherical array, 5 MHz, 800 nm excitation	1	-	• Visualization of vascular anatomy with submillimeter spatial resolution.
PAM <i>Kyoto University Canon</i>	[50]	Rectangular detector array, 1 MHz, multiple wavelengths (1064 , 825, 797, 756 nm) for excitation	-	27	• 8 of 12 malignancies visible (not receiving systemic therapy). • 7 of 9 malignancies visible (receiving systemic therapy). • 5 of 5 DCIS¹ visible.
PAM <i>University of Twente</i>	[56]	Forward mode, detector array (590 elements), 1 MHz (130% bandwidth), 1064 nm excitation, 4x4 cm ² FOV	-	17	• 10 of 10 malignancies visible (See further Chapter 4).
PAT <i>OptoSonics</i>	[48]	2 MHz detector elements (512) in spiral scan configuration, 756 nm excitation	4	-	• In the four human volunteers, the vasculature was well visualized throughout the breast tissue, including to the chest wall.
PAM <i>University of Twente</i>	[57-59]	Forward mode, detector array (590 elements), 1 MHz (130% bandwidth), 1064 nm excitation, 9x8 cm ² FOV	-	43	• 32 of 33 malignancies visible (see further Chapter 7). • PA appearance can be largely related to tumor vasculature (see further Chapter 6). • 3 of 4 cysts visible with specific signature appearance (see further Chapter 5).

¹ Ductal Carcinoma in Situ

Figure 1.7 Schematic of the Twente Photoacoustic Mammoscope (PAM1) [51] © Institute of Physics and Engineering in Medicine.

1.6.3 First clinical measurements using PAM1

First studies with PAM1, showed the potential of the technique for breast imaging. In 2004 and 2005 it was seen that spheres with a diameter of less than 2 mm could be detected at a depth of 30 mm [51, 60]. In 2007, the first peer-reviewed results of NIR PA imaging of breast cancer in human subjects were published [54]. Thirteen subjects participated in this study, but seven of the measurements were not acceptable for further analysis due to technical and methodological problems. Improvements in the study design and inclusion methodologies made it possible to get five technical acceptable measurements on patients with breast malignancies. Of those, four cases revealed a higher PA contrast associated with tumor related vasculature [54]. These results demonstrated that NIR PA imaging has potential in the diagnosis of breast cancer [61] but that improvements to the study protocol should be made.

In order to get more information on the feasibility of PA breast cancer imaging, there was need for more patient data from carefully designed clinical studies comparing various imaging modalities. It needed to be investigated if the positive results could be repeated in a larger population. Questions that were pending from the previous studies were for example: what PA parameters can provide information on the presence of malignancy and how do patients experience the measurements? Furthermore, to get more information about the possible added value of PA mammography it was necessary that the PA images were compared with the results from conventional imaging and pathology outcomes.

1.7 Outline of this thesis

In 1983, two Australian surgeons observed the famous painting ‘Bathsheba at her bath’ (Rembrandt van Rijn, see Figure 2.1, Chapter 2) and noted, amongst others, a bluish shade on Bathsheba’s left breast. Based on this discoloration, the surgeons hypothesized that Bathsheba’s model might have suffered from breast cancer [62]. **Chapter 2** describes the results of Monte Carlo simulations, which were applied to answer the question if it is optically possible that breast cancer causes a bluish shade on the overlying skin. The painting of Rembrandt van Rijn is used as model to provide insights into the altered light-tissue interactions as a consequence of the increased vascularization at malignant sites.

In **Chapter 3**, a review is given on imaging techniques that aim at visualizing this tumor vascularization for detection and diagnosis of breast cancer. In addition to dynamic contrast enhanced magnetic resonance imaging (DCE-MRI), contrast enhanced digital mammography (CEDM) and contrast enhanced ultrasound (CEUS), also light-based techniques are described: diffuse optical tomography (DOT) and PA imaging. The latter forms the basis of this thesis.

A new clinical study was designed using PAM (Section 1.6.2). The clinical study was performed at the center for breast care of the Medisch Spectrum Twente in Oldenzaal. PA mammography measurements were embedded in the normal diagnostic routine as described in Figure 1.4. Based on the BI-RADS classification (Table 1.1) of the lesion, the lesion size and lesion depth, it was decided whether or not to ask the patient for cooperation to the study. PAM measurements were then performed in between conventional imaging and the ultrasound guided biopsy procedure (Figure 1.8). The focus of the study was primarily on patients with lesions that were highly suspicious for malignancy (**Chapters 4, 6 and 7**).

Imaging results were retrospectively compared to x-ray and US images and if applicable to results from MRI and histopathology. Due to technical constraints, in the first phase of the clinical study measurements were performed within a limited field of view (FOV) of $4 \times 4 \text{ cm}^2$ (25 minutes scan duration). In this study, the focus was therefore on palpable lesions between approximately 1 and 3 cm in size. To make first statements about the potential of the technique and the photoacoustic hallmarks of breast malignancies, mainly BI-RADS 5 lesions were measured. The results of ten measurements on malignancies and two negative controls on cysts are described in **Chapter 4**.

The promising results obtained in the first phase of the clinical study, motivated the continuation of the study and the associated modifications to the system. The pending questions at the end of the clinical study showed the need for large field of view measurements. Therefore, the system was updated in order to allow for measurements on palpable and non-palpable lesions in a region of interest of $9 \times 8 \text{ cm}^2$ with 10 minutes scan duration. Meanwhile, the photoacoustic appearance of benign cysts in the current imaging system was investigated using simulations and phantom and patient measurements (**Chapter 5**). In the continuation of the clinical study, the focus was on lesions which were suspect to highly suspect (BI-RADS 4, 5) for malignancy. The increased field of view

allowed us to perform more standardized measurements and to make better comparison with histopathology and MRI and conventional imaging possible. Results of these comparisons are described in **Chapter 6** and **Chapter 7**. In **Chapter 8** the results are discussed and recommendations for future research and future equipment are given.

Figure 1.8 The photoacoustic mammography measurements are embedded in the normal diagnostic routine.

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2

Monte Carlo simulations shed light on Bathsheba's suspect breast¹

In 1654, Rembrandt van Rijn painted his famous painting Bathsheba at her Bath. Over the years, the depiction of Bathsheba's left breast and especially the presence of local discoloration, has generated debate on whether Rembrandt's Bathsheba suffered from breast cancer. Historical, medical and artistic arguments appeared to be not sufficient to prove if Bathsheba's model truly suffered from breast cancer. However, the bluish discoloration of the breast is an intriguing aspect from a biomedical optics point of view that might help us ending the old debate. By using Monte Carlo simulations in combination with the retinex theory of color vision, we showed that is highly unlikely that breast cancer results in a local bluish discoloration of the skin as is present on Bathsheba's breast.

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2.1 Introduction

Rembrandt van Rijn (1606-1669) is one of the most celebrated portraitists in the history of art. He is famous for his depiction of emotion, attention to detail and the use of dramatic contrast of light and shadow. Rembrandt was, via his paintings, a great storyteller and he favored an almost disturbing realism in his often biblically themed work [1]. In 1654, Rembrandt painted *Bathsheba at her Bath* (Figure 2.1a, Louvre, Paris) depicting the story of the seduction of Bathsheba by King David from the second book of Samuel (11:2-4). Three centuries later, Braithwaite and Shugg [2] noted that Bathsheba's left breast in the painting appeared suspect. They described the condition of *peau d'orange* (where the skin takes on the texture of an orange peel) and skin discoloration. Further, they reported the presence of axillary fullness, as if the associated lymph nodes in the underarm were swollen, and a distortion in the symmetry of the breasts. The authors hypothesized that, based on these visual features, Rembrandt's Bathsheba was suffering from breast cancer [2].

The suggestion of Bathsheba's model having breast cancer has been generally accepted ever since and the painting is often used as a symbol for breast cancer. It even became the title of a book about the history of breast cancer in women [3].

But it was more: it was the beginning of an 'epidemic' of breast cancer among models of famous artists [4]. After Bathsheba, several other paintings were reported to represent signs of breast cancer [4-8]. One well-known example is *La Fornarina* (Figure 2.1c-d, Palazzo Barberini, Rome) from Raphael Sanzio da Urbino (1483-1520) of which the presence of deformation of the breast and blue skin discoloration led to the on art diagnosis of advanced breast cancer [5].

The suggestion of the presence of breast cancer in old paintings has also generated debate. Using historical, medical and artistic arguments, several authors have challenged these diagnoses [9-14]. Until now, the true answer to the question whether or not Rembrandt's Bathsheba suffered from breast cancer is unknown. However, the knowledge of light tissue interactions provides us one fundamental question whose answer could possibly help to end the debate: is it optically possible that a breast cancer mass manifests itself as a dark bluish shade on the skin as visible on Bathsheba's breast?

Breast cancer is generally associated with a pronounced vascularization caused by angiogenic processes responsible for growth and metabolism of the cancerous mass. This phenomenon is nowadays attracting attention for diagnosis [15] and therapy [16]. The abundance of hemoglobin in cancerous tissue makes it tempting to draw an analogy between the blue breast discoloration and the everyday appearance of blue skin overlying veins. It is known that blood is red because hemoglobin is a strong absorber of primarily blue and green light (Figure 2.2b); still one can experience the blue appearance of the veins on the ventral side of the wrists (Figure 2.2a). Using experiments and Monte Carlo simulations in combination with the retinex theory of color vision [17], Kienle et al. [18] succeeded in solving this contradiction.

Figure 2.1 a) *Bathsheba at her bath* as painted by Rembrandt van Rijn in 1654. b) The lower outer quadrant of Bathsheba's left breast shows deformation, peau d'orange, and discoloration, which are suggested to point at the presence of locally advanced breast cancer [2, 3]. c) *La Fornarina* from Raphael Sanzio da Urbina. d) A detail of *La Fornarina*'s left breast shows dimpling of the skin, pointing of the finger and skin discoloration [5], which are also hypothesized to indicate the presence of a breast cancer mass. All figures are reprinted, with permission, from the Bridgeman Art Library.

The blue appearance of a vein is not the consequence of the greater remission of blue light above a vein. Rather it is the greater decrease in red remission above the vessel compared to the lower decrease in remission of green and blue light [18]. Red light penetrates deeper into tissue than blue and green light as a consequence of the lower tissue optical absorption and scattering for longer wavelengths. If the vein is at an optimum depth, red light is absorbed by the hemoglobin, leading to a fall in the reflectance of red light above the vessel. Blue and green light, which do not reach the vessel, will not suffer from an appreciable change in remitted light [18].

This greater decrease in red-remission in itself does not explain the blue color of the skin overlying vessels, since at every position the most light is reflected in the red regime. According to Land's retinex theory of color vision [17, 20], it is, however, not purely the composition of the light from one area (the skin above the vessel) that specifies the color. One must also include the remission from other areas around it (the skin without vessel), since this information is used in higher order mental processing of the perceived color. In the retinex theory [18, 20], the relationship between spectral light remitted from an object and its color as perceived by a human observer is determined by a trio of numbers. These three lightness values (R_λ to be defined later in Section 2.2.3) are each computed for a

single wave band (short, middle and long), by comparing the reflected light from the skin above the vessel with the reflected light from the adjacent skin. Together, the lightness values specify a point in the so-called retinex three space that corresponds to a certain perceived color (see Figure 2.2c). The most positive (or least negative) lightness value determines the dominance of the associated color in the total color perception. If the dominance of one lightness value is not pronounced, the color appearance is less distinct. For the blood vessels there is hardly any difference in reflectance of green and blue light comparing areas with and without vessels. Lightness values for these two wavebands are therefore close to zero. However, the difference in red-remittance between the area with and without vessel is large due to the deeper penetration of the red light and absorption by the vein, as explained above. The lower reflectance above the vessel will lead to a negative lightness value (this will be explained further in Section 2.2.3) in the red waveband. Therefore, the contribution of the red light to the perceived color is minor and the blue and green are more dominant, resulting in the characteristic blue-green hue (Figure 2.2c, circle) on the skin above the vessel (Figure 2.2a).

Figure 2.2 **a)** A vein under the skin can appear blue, though blood is red. **b)** This blue appearance is the consequence of the deeper penetration of red light as a consequence of the lower hemoglobin absorption at wavelengths above 600 nm. The presence of a vessel influences the remission of red light, while the remission of green and blue light will be less affected by the presence of the vessel (data obtained from [19]). **c)** According to Land's theory of color vision, this color selective decrease in remission will result in a shift in retinex three space towards the blue-green colors. Here, R_s is the lightness value in the short wavelength range; R_m is the lightness value in the middle wavelength range and R_l is the reflectance in the long wavelength range. The black circle indicates the position where R_s and R_m are 0 (no change in reflectance of blue and green light between the skin with and without vessel) and R_l is negative because of the absorption of the red light by the vessel. The schematic of the retinex three space is based on [20], with permission from Elsevier.

Based on this theory, Kienle et al. [18] explained that the bluish appearance of the skin overlying the vein is due to the decrease in reflectance of the longer wavelengths above the vessel, as compared to the reflectance of the surrounding skin. This work beautifully explained the blue appearance of the small and superficial veins in skin. In the context of Bathsheba's breast, the following question arises: can a much deeper, bigger and less absorbing breast carcinoma cause a comparable blue shade on the skin?

In this chapter, we use Monte Carlo simulations in combination with the retinex theory of color vision to investigate if breast cancer masses at different depths can be perceived by the human eye and if so, with which color such regions will be manifested. By doing this, we want to answer the ever-pending question whether it is likely that the discoloration of Bathsheba's breast is the consequence of a breast cancer mass.

2.2 Materials and methods

2.2.1 Monte Carlo simulations

Monte Carlo simulations were performed using Montcarl 20.12 (MedPhys Software and Services [21]).

Figure 2.3 represents the model that is used for the Monte Carlo simulations in this study. The breast was modeled as two layers representing skin and breast tissue with thickness 2 and 60 mm respectively. The tissue layers were infinite in x and y direction. A spherical structure with radius r (3 or 5) mm at depth d (0.5; 1.0; 1.5; 2.0; 2.5; 3.0 or 3.5) mm under the surface representing the carcinoma was placed in the light scattering medium. The spherical structures are further referred to as 'carcinoma spheres'. The most superficial carcinoma spheres were partly embedded in the skin layer.

Figure 2.3 Multi-layered Monte Carlo model including carcinoma sphere.

For all simulations, the incident beam was square with a side of more than 80 mm entering through a beam diaphragm with a radius of 20 mm. The diaphragm was implemented in order to use the central homogeneous part of the beam while keeping the illumination area always much larger than the diameter of the carcinoma sphere and the average penetration depth of the photons.

The photons were incident perpendicular to the optically turbid medium representing the breast. For each combination of depth and radius of the carcinoma sphere, simulations were performed with three wavelengths: 450 nm, representing the blue region; 550 nm for green light; and 633 nm for the red wavelength region. Kienle et al. [18] proved that the depiction of these three wavelengths gives a reasonable approximation of color perception from human skin.

The optical properties of the skin, breast tissue and carcinoma sphere were defined for all three wavelengths. The absorption (μ_a) and scattering (μ_s) coefficients for the skin layer were the same as used in the model of Kienle et al. The μ_s for both breast tissue and breast cancer tissue at the three wavelengths were derived from *ex vivo* measurements reported by Ghosh et al.[22]. For μ_a we used the *in vivo* measured concentrations of Hb, HbO₂ and beta carotene (BC) [23] in combination with their molar extinction coefficients [19] rather than *ex vivo* measurements. For all tissue types, the Henyey-Greenstein (HG) phase function was used to calculate the scattering with the anisotropy factor g chosen as 0.9 for the skin [18], 0.88 for breast tissue and 0.96 for breast cancer tissue [18, 22]. The optical properties of the model are listed in Table 2.1.

For each combination of depth and size of the inclusion, and for each wavelength, photons were injected in the sample. A rectangular detector, having a numerical aperture (N.A.) of 0.5 was defined on top of the sample, extending for 20 mm and 30 mm in x direction for the 3 and 5 mm carcinoma spheres respectively, and for the diameter of the sphere in y direction (see Figure 2.3). The number of photons, either specularly or diffusely reflected from the tissue slab at the detector site, was stored. For each detected photon, the x- and y-coordinates at detection were saved. The simulation was terminated when the number of detected photons was 5,000,000 for the 3 mm sphere or 12,500,000 for the 5 mm sphere.

Table 2.1 Optical properties per wavelength and tissue type.

		Wavelength (nm)		
		450	550	633
Skin [18]	μ_a (mm ⁻¹)	0.18	0.15	0.025
	μ_s ' (mm ⁻¹)	3.00	2.00	1.00
	g	0.90	0.90	0.90
Breast [19, 22, 23]	μ_a (mm ⁻¹)	2.65	1.16	0.028
	μ_s ' (mm ⁻¹)	2.17	1.82	1.57
	g	0.88	0.88	0.88
Breast Carcinoma [19, 22, 23]	μ_a (mm ⁻¹)	5.71	2.76	0.130
	μ_s ' (mm ⁻¹)	3.15	2.84	2.62
	g	0.96	0.96	0.96

2.2.2 Analysis

Values for all the pixels with the same 'x' coordinate (see Figure 2.3), were summed and then displayed in a graph. In this way, the remission profile above the carcinoma sphere could be observed. The summation was used to smoothen the curve and to give the best apparent difference between regions with and without inclusion. To get a statistically sufficient number of photons, the number of detected photons per unit detector area was kept constant for all simulations. This resulted in a different number of emitted photons for the three wavelengths used. Therefore, a correction factor was applied to normalize the results and to make inter-wavelength comparison in terms of reflectance possible.

A Gaussian fit to the profile was performed using the Levenberg-Marquardt least chi-squared algorithm in Origin 7.5 (OriginLab) to determine the reflectance above the breast with and without the carcinoma sphere. The baseline of the Gaussian fit was used as an estimate for the number of detected photons above the breast without carcinoma sphere (I_{br}^λ) and the minimum of the fit was used as an estimate for the number of detected photons above the center of the carcinoma sphere (I_{ca}^λ). If the Gaussian fit did not result in a local minimum above the sphere, it was assumed that there was no difference in reflectance between the two regions and the curve was fitted to a constant value to obtain both I_{br}^λ and I_{ca}^λ .

2.2.3 Retinex theory of color vision

Analogous to the method of Kienle et al. [18], in this study the equation for calculation of the lightness values within the retinex theory of color vision was simplified to Eq. (2.1):

$$R_\lambda = \log_{10}(I_{ca}^\lambda / I_{br}^\lambda) \quad (2.1)$$

with R_λ the lightness value for the wavelength chosen; and I_{ca}^λ and I_{br}^λ the number of detected photons above the center of the symptomatic and above the non-symptomatic breast respectively. In this way, for each depth and size, three lightness values were obtained, representing the short, middle and long wavelength regimes. Since the μ_a of the carcinoma sphere was higher than the μ_a of the breast tissue (see Table 2.1), this always led to negative lightness values (less remitted photons above the carcinoma) if the photons were able to reach the carcinoma sphere. Moreover, in many cases the absolute lightness values were expected to be relatively small. From the obtained (negative) lightness values, the colors could be determined as follows [18]: the red colors in the retinex three space (Figure 2.2c) were found in the region where R_{450} and R_{550} are small (negative) and R_{633} is great (less negative or more positive); accordingly, the blue and green colors were obtained at great values of R_{450} and R_{550} respectively and for small values for the other two wave bands. If the dominance of one R value was not pronounced, the color appearance was less distinct. If the R values for all three wavelengths were similar, turquoise-gray colors could be found in the retinex three space [18].

2.2.4 Additional simulations

In order to verify the results from the simulations, an extra set of simulations was performed on vessel mimicking structures. The model in Figure 2.4a was used to repeat the simulations performed by Kienle et al. For this, a cylindrical structure with radius r (0.25 mm) and optical properties of venous blood (oxygen saturation 50%) was embedded at depth d (0.04; 0.08; 0.20; 0.35 or 0.50) mm in a medium mimicking the skin. A rectangular detector extending for 16 mm in x-direction and 6 mm in y-direction was used to detect the reflected photons.

The model in Figure 2.4b was used to perform simulations on a breast carcinoma that was infinitely large in x and y direction. Here, the carcinoma was modeled as a complete layer of carcinoma tissue (thickness 6 mm) at a depth d (0.5 -5 mm) below the skin. The number of photons that needed to be emitted to detect 5,000,000 photons at a rectangular detector above the skin was compared for the situation without cancer layer and with the cancer layer at different depths. The number of emitted and reflected photons for the cancerless model was used to calculate the total reflectance of the breast tissue. For each depth of the carcinoma layer, the average of the remission profile was estimated to obtain I_{ca}^λ . A simulation in the absence of a carcinoma layer was used to retrieve I_{br}^λ and to calculate R_λ .

Figure 2.4 Monte Carlo models for a vessel structure (a) and cancer layer (b).

2.3 Results

2.3.1 Venous blood vessels

In Table 2.2, it can be seen that the results of the simulations on small and superficial blood vessels (see MC model Figure 2.4a) show comparable lightness values as observed by Kienle et al [18].

Table 2.2 Results of simulations (lightness values and colors) on a small vessel below the skin.

Cylinder, radius 0.25 mm				
D(mm)	R_{450}	R_{550}	R_{633}	Color
0.04	-0.85	-0.81	-0.37	(Turquoise)** $\rightarrow$ red
0.04 [18]*	-0.82	-0.88	-0.34	(Turquoise) $\rightarrow$ red
0.08	-0.62	-0.66	-0.33	(Turquoise) $\rightarrow$ red
0.20	-0.35	-0.41	-0.25	Turquoise $\rightarrow$ red
0.20[18]*	-0.31	-0.38	-0.26	Turquoise $\rightarrow$ red
0.35	-0.17	-0.23	-0.18	Turquoise
0.50	-0.10	-0.15	-0.14	Turquoise $\rightarrow$ blue
0.50[18]*	-0.07	-0.12	-0.13	Turquoise $\rightarrow$ blue
Measurement *	-0.06	-0.10	-0.16	Blue
d~0.50 [18]				

* Results from current simulations are compared with results from previous simulations and measurements by Kienle et al. [18]. The latter are shown in italics.

**Colors within brackets are less pronounced.

2.3.2 Depth of cancer sphere

Figure 2.5 shows for every x-position of the detector the number of remitted photons from a breast-mimicking medium with a 3 mm carcinoma sphere embedded at different depths. The reflectance is affected by the presence of the carcinoma sphere, as can be seen by the decrease in reflectance above the cancer (Figure 2.5). In all cases, the remission in the red regime is highest, regardless of the presence and depth of the carcinoma sphere. For very superficial spheres (depth $d=0.5$ mm) the blue and green remission are affected more by the presence of the absorbing structure than the red remission (Figure 2.5a). This is indicated by the more negative lightness values (R) for 450 nm and 550 nm than for 633 nm as can be seen in Table 2.3. Therefore, according to the retinex theory of color vision, the skin above the sphere would be perceived as red.

Figure 2.5 Remission curve above a 3 mm radius carcinoma sphere at a depth of **a)** 0.5 mm, **b)** 1 mm, **c)** 1.5 mm and **d)** 3.5 mm. The remission curves are visualized for all three wavelengths. Symbols indicate the results from the simulations and the lines indicate their (Gaussian or constant) fit. Blue (dots)=450 nm; green (stars)= 550 nm; and red (crosses)=633 nm.

For somewhat deeper spheres ($d=1$ mm) the blue remission is affected less than the red and green remission (Figure 2.5b) and the consequence is a bluish shade over the skin as is indicated by the least negative lightness value for 450 nm (Table 2.3). Upon the illumination of even deeper spheres ($1 \text{ mm} < d < 3 \text{ mm}$), the blue and green remission are not affected by the presence of the sphere (Figure 2.5c), giving rise to a lightness value of 0.

Apparently, the sphere is beyond the penetration depth of the photons in these wavelength regions. However, red photons can still reach the spheres as results in a decrease in red remission above the sphere with respect to the adjacent regions. The result is that spheres at this depth cause a blue-green hue on the skin. Beyond a depth of 3.0 mm even the red light is not affected by the sphere and the sphere would be unperceivable to the human eye (Figure 2.5d).

Table 2.3 Simulations on the medium with carcinoma spheres. Results (lightness values and colors) for the simulations on carcinoma spheres with a radius of 3 mm are given.

Sphere, radius 3 mm				
Depth (mm)	R ₄₅₀	R ₅₅₀	R ₆₃₃	Color
0.5	-0.06	-0.08	-0.04	(Red) → turquoise
1.0	-0.01	-0.02	-0.03	Turquoise → (blue)
1.5	0	0	-0.02	Blue-green
2.0	0	0	-0.01	(Blue-green)
2.5	0	0	-0.01	(Blue-green)
3.0	0	0	0	Unperceivable
3.5	0	0	0	Unperceivable

The lightness values are much smaller than the lightness values in the simulations (and measurements [18]) on vessels which are known to cause a bluish shade on the skin (Table 2.2).

2.3.3 Size of the carcinoma model

The effect of the size of the carcinoma sphere on the apparent color was investigated. Tables 2.3 and 2.4 compare the results of the spheres with a radius of 3 and 5 mm respectively. The results are comparable, except for the shallowest position where the apparent color of the larger sphere is blue-shifted with respect to the smaller sphere. The depth at which the cancer is unperceivable is independent of the size of the sphere in this range of diameters.

Table 2.4 Simulations on the medium with 5 mm carcinoma spheres. Results (lightness values and colors) for the simulations on carcinoma spheres with a radius of 5 mm are given.

Sphere, radius 5 mm				
Depth (mm)	R ₄₅₀	R ₅₅₀	R ₆₃₃	Color
0.5	-0.05	-0.07	-0.05	Turquoise
1.0	-0.01	-0.02	-0.03	Turquoise → (blue)
1.5	0	0	-0.02	Blue-green
2.0	0	0	-0.01	(Blue-green)
2.5	0	0	-0.01	(Blue-green)
3.0	0	0	0	Unperceivable
3.5	0	0	0	Unperceivable

Table 2.5 gives the results of simulations on complete layers of carcinoma tissue (thickness 6 mm, see MC model Figure 2.4b) at different depths. These layers mimic a breast carcinoma mass which is infinitely large with respect to the viewing angle of the eye. This situation results in more negative lightness values at shallow depths as compared to the ‘small’ spheres, making the perceived color more pronounced. The carcinoma layer is perceivable until a depth of 5 mm, while the spheres are not perceivable beyond 3 mm.

Table 2.5 *Simulations on the medium with carcinoma layers. Results (reflectance, lightness values and colors) for the simulation on an infinitely large breast carcinoma, modeled as a complete layer (thickness 6 mm) of cancerous tissue are given.*

	Layer, thickness 6 mm						
	Reflectance (%)			Lightness Value (R)			Color
Depth (mm)	450 nm	550 nm	633 nm	450 nm	550 nm	633 nm	
No layer	32.4	28.6	48.5	-	-	-	-
0.5	22.0	16.8	37.9	-0.17	-0.23	-0.11	Red→ (turquoise)
1.0	30.0	24.4	40.0	-0.04	-0.07	-0.08	Blue
1.5	31.9	27.4	41.6	-0.01	-0.02	-0.07	Blue-green
2.0	32.4	28.5	42.8	0	0	-0.05	Blue-green
2.5	32.4	28.6	44.6	0	0	-0.04	Blue-green
3.0	32.4	28.6	45.8	0	0	-0.03	Blue-green
3.5	32.4	28.6	46.6	0	0	-0.02	Blue-green
4.0	32.4	28.6	47.2	0	0	-0.01	(Blue-green)
4.5	32.4	28.6	47.6	0	0	-0.01	(Blue-green)
5.0	32.4	28.6	47.9	0	0	-0.01	(Blue-green)
5.5	32.4	28.6	48.1	0	0	< -0.01	Unperceivable
6.0	32.4	28.6	48.3	0	0	< -0.01	Unperceivable

2.4 Discussion

Over the years, there has been much debate on whether Rembrandt's Bathsheba suffered from breast cancer. Until now, the discussion mainly focused on historical, medical and artistic arguments. In this work, we investigated the problem from a biomedical optics perspective for the first time. To answer the question if light interactions with cancerous tissue can cause a discoloration of the skin as present in the old paintings, we performed Monte Carlo simulations on simple models of breast cancer. The suitability and optical properties of the model are discussed in supplementary information [18, 19, 22-25].

The simulations on carcinoma spheres show that, as a consequence of wavelength dependent absorption and scattering, breast cancers can indeed appear slightly blue to the human observer as long as their surface is between a depth of 1 and 3 mm. Beyond a depth of 3 mm, most photons in the visible wavelength region do not reach the carcinoma which make the malignancy unperceivable. If closer than 1 mm, the mass would appear red. Layers of cancerous tissue, which mimic very large tumors, are perceivable beyond a depth of 3 mm, but will be unperceivable beyond 5 mm as well.

Ansari et al. [26] recently investigated the distance of breast cancer from the skin in 233 patients. Shallow lesion depths were reported, but the average (and median) distance from the skin was more than 9 mm, with more than 75% of the tumors beyond 5 mm in depth. This is highly comparable to the numbers Cunningham et al. [27] have published earlier. Although not impossible, it is very unlikely that a malignancy is positioned shallower than 3 mm, but meanwhile not closer than 1 mm from the skin or even invading through the skin.

Further, the lightness values of the cancer spheres are much smaller than the lightness values obtained for vessels in the skin, which means that the color difference between the cancer and the environment is less pronounced than for the blood vessels. This is largely due to the lower μ_a of the carcinoma, compared with the blood vessel. So, while a breast carcinoma mimicking mass can cause a bluish shade on the skin, this is only the case for very superficial malignancies and the bluish shade is much less pronounced than for blood vessels.

We can with some certainty conclude that the pronounced discolorations on Bathsheba's skin are not the direct consequence of the presence of a breast cancer lump. One should, however, be aware that in rare cases breast cancer can also lead to inflammatory type reactions of breast tissue by blocking of the lymph drainage. This inflammatory breast cancer is characterized by swelling, *peau d'orange* and discoloration of the skin [28]. However, the skin discoloration is diffuse, visible in at least one third of the breast and associated with a pronounced increase in breast size [28], which is not the case in Bathsheba's Breast.

In general, great care must be taken to interpret a painting of more than 350 years old without a comprehensive knowledge of the painting and varnish layers and their evolution in time. Discolorations can occur due to aging or other reasons, and only scientific analyses, for example by the use of multispectral techniques [29, 30], can provide us the details that would indicate if such a blue shade was intentionally painted by the artist or not. Such analyses have not yet been performed on the painting as far as authors know. However, the present work shows that, whether painted intentionally or not, the pronounced bluish discoloration visible on Bathsheba's left breast is unlikely to originate from an underlying breast cancer lump.

2.5 Conclusions

Rembrandt van Rijn's depiction of the biblical figure Bathsheba has generally been adopted as a symbol of breast cancer due to visual features purportedly symptomatic of the disease, including a discoloration on her breast. Monte Carlo simulations show that a bluish discoloration will appear only if the surface of a breast malignancy lies between 1 and 3 mm depth. Deeper lesions will not be perceivable, and more superficial lesions will appear red. However, from clinical practice it is known that most carcinomas occur at larger depths in the breast. Further, even if a malignancy is located favorably, the bluish appearance will be subtle and much less pronounced than the appearance of a vein. We can conclude that

from tissue optical considerations it is unlikely that Rembrandt's Bathsheba suffered from breast cancer.

2.6 References

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Supplementary information

Here we discuss the suitability of the model and the assumptions which were made.

Model

In reality there will be many tissue types associated with the breast, comprising amongst others: dermis, epidermis, subcutaneous tissue, fatty tissue and glandular tissue. Since malignancies develop in glandular tissue, and the layer of fatty tissue does not have the same thickness everywhere, we used a simple two-layer model consisting of one layer of skin-tissue (comprising dermis, epidermis and subcutaneous fat) and a bulk layer of glandular tissue.

The shape of the breast lump was modeled by either a sphere or a complete layer of cancerous tissue. The optical properties were assumed to be homogeneous within the different structures. We believe that this simplified model is sufficient to answer the questions posed and a more complex model will possibly have negligible influences on the results and conclusions.

Optical properties

In literature there are not much *in vivo* data on tissue optical properties in the visible wavelength region. The optical properties from breast and breast cancer tissue were obtained from two different studies. Data from Ghosh et al. [1] were used for μ_s and g , while data from van Veen et al [2], in combination with the extinction coefficients of (oxy)hemoglobin and beta-carotene [3] were used for μ_a . The combination of data as we applied it, was the only justifiable way to get a good representation of *in vivo* optical properties. The total reflectance from the breast tissue as determined with our simulations (Table 2.5) is comparable to the reflectance of the skin in Kienle et al's simulations and measurements (see Table s2.1). Since the skin of the breast has a comparable color as the skin of the wrist, these results indicate the applicability of the optical properties we choose.

Table s2.1 Reflectance from skin and breast tissue.

	Reflectance (%)		
	450 nm	550 nm	650 nm
Measurements skin [4] (approximation from graph)	35	30	48
Simulations skin [4] (approximation from graph)	32	29	47
Simulations on breast	32	29	49

One might argue that the absorption coefficient of the breast tissue in the red-regime is relatively high with respect to other values present in literature [5, 6]. This possibly influences the penetration depth of the photons and therefore also the depth at which a structure can be perceived with a blue tint. To test the influence of the relatively high μ_a for the breast tissue at 633 nm on the perceptibility of the carcinoma sphere, we repeated

simulations with a lower, though extreme, value for μ_a . Results show that the effect of lowering μ_a is negligible, leading to an increase in perceptible depth by only 0.5 mm.

Detector

Our detector had a numerical aperture (N.A.) of 0.5. With a pupil diameter of 8 mm, the N.A. of 0.5 corresponds to looking at an object at a distance of 8 mm. In realistic situations the distance between the object and our eye is much bigger, making the N.A. much smaller (close to 0.04 for viewing an object at 10 cm distance). However, with such a small N.A., simulations required the injection of large, impractical, numbers of photons. Therefore for three different depths, the influence on variations in N.A. was investigated. There are no differences in the N.A. from 0.7 to 0.1 for the 3 mm spheres (see Table s2.2). We assume that a further decrease in N.A. would not make a difference as well and this makes it justified to choose an N.A. of 0.5 for our detector.

Table s2.2 *The effect of the N.A. on the apparent color of a 3 mm diameter sphere.*

depth	N.A.	R ₄₅₀	R ₅₅₀	R ₆₃₃	Apparent color
0.5 mm	0.7	-0.06	-0.08	-0.04	Red
	0.5	-0.06	-0.09	-0.04	Red
	0.3	-0.06	-0.09	-0.04	Red
1.0 mm	0.7	-0.01	-0.02	-0.03	Turquoise → blue
	0.5	-0.01	-0.02	-0.03	Turquoise → blue
	0.3	-0.01	-0.02	-0.03	Turquoise → blue
	0.1	-0.01	-0.02	-0.03	Turquoise → blue
2.0 mm	0.7	0	0	-0.01	Blue-green
	0.5	0	0	-0.01	Blue-green
	0.3	0	0	-0.01	Blue-green

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3

Imaging tumor vascularization for detection and diagnosis of breast cancer²

Breast cancer is one of the major causes of morbidity and mortality in western women. Current screening and diagnostic imaging modalities, like x-ray mammography and ultrasonography, focus on morphological changes of breast tissue for early detection of the disease. However, these techniques still miss some cancers and often falsely detect cancer. The sensitivity and specificity for detecting the disease can probably be improved by focusing on the consequences of tumor angiogenesis: the increased microvessel density with altered vascular characteristics. In this review, various techniques for imaging breast tumor vasculature are discussed.

Dynamic contrast enhanced magnetic resonance imaging is the most-used imaging modality in this field. It has proven high sensitivity, but low specificity and cannot be applied in all women. Moreover, it has problems with detecting ductal carcinoma in situ (DCIS). Two other techniques under investigation for their potential are contrast enhanced digital mammography and contrast enhanced ultrasound. The first can detect DCIS, but requires the use of ionizing radiation and while the latter provides real-time information about true intravascular blood volume and flow, it still has difficulties with discriminating benign from malignant tissue. Moreover, these three imaging modalities all require the injection of contrast agents. Two relatively new techniques that do not use external contrast agents are diffuse optical imaging and photoacoustic imaging. Both visualize the increased concentration of hemoglobin in malignant tissue and thereby provide a high intrinsic contrast. Especially photoacoustic imaging is a promising technique for breast imaging, since it combines high ultrasound-like resolution with optical contrast and it is safe and non-invasive.

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3.1 Introduction

Although the death rates of breast cancer have decreased with approximately 40% in the last 20 years [1, 2], breast cancer is still one of the major causes of morbidity and mortality in western women aged over 40 years [3, 4]. Worldwide, every year more than 1,300,000 women are diagnosed with breast cancer and about 450,000 women die from this disease [2, 3]. The decrease in death rates is, in addition to an increased specialization of care and widespread availability of modern treatments, most likely due to the screening programs that started in the late 1980's [2]. Screening programs focus on an early detection of breast cancer, since the earlier the stage of the disease at detection, the earlier interventions can be applied and the better the survival chances [1]. Currently, the imaging modalities that are mostly used for screening and diagnostic purposes are x-ray mammography (MMG) and ultrasonography (US). These modalities visualize anatomic features of breast tissue in order to discriminate malignant from benign tissue. However, both in screening and diagnostic imaging programs, breast cancers are regularly missed and often falsely detected [5-7]. The sensitivity numbers for screening mammography lie approximately between 70% and 90% [8-10] and, although the numbers found in literature are highly varying, the specificity mostly does not exceed 90% [8, 10], resulting in positive prediction values around 20% or lower in certain patient groups [11, 12]

While the use of ultrasonography as an adjunct to x-ray mammography can improve the sensitivity in women with dense breasts from 73% to 85%, the combined specificity (40%) in this population is even lower than that of x-ray mammography alone [8, 13]. This might be an indication that focusing mainly on anatomical features is not sufficient for early breast cancer detection. Besides, tissue architecture does not provide enough information about the nature of the tumor, for example whether it is prone to become invasive, to grow or to metastasize.

For a breast tumor to develop and grow, certain functional changes to breast tissue are required. Those functional changes occur well before any morphologic alterations are visible on conventional x-ray mammography or ultrasound [14, 15]. One example of such a change is the increase in vascularization that is required for a tumor to grow beyond a few millimeters [16]. The best-known imaging modality that aims at visualizing this tumor vasculature is Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI). In breast cancer diagnosis, DCE-MRI is mostly used as an additional imaging modality when the combination of x-ray mammography and US does not provide sufficient information or cannot appropriately be applied. The sensitivity of DCE-MRI has proven to exceed the sensitivity of x-ray mammography and US, applied solely as well as in combination [17-20]. However, MRI has quite some limitations that make it inappropriate as a first-line imaging modality. MRI suffers, for example, from a low specificity and high costs and cannot be applied in, amongst others, pregnant women or women with metallic implants

[18]. The high sensitivity of DCE-MRI proves that imaging the tumor's vasculature can indeed provide information about the malignant characteristics of tissue. However, there is a search for a new modality that overcomes the limits of the currently used methods.

In this review, we will provide an overview of various breast imaging modalities that aim at visualizing the tumor's vasculature. Amongst these are dynamic contrast enhanced MRI (DCE-MRI), contrast enhanced digital mammography (CEDM), contrast enhanced ultrasound (CEUS) and diffuse optical imaging (DOI). We will highlight their working principles, merits and costs, while introducing a relatively new, non-invasive imaging technique that has potential in this field, namely photoacoustic mammography (PAM).

3.2 Tumor angiogenesis

Angiogenesis is the process in which new blood capillaries are developed by sprouting and branching from existing ones [21-23]. Since the diffusion limit for oxygen is only 100-200 μm [21, 23], rapidly expanding tumors, which are dependent on oxygen diffusion from the normal vasculature, become hypoxic and acidic and eventually die [21, 22]. Therefore, gaining access to the host vascular system by the formation of blood vessels is one of the critical factors for tumor survival and expansion [21, 24].

Tumor angiogenesis is linked to a switch in the equilibrium between positive and negative angiogenic regulators [24]. Before this angiogenic switch tips the balance to the side of the pro-angiogenic factors, tumors are in their dormant phase and no angiogenic processes occur. Due to the lack of oxygen and nutrients, the cellular proliferation rate is balanced by the apoptotic rate, and the tumor is unable to grow beyond a few millimeters [14, 21]. However, the starving and hypoxic tumor cells express high levels of (amongst others) hypoxia inducible factor (HIF), which induces the release of pro-angiogenic growth factors like vascular endothelial growth factors (VEGFs) [22, 25]. These pro-angiogenic growth factors switch the equilibrium to the angiogenic side and initiate the formation of new capillaries [21]. Until this stage, angiogenesis is a normal physiological process that also occurs in wound healing [26, 27]. In the normal angiogenic processes, there is a tight balance between pro-angiogenic and anti-angiogenic signals, allowing the new vessels to mature and become stable [24, 25]. On the contrary, in tumor angiogenesis, the loss in balance between positive and negative signals causes the vessel-growth to occur without stopping, enabling the constant growth of new tumor blood vessels. This supports rapid cell proliferation and permits a tumor to expand in mass [24, 25]. The resulting irregular and tortuous shape of the vessels and the abundant presence of shunts cause an abnormal and inefficient perfusion. The fact that tumor-associated capillaries lack sphincters and pericytes makes them largely independent from the normal mechanisms of flow-regulation. Moreover, fenestrated endothelial cells and tumor cells form the vessel lining and a basement membrane is missing, which causes tumor vessels to be more permeable than normal capillaries [21, 23, 24].

Imaging of tumor angiogenesis can focus on various stages of the angiogenic process. In molecular imaging techniques, the focus is on the molecules that are involved in angiogenesis [28]. In this review, the focus is on imaging techniques that visualize the results of the angiogenic processes: an increase in vascularization with altered vascular properties. Currently, the gold standard for measuring this end product of angiogenesis is a histological estimation of microvessel density (MVD) in which the average number of microvessels within a microscopic field is quantified after immunohistochemical staining with for example anti-CD31, anti-CD34 or anti-factor VIII/von Willbrandt factor [29]. Assessing the microvessel density is an invasive method and, besides, cannot be used to determine the vascular properties. Moreover, MVD measures will often overestimate or underestimate the degree of angiogenesis, since tumors are heterogeneous and MVD measures will vary according to the location from which the biopsy is taken [29, 30]. Therefore, the search is for a non-invasive method that will provide clinically relevant information about the tumor's vasculature.

3.3 Magnetic Resonance Imaging (MRI)

The gold standard and most-used modality for imaging tumor vascularization is currently, dynamic contrast enhanced MRI (DCE-MRI), which is often used as an additional method to x-ray mammography and ultrasonography [31] and is usually referred to as breast MRI. In DCE-MRI, a contrast agent that shortens the T1 recovery time (the spin-lattice relaxation time) is injected into the blood stream. This contrast agent is mostly a small molecular weight gadolinium-containing compound [32]

In areas with greater vessel density, contrast enhancement can be observed by using the appropriate imaging parameters. Morphologic information, for example the type of enhancement, mass shape and margins and the internal architecture, are of importance for differential diagnosis [33] as can also be seen in Figure 3.1a. Since the structure and permeability of the tumor vasculature are altered, the local kinetic behavior of the contrast is also altered [33]: the low molecular weight contrast media that leak slowly from the normal vasculature, diffuse more rapidly out of the tumor vessels. This results in the characteristic contrast dynamics associated with tumor vascularization: a rapid initial wash-in of contrast followed by wash-out on delayed images [29, 33] as can be seen in Figure 3.1b. Benign lesions are more associated with an initial increase in contrast followed by either progression or stabilization of contrast enhancement on delayed images [34]. Indeed, contrast enhancement and kinetic parameters in DCE-MRI have been shown to correlate with histopathologic measures for neo-angiogenesis [35]

The advantage of MRI is that it does not make use of ionizing radiation. Besides, DCE-MRI exhibits an excellent soft tissue resolution (voxel size $< 1 \text{ mm}^3$) [33] and uses a tomographic imaging principle [18, 19]. DCE-MRI has a very good sensitivity for invasive carcinoma compared to x-ray mammography and ultrasound imaging [18, 34, 36] with sensitivity numbers near 100% being reported [18, 37]. The sensitivity is not impaired by

the density of the fibroglandular tissue, nor by scar tissue, radiation therapy and the presence of breast implants [33]. However, the enhancement characteristics of DCE-MRI are unreliable for ductal carcinoma in situ (DCIS). Only about 70% of DCIS lesions show the characteristic rapid enhancement [33, 36, 38]. In fact, the kinetics of the contrast enhancement of DCIS sometimes correspond more to benign lesions than to malignancies, which lowers the sensitivity of the technique for diagnosing DCIS [33, 36]. However, the sensitivity for high-grade DCIS is still higher for MRI than for x-ray mammography [39] and in a recent study, MRI has proven to be able to detect 80% of the DCIS that was occult on x-ray mammography [38]

Figure 3.1 Example of a Dynamic Contrast Enhanced MRI image. **a)** T1 weighted image after gadolinium injection. This image shows a region with contrast enhancement (arrow) close to the nipple of the patient's right breast. The area is suggestive for the presence of an infiltrating malignancy. **b)** Dynamic contrast enhancement curve. This figure shows the percentage contrast enhancement (vertical axis) in the suspicious area as a function of time (horizontal axis, in minutes after contrast injection). The rapid wash-in (to 151% at four minutes) and a 20% wash-out (to 121% at seven minutes) of contrast in this region are suggestive for the presence of malignancy.

There are numerous disadvantages with respect to MRI breast cancer imaging. Firstly, the method cannot be used in claustrophobic patients, pregnant patients and in patients with pacemakers or implanted non-titanium metallic clips [18]. Secondly, it is a time-consuming and expensive method with a low throughput. An MRI investigation costs about five times the amount of a screening x-ray mammography [5, 40]. Thirdly, the high signal intensity of fat may obscure the lesion of interest. To overcome this, specific fat suppression sequences should be used, but they require an increased scanning time [34]. Finally, for DCE-MRI the intravenous application of a paramagnetic contrast agent is mandatory [5] and, although gadolinium has proven to be safe, every injection of contrast agent is associated with risks of adverse reactions [41].

Another disadvantage of DCE-MRI is its low specificity with numbers below 50% reported [42, 43]. Not only the number of false positives is high, but with DCE-MRI a risk for overestimation in size, stage and ductal spread exists [5, 18, 36, 44, 45]. The lack in

specificity of breast DCE-MRI is mainly caused by the fact that the contrast in DCE-MRI not only depends on the local amount of contrast agent and therefore on the vessel density, but also on the pulse-sequence settings and the characteristics of the contrast agent. Specifically the T1 contrast of the pulse-sequence used, the baseline T1 recovery of different tissues and of the contrast agent, and the diffusion of the contrast agent are of influence on the measured contrast [30]. The tissue even shows highly variable enhancement in normal breast parenchyma, which is referred to as background enhancement and depends on the patient's hormonal status [33, 34, 45, 46]. By far the most important source of false positive diagnoses is the fibroadenoma. This benign tumor demonstrates signal enhancement patterns similar to that of invasive cancer [47, 48] reflecting the significant overlap in the number of microvessels between invasive ductal carcinomas and fibroadenomas [48]. Although there are differences in the microvessel distribution within the lesions [48, 49], the fact that the MRI contrast agents leak out of the vasculature makes it very difficult to differentiate intravascular from extravascular contrast and therefore to visualize the small differences in microvessel distribution. Recently it has been shown, however, that special image sequences might be able to improve the diagnostic specificity of DCE-MRI [50].

3.4 Contrast enhanced digital mammography (CEDM)

X-ray mammography is the only breast screening modality that has been proven to reduce the death rates from breast cancer [51]. However, this morphology sensitive technique suffers from quite some limitations. Breast cancers are still missed by mammography [51, 52] and its accuracy is limited in dense breasts where it suffers from a low contrast between benign and malignant tissue [53, 54]. Imaging tumor angiogenesis with mammography has been investigated more than twenty years ago, using a technique called digital subtraction angiography (DSA) [54, 55]. In this technique, a contrast medium (usually iodine) was intravenously administered, followed by a set of radiographic views of the breast using an image intensifier system. It was thought that the presence of a blush (a localized prolonged retention of contrast medium) accompanied by abnormal vessel characteristics was indicative for malignant lesions [54, 55]. Indeed, abnormal tumor vasculature could be visualized with both planar DSA and contrast enhanced x-ray computed tomography (CT). The results of these techniques have been in close agreement with biopsy and pathologic findings [53-56]. However, both techniques did not prove to be clinically useful [52], mainly because of the high radiation exposure [54, 55], the lengthy imaging duration and implementation problems [57].

Digital mammography systems have been developed to enable rapid imaging and image processing. Those systems are therefore able to employ sophisticated techniques for imaging and localization of early breast cancer [57-59]. One of these techniques is contrast enhanced digital mammography (CEDM) in which, like in DCE-MRI and DSA, an intravenous contrast agent is administered to enhance malignant breast lesions [51]. The

most-used contrast agent is iodine, which provides a highly differentiating attenuation compared to tissue. Besides, iodine is non-toxic and has proven to be useful and safe in other vascular imaging procedures [57, 58].

Two different techniques of CEDM exist: the dual-energy technique and the temporal or dynamic subtraction technique [51, 52, 56, 58, 59]. In the temporal subtraction technique, the high-energy images are obtained before and at varying time points after the injection of contrast medium (Figure 3.2). Afterwards, the pre-contrast image is subtracted from the post-contrast images [59]. This technique can be compared with DCE-MRI and DSA, since it makes it possible to analyze the kinetic behavior of the contrast agent at the location of the lesion [59]. The images are acquired over several minutes with only slight breast compression, which makes the method highly prone to motion and displacement artifacts. Since the pre-contrast and post-contrast images need to be registered with high accuracy, motion artifacts have an impact on the final contrast [56, 57, 59, 60]. To overcome this problem, the breast can be more tightly compressed, which is uncomfortable for the patient and might also limit the blood flow and affect contrast enhancement [51, 60]. Since the breast cannot be repositioned, only one view can be obtained with a single injection [51, 52]. Therefore, the 3D location of an abnormality cannot be estimated with temporal CEDM [51].

In dual-energy CEDM, images are obtained by using energies both below and above the k-edge (33.2 keV) of the contrast agent [51, 52, 59, 60]. This technique makes use of the energy dependence of the x-ray attenuation through different ‘materials’, which are in this case iodine and soft tissue (Figure 3.3). Usually, for the low energy source a molybdenum target at peak-voltages of 22-33 kV is used in combination with a molybdenum filter; for the high-energy source, this is a rhodium target at 44-49 kV in combination with a rhodium filter [52]. A weighted subtraction of the two images is applied to minimize the visibility of breast tissue, while maximizing the visibility of iodine contrast agent. Therefore, this technique enhances the areas with contrast uptake [52, 59]. Compared to the temporal technique, the major advantage of dual-energy CEDM is its reduced sensitivity to motion artifacts, since the time between the different exposures is very short [59]. Because this technique does not require matching of pre-contrast and post-contrast images, multiple views can be obtained, making a more exact localization of the lesion possible [51, 52, 59]. Besides, contrast injection can be performed before breast compression is applied, circumventing the problem of influencing the vascular dynamics by breast compression [51, 61]. The fact that low and high energy views are required makes that the radiation exposure is higher for dual energy CEDM than for temporal CEDM [59]. Therefore the number of images performed after contrast injection is limited and no information about the kinetics of enhancement can be obtained [59]. Finally, since iodine contrast is present in both the low and high energy images, this approach can cause a small loss of iodine signal after the weighted subtraction techniques [51].

Figure 3.2 The principle of temporal subtraction contrast enhanced digital mammography (CEDM). High energy images are obtained before and at various time points after the injection of contrast medium. Afterwards, the pre-contrast image is subtracted from the post-contrast images. Reprinted and adapted with permission from [59].

Figure 3.3 The principle of dual energy subtraction contrast enhanced digital mammography (CEDM). Images are obtained by using energies both below and above the k -edge of iodine. The subtraction of the two images shows the iodine enhancement. Figure reprinted and adapted with permission from [59].

Preliminary studies have shown that CEDM might be a useful (adjunctive) technique for diagnosing breast cancer [62-64], especially in women with dense breasts [53], showing

sensitivity numbers above 80% and excellent correlations between histological tumor size and the size of contrast enhancement [59]. Specificity numbers vary between 60% (looking only at enhancement) and 90% (including morphologic information) [51, 58]. The fact that especially the low-energy source can also visualize microcalcifications makes the dual-energy CEDM potentially more sensitive to DCIS detection than DCE-MRI [52]. Because of the widespread availability of digital mammography, CEDM will become highly accessible and relatively inexpensive, especially when it is compared to MRI [53]. A recent study on the performance of CEDM, showed detection rates of known primary tumors comparable to DCE-MRI [62], however it remains to be seen if CEDM can exceed the sensitivity and resolution of MRI either in diagnosis or screening [51, 52, 59]. The most important difference with MRI is that CEDM is a projection technique; even with the injection of contrast agents, superimposition of adjacent breast tissue might influence sensitivity and specificity numbers. Therefore it is not expected that ordinary CEDM will replace MRI in the near future [51].

Digital breast tomosynthesis (DBT) is another relatively new digital x-ray technique in which a stationary detector and a moving x-ray source are used to obtain different projections under various angles [58, 61]. In this way, fast three dimensional information can be obtained [51]. Combining CEDM and digital breast tomosynthesis potentially overcomes most of the limitations of the former used in isolation. Both breast cancer morphology and vascular information can be provided, superimposition problems may be overcome and less compressive force needs to be applied. Indeed, preliminary studies proved the possibility of combining both techniques [56, 65] and statistically significant correlations between contrast agent kinetics for contrast enhanced breast tomosynthesis and DCE-MRI could be observed in a limited number of patients [65]. However, this combined technique is still very new and requires further examination before conclusions about its potential can be drawn [56].

3.5 Doppler ultrasound imaging and contrast enhanced ultrasound imaging (CEUS)

Breast ultrasound imaging is widely used as an adjunct to mammography. Ultrasound is an excellent method for assessing palpable abnormalities, differentiating between cystic and solid lesions, and classifying solid masses. Moreover, ultrasound imaging is the method of choice to guide core needle biopsies [19] and can improve the characterization of solid breast lesions as benign or malignant [66]. However, the specificity of this technique is quite low, it has limited ability to distinguish small lesions from fat lobules and suffers from an examiner dependent accuracy [67].

In an attempt to improve the diagnostic accuracy of ultrasound, tumor vascularity has been assessed non-invasively with both color Doppler and power Doppler ultrasonography [68, 69]. Color Doppler ultrasonography is based on the Doppler frequency shift between the transmitted and received acoustic pulse due to the movement of the blood cells through

the measured volume [70]. This method can evaluate blood flow and it is therefore useful in the differential diagnosis of breast lesions [70, 71]. However, the technique suffers from an angular dependency and poor signal to noise ratios [70], making it often unable to evaluate flow signals from small vessels (< 1 mm) in which the flow is low (3-5 cm/s) [69]. This flow approximately equals the tissue motion velocity, making the technique highly sensitive for motion artifacts caused by for example patient breathing [72, 73].

To overcome these limitations, power Doppler imaging was introduced in which the color map shows the integrated power of the Doppler signal. Power Doppler imaging is less prone to noise artifacts since noise has a very low power with respect to the information containing signal [70]. Power Doppler ultrasound is also much less angular dependent since the power in the Doppler spectrum is related to the number of red blood cells producing the Doppler shift [70]. These aspects increase the sensitivity of Doppler ultrasound: smaller frequency shifts and smaller numbers of intravascular reflectors can be measured [69]. Indeed, power Doppler ultrasonography has higher sensitivity ($>75\%$) and specificity ($>75\%$), and therefore higher diagnostic accuracy than color Doppler ultrasonography in the evaluation of palpable breast lesions [68, 69, 74]. Although ultrasound Doppler images are on average statistically different between benign and malignant lesions, groups overlap and consequently the technique has a weak diagnostic performance [66, 75]. Power Doppler ultrasound imaging still exhibits a low sensitivity for detecting flow in small tumor vessels (< 200 μm) and cannot distinguish small Doppler signals from background disturbances [66, 69, 71]. Despite the fact that Doppler ultrasound does not show the desired high predictive values and can therefore only be used as complementary technique to the ultrasound B-mode evaluation, it can be valuable in the investigation of (benign) breast conditions, for example in the evaluation of fibroadenoma, gross cysts or suspicious axillary lymph nodes [76]. This value can further be exploited by the combination of Doppler ultrasound with ultrasound elastography [77],

A newer approach in ultrasound imaging of angiogenesis is contrast-enhanced ultrasound (CEUS). Ultrasound contrast agents with microbubbles can be used to increase vascular signals in ultrasonography [73]. The micron-sized, gas-filled microbubbles are able to produce a 15-25 dB increase in the echo intensity of the blood flow signal [66]. This improves the Doppler assessment of the presence, morphology and flow of tumor microvessels in the breast [67, 71, 73]. The first microbubbles were simple air bubbles wrapped in an outer shell of protein or lipid, while current microbubbles encapsulate insoluble gas to make them remain more stable in the blood [78]. Microbubbles not only reflect sound at the fundamental (incoming) frequency, but also generate higher harmonics. This makes them suitable for harmonic imaging in addition to color and power Doppler ultrasonography [78].

Differences in contrast enhancement intensity and kinetics have been observed between benign and malignant lesions [79-81] making the technique potentially valuable for the differentiation of benign from malignant lesion, especially when combined with

conventional US imaging [79]. CEUS made detection of smaller vessels possible and improved the diagnostic accuracy, sensitivity and specificity of Doppler ultrasonography [30, 66, 69, 71, 73, 78]. Sensitivity and specificity numbers both exceeding 95% are reported [69]. One advantage of CEUS is that it is a real-time imaging method that is relatively inexpensive and readily repeatable [78]. The major difference with other contrast-enhanced imaging techniques, like DCE-MRI and CEDM, is that the microbubbles are true intravascular contrast agents: they do not diffuse into the extravascular compartment. Therefore, CEUS is a useful technique for measuring true blood flow and volume [30, 71, 82]. Especially with the use of newer contrast imaging techniques (like pulse inversion harmonic imaging and intermittent power Doppler), the enhancement parameters can give information about the microvessel density, the amount of shunting of the vessels and the heterogeneity of the vasculature [30, 66, 67, 73]. An example of a CEUS image can be seen in Figure 3.4.

Figure 3.4 Contrast US before and after contrast medium injection. 23mm ductal infiltrative carcinoma. **a)** B mode sonography. Spiculated mass, classified as highly suspicious for malignancy. Infiltrative feature is suspected because of the echogenicity of the surrounding fat (arrowhead). Microcalcifications are also visible within the tumor (arrow). **b)** Same patient. Contrast US including B mode and contrast mode (SonoVue®). The tumor is strongly enhanced after injection. Vessels are located in the peripheral area of the lesion. An area of the lesion remains poorly enhanced (arrow) which corresponds to necrosis. **c)** Same patient. Contrast US with CPS®, contrast mode only. Figure reprinted with permission from [73].

On the other hand, CEUS cannot provide any information on the permeability of the tumor capillaries [30, 71] and it presents some more limitations. Firstly, the microbubble contrast agents are present in the tumor vessels for a short time only, which hampers full breast scanning within the few minutes before the contrast agent washes out. Therefore, often multiple doses of contrast material are required to visualize all vascular phases of the contrast agent [30, 71, 78]. Secondly, CEUS cannot provide information about the entire breast like in DCE-MRI [30, 78]. Thirdly, the assessment of breast CEUS is subjective and not all studies have shown significant correlations between the CEUS image and histological microvessel density. The higher specificity of this technique sometimes occurred at the cost of a weaker sensitivity [66, 71, 73]. Finally, as was the case with DCE-MRI, contrast-enhancement can also be observed in some benign lesions, like hypervascularized inflammations and fibroadenoma. Those lesions may be mistaken for malignancy, while on the other hand low-grade hypovascular carcinoma might be diagnosed as benign tissue [69, 73].

3.6 Diffuse optical imaging (DOI)

Far-red and near infrared (NIR) light (650-1100 nm) can travel across several centimeters of breast tissue due to low absorption and scattering coefficients of soft tissue in this wavelength region [83]. The diffuse transmittance or reflectance of NIR light can provide information about the local absorption and scattering coefficients of tissue [84, 85]. Hemoglobin is a strong intrinsic absorber of NIR light, which causes the absorption coefficient of blood vessels to be much higher than that of surrounding glandular and adipose breast tissue [86]. Because of the angiogenic processes, the local concentration of hemoglobin is increased at malignant sites. Moreover, the oxygen saturation is decreased in rapidly expanding tumors due to their hypermetabolism [40]. Tissue scattering can be increased by an increase in the number density and size of subcellular organelles [40] and both are altered in malignancies. Consequently, the absorption and scattering properties of light at the NIR wavelengths can give information about cell sizes, blood dynamics, total hemoglobin concentration and blood oxygen saturation parameters, which could reveal the presence of breast malignancies [40, 87, 88].

Diffuse optical imaging (DOI) can be applied using three distinct methods: continuous wave, time domain or frequency domain imaging. In the continuous wave approach, the transmission of continuously delivered NIR light across the breast is measured. This technique is relatively inexpensive and rather compact, but it suffers from some disadvantages. The detected intensity is, for example, highly dependent on surface coupling and measurements of the intensity at a single wavelength alone are not sufficient to differentiate absorption from scattering interactions [83, 89, 90]. An example of a continuous wave DOI system for breast imaging is the Philips optical mammoscope [90], which was already tested for its feasibility in the late 1990's and proved to be able to detect a variety of inhomogeneities. However, the specificity of this technique is still poor [88,

90], with false positive rates up to 22% being reported in a group of women with proven breast malignancies [88].

In frequency domain measurements the light intensity is modulated at tens to hundreds of MHz. In this case, information about the optical properties can be calculated from amplitude decay and phase shift measurements of the signal [83]. In time domain methods, picosecond-pulsed laser light is used to measure the temporal distribution or times of flight of photons that exit the breast tissue to obtain information about optical properties of tissue [83, 91]. Time-domain and frequency domain measurements can theoretically express the same information, but both have their advantages and disadvantages. Frequency domain systems are relatively inexpensive, easy to develop and provide fast temporal sampling. In time-domain imaging, the information content of the signal is greater than can currently be measured in the frequency domain: the frequency content of the time of flight distributions extends to several GHz and it is not yet possible to modulate high-intensity sources at such high frequencies [89]. Moreover time-domain systems are far more sensitive than the frequency domain systems, making imaging at higher depths possible [89].

DOI for mammography can be divided in transillumination approaches and the so-called tomographic principles [83, 89]. In transillumination or planar imaging, source and detector pairs are arranged on opposite sides of the breast in order to obtain two-dimensional projection images of the tissue. This approach has advantages of easy implementation, easy reconstruction and comparable views to conventional mammographic images. However, this technique does not provide detailed information about the 3D spatial localization of the specific absorbers and usually requires breast compression [83]. Currently, most research focuses on diffuse optical tomography (DOT), which applies DOI in a tomographic manner, usually in time [84, 91] or frequency domain [40, 92]. Sources and detectors are placed over the available surface in order to record signals from multiple lines-of-sight across the entire volume [89]. Hereby, 3D maps of the optical properties of the breast can be obtained [83, 93]. The disadvantages are that more powerful sources and more sensitive detectors are required, as are non-linear reconstruction algorithms [93].

In recent studies with respect to breast cancer diagnosis, it has been shown that DOI is able to differentiate between benign and malignant tissue optical properties as can be seen in Figure 3.5. In some cases, malignant lesions that were smaller than 1 cm in diameter could be discriminated from normal glandular tissue [40]. This was primarily due to the higher hemoglobin contents, causing more light to be absorbed. However, also the scattering of light and the oxygen saturation suggested good discriminatory power [40, 87, 88, 94]. Advantages of DOI are the use of non-ionizing low energy electromagnetic radiation, continuous data acquisition and low price compared to MRI and x-ray mammography [83, 87]. Furthermore, DOT measurements can be applied with the patient in a prone position with reasonably good coverage of the breast, using only little breast compression [95, 96]. Molecular level information and submicroscopic structural information about the tissue function can be obtained with DOI and diffuse optical

spectroscopy (DOS) [40, 95]. In the optical imaging techniques, breast density seems not to be an issue, so it can also be used in younger women [87]. The greatest strength of the optical imaging techniques is that the absorption of NIR light by hemoglobin can provide one of the highest intrinsic biological contrasts possible. Sometimes the contrast reaches levels up to 200%, compared to only 10% increase in intensity for malignant with respect to normal tissue in x-ray mammography [97].

Figure 3.5 Breast MRI and frequency domain DOI of a 53 year old women with a 2.2 cm infiltrating ductal carcinoma. **a)** Sagittal DCE-MRI image and **b)** axial DCE-MRI image. The gadolinium contrast enhancement visualizes the malignancy. **c-e)** DOI images obtained from multispectral analysis of frequency domain data [40] representing relative total hemoglobin concentration, relative oxygenated hemoglobin and relative tissue scattering. The higher contrast in the indicated areas in Figure c, d and e, indicates that, amongst others, the relative total hemoglobin concentration, the relative oxygenated hemoglobin and the relative tissue scattering can give information about the presence of a malignancy. Image reprinted with permission from [40].

A disadvantage is that the optical mammography techniques have low spatial resolutions. Due to multiple scattering of NIR light, the achievable resolution is degraded to near 5 mm within a few centimeter of tissue and worsens with depth [86, 97]. In order to moderately improve the spatial resolution, the number of sources and detectors should be (impractically) large [40]. Another disadvantage of the DOT techniques is the difficulty of image reconstruction. An accurate quantification of optical properties relies heavily on the reconstruction algorithm and often a-priori information about the tumor and the surrounding tissue is required [40, 86].

The clinical applicability of the optical imaging techniques is limited by the heterogeneity of the optical properties of breast tissue between and even within patients. Tumor tissue exhibits heterogeneous levels of vascularization and oxygen saturation. The interpatient variations and variations throughout the different phases of the menstrual cycle can exceed the differences between benign and malignant lesions [98, 99]. Static, single shot optical images do neither provide information about the altered vascular characteristics nor about the mechanical changes of the malignant breast tissue. Therefore, some research centers investigate the application of dynamic imaging techniques to characterize the optical properties of the tissue. In dynamic optical imaging, external dynamic stimuli are introduced to measure relative changes in the tissue optical properties. Stimuli can be chemical, mechanical or physiological [99, 100]. Although only few studies to the use of dynamic stimuli in DOI of malignancies were applied, the results are promising: the external stimuli made better differentiation between benign and malignant lesions possible [87, 98, 99, 101]. Because of the disadvantages of diffuse optical imaging for detection of malignancies, such as the low spatial resolution and poor discriminative power, most studies currently focus on spectroscopic investigation of the tissue in order to investigate and predict the disease response to neoadjuvant chemotherapy rather than on diagnosis of breast cancer [102, 103].

Another approach is the use of fluorescent contrast agents in DOT [104]. Those fluorescent agents may improve lesion contrast and might therefore be useful in the early detection of breast cancer [105, 106]. It has been shown that DOT using a low concentration of a non-specific fluorescent contrast agent (Indocyanine Green [105] or Omocyanine [106]) is feasible and safe for breast cancer visualization in patients [105-107]. However, the technique requires the injection of fluorescent agent prior to the study. Moreover, since the contrast agents are non-specific, normal tissue could also show contrast enhancement. Therefore, development of probes with more specific binding to relevant tissue components is required to make diffuse optical imaging using fluorescent contrast agents clinical applicable [106].

3.7 Photoacoustic imaging (PAI)

As described in the previous section, the interactions of NIR light with tissue can provide a wealth of information about the tissue constituents, the presence of hemoglobin and the

oxygen saturation of the blood. Therefore, imaging techniques that are based on the absorption of NIR light in tissue can provide high contrast between vascularized and non-vascularized tissue. However, in pure optical based imaging techniques the spatial resolution degrades significantly with depth [108]. Photoacoustic imaging (PAI) is an upcoming technique that overcomes this limitation by combining optical excitation with ultrasound detection, exploiting the thermoelastic effect that occurs upon absorption of pulsed light [109-111]. With photoacoustic imaging, laser light in the NIR region is used for irradiating the tissue, like in optical imaging. The optical absorption of the pulsed laser light causes a local temperature increase of the tissue, followed by a thermal expansion leading to a pressure rise. The pressure relaxes as an ultrasound wave that propagates with minimal distortion to the surface where it can be detected using appropriate wideband ultrasound detectors [108, 109, 112, 113]. The time-of-flight, amplitude and peak to peak time of the photoacoustic signal depend on the amount of absorption of the laser light and on the size and position of the absorbing objects [114]. Those aspects are dependent on the composition of the tissue and on the local amount of vascularization and blood oxygenation. Therefore, the increased concentration of hemoglobin provides the main source of optical (and therefore photoacoustic) contrast for breast malignancies relative to normal tissue [115]. Because the detected signal is not the highly scattered light, but the much less scattered ultrasound, the resolution of PAI is superior to that of DOI. With PAI, one can potentially combine optical contrast with ultrasound resolution, without limitations of breast density, magnetic compatibility problems and the use of contrast agents or ionizing radiation [112, 115, 116]. This technique allows the future combination of pure ultrasound and photoacoustic measurements in one system [117-119], providing both morphologic and functional information about the breast tissue, while with photoacoustic spectroscopy information about the oxygen saturation of the blood can be obtained [120-122]. Analogous to diffuse optical imaging, also in photoacoustic imaging external contrast agents can be used for better imaging of the tumor's vasculature [123, 124]. More specific and higher absorption of the NIR light at tumor vasculature can result in detection of smaller and deeper tumors. Recent studies with animal models show the possibilities of using external contrast agents for imaging tumor vascular networks. Examples of photoacoustic contrast agents are dyes such as Evans blue [125] and nanoparticles such as gold nanorods [126, 127], gold nanobeacons [128] and carbon nanotubes [129].

Although it is possible to generate photoacoustic signals with intensity modulated continuous wave lasers [130], pulsed lasers are mostly used. Photoacoustic imaging using a pulsed laser usually has a higher signal to noise ratio and makes it possible to directly calculate the distance of the photoacoustic source from the detector [113]. Different imaging configurations can be used for photoacoustic imaging. With one technique a focused ultrasound transducer is used for time-resolved detection of the photoacoustic waves. By scanning the transducer over the tissue, 3D information of the underlying tissue can be obtained [109, 131]. This technique is the easiest to implement, however it takes

very long to scan large volumes and is therefore not useful for scanning the whole breast. For breast imaging, it is more convenient to use an array of unfocused ultrasonic transducers [109, 131, 132].

The maximum imaging depth is determined predominantly by light penetration in the breast, but is also influenced by the attenuation of the ultrasound. Mostly, light in the NIR region is used; NIR light penetrates relatively deep into tissue, while the absorption contrast of hemoglobin with respect to tissue remains high. The disadvantage of photoacoustic imaging is that, due to optical scattering and absorption, the maximum imaging depth is limited to about ~5 cm (with a resolution < 1 mm) [109]. Since the attenuation of ultrasound increases with frequency, human tissue serves as a low pass filter: the ultrasound attenuation changes the amplitude and waveform of signals from more deeply positioned sources. By decreasing the central frequency of the ultrasound detector, one can detect signals from deeper structures. However, only detecting lower frequencies also degrades the spatial resolution of the system. Therefore, the interplay between detector frequency and bandwidth highly influences the detection characteristics of the photoacoustic system [108, 109, 113, 133].

In the last years, several groups have studied the clinical utility of photoacoustic imaging in breast cancer diagnosis. Special instruments with different configurations have been developed to get the optimal resolution, imaging depth and contrast in breast tissue phantoms, small animals and healthy volunteers [114, 116, 134-141]. An example of the latter is shown in Figure 3.6 in which the microvasculature of the healthy breast can be clearly visualized with both high contrast and excellent resolution.

Figure 3.6 Maximum Intensity Projection of the photoacoustic tomography images of the breast of a 57 year old volunteer. **a)** Lateral projection and **b)** anterior-posterior projection. The square in Figure **a)** represents a $1 \times 1 \text{ cm}^2$ area. In this figure, a submillimeter vessel is visible to a depth of 40 mm (hollow arrow) beneath the front of the breast (two arrows). The images are obtained by using an 800 nm pulsed laser source and 128 5 MHz ultrasound detectors positioned in a hemispherical configuration. Imaging reprinted with permission from [136].

Also different clinical studies have been performed that showed the possibility to produce high-resolution clinical photoacoustic images suitable for the morphologic analysis of the tumor angiogenesis. [115, 142-145] An example of this can be seen in Figure 3.7. For more results regarding photoacoustic imaging of breast cancer, see Chapter 4, 6 and 7 of this thesis.

Figure 3.7 X-ray (a), ultrasound (b) and photoacoustic (c) images of a 50 year old woman with a palpable breast lesion. The photoacoustic image is obtained with the Twente Photoacoustic Mammoscope (PAM) in a clinical pilot study in 2006. A 1064 nm pulsed laser source is used in a transillumination approach with a planar array of 590 1 MHz ultrasound detectors. Both the x-ray and ultrasound images were highly suspicious for malignancy. The photoacoustic image also showed a region with higher signals, indicating a region with higher vascularity. The lesion was histopathologically determined as an infiltrating ductal carcinoma. Image reprinted with permission from [146].

There are considerable challenges evidenced by optical attenuation in the skin and breast tissues, acoustic artifacts and sensitivity and bandwidths of the transducer array [115, 132]. The only clinical studies that have been performed, use small patient groups [115, 132, 142, 144, 146]. Therefore, although the first clinical results using this technique are promising, it needs to be investigated if the positive results can be repeated in larger populations in order to get more information about the feasibility of photoacoustic breast cancer imaging.

3.8 Discussion

Techniques that focus on an altered tissue morphology, such as x-ray mammography and ultrasonography are often not able to discriminate benign from malignant lesions and often miss breast cancers. In order to improve the diagnostic accuracy of breast cancer detection, we believe that imaging modalities should focus on the results of tumor angiogenesis: a locally increased, but heterogeneous, microvessel density (MVD) with irregular and highly permeable vessels. This review described the use of Dynamic Contrast Enhanced - Magnetic Resonance Imaging (DCE-MRI), contrast enhanced digital mammography (CEDM), ultrasound techniques, diffuse optical imaging (DOI) and photoacoustic imaging (PAI) for imaging the breast tumor's vasculature. In Table 3.1, the advantages and

disadvantages per image modality are shown. These properties will be discussed in more detail in following sections.

Table 3.1 This Table summarizes the advantages and disadvantages per imaging technique that is described in this review.

	DCE-MRI	CEDM	Doppler US	CEUS	DOI	PAI
Resolution	++	++	+	+	-	+
Contrast	++	+	-	+/-	++	++
Sensitivity	++	+/-	+/-	+	*TBD	*TBD
Specificity	-	+/-	+/-	+/-	*TBD	*TBD
Applicability	+/-	+/-	+	+	+	+
Ionizing Radiation	N	Y	N	N	N	N
Contrast Agents	Y	Y	N	Y-	N	N
Assessment of MVD	+	+	+	+	+	+
Assessment of vessel characteristics	+	+	-	-	-	-
Full 3D breast scanning	+	-	-	-	+	+
Duration	-	+	+	+	-	-
Costs	-	+/-	+	+	+/-	+/-

*TBD: those properties are yet to be determined; ++ very good; + good; +/- reasonable; - poor; N= no requirement of ionizing radiation/contrast agents; Y=requirement of ionizing radiation/contrast agents.

3.8.1 Sensitivity and Specificity

Sensitivity and specificity numbers for various techniques are shown in Table 3.2.

Table 3.2 Sensitivity and specificity for diagnostic breast imaging. The performance of the conventional imaging methods, digital x-ray mammography(MMG) and US, are compared with the performances of DCE-MRI, CEDM and CEUS. Different studies are reported to show the high variation in sensitivity and specificity numbers. Sensitivity and specificity numbers for DOI and PAI are yet to be determined. *Specificity numbers not reported.

Technique:	Study	Number of patients	Sensitivity	Specificity
<i>x-ray (digital)</i>	Jensen 2010 [149]	93,585 (United States)	85.0%	93.2%
<i>mammography</i>	Jensen 2010 [149]	51,313 (Denmark)	82.4%	98.6%
<i>Ultrasonography</i>	Houssami 2005 [150]	480 (without x-ray knowledge)	79.4%	88.4%
	Houssami 2005 [150]	480 (with x-ray knowledge)	87.1%	85.2%
<i>Dynamic Contrast Enhanced Magnetic Resonance Imaging</i>	Bluemke 2004 [151]	821 (BIRADS 4-5)	88.1%	67.7%
<i>Contrast Enhanced Digital Mammography</i>	Dromain 2006 [59]	20	80%	*
	Diekmann 2009 [152]	80 (suspicious lesions on MMG/US or MRI)	62%	70%
<i>Contrast Enhanced Ultrasound</i>	Caproni 2010 [153]	39 (BIRADS 3-5 at MMG or US)	91%	73%
	Sorelli 2010 [147]	15	100%	37.5%
	Zhao 2010 [67]	15	86.6%	96.8%
	Liu 2008[71]	71 (combined with US)	95.3%	88.3%
		104		

Obviously, the values are highly varying throughout the literature. For example, Sorelli et al. [147] reported a sensitivity of 100% and a specificity of 37.5% for contrast enhanced ultrasound in the evaluation of palpable breast lesions, while Zhao et al. [67] reported a sensitivity of 86.7% and a specificity of 96.7% for this same technique. The variation in sensitivity and specificity can be caused by: 1) differences in the way the technique is being performed [37]; 2) different evaluation criteria [42]; 3) different study-populations [147-149]; and 4) the difference in the physician's knowledge about earlier imaging results [150]. Therefore, the sensitivity and specificity numbers given in this review are only rough indicators of the performance of a technique.

3.8.2 *Diagnosis*

When referring to sensitivity and specificity numbers, one should differentiate between screening and diagnostic imaging techniques. For diagnostic imaging of the breast's vasculature, dynamic contrast enhanced MRI (DCE-MRI) is the only technique that finds widespread clinical use. Indeed, it has been proven that the sensitivity of breast cancer diagnosis increased when DCE-MRI was used as an adjunct to x-ray mammography in case the mammogram proved to be inconclusive [154]. In addition to DCE-MRI, CEDM and CEUS also proved to be useful adjuncts to conventional imaging techniques [53, 67]. However, they do not (yet) find widespread clinical use, because of several disadvantages with respect to DCE-MRI. Sensitivity numbers of both CEDM and CEUS do not exceed those of DCE-MRI, while CEDM does require the use of ionizing radiation and both, currently, cannot provide a full 3D view of the breast. Diffuse optical imaging (DOI) and photoacoustic imaging (PAI) are still in early phases of development, but preliminary promising results on breast cancer diagnosis have been published [115, 146, 155] and will also be shown in later chapters of this thesis. Those techniques have the advantages that they do neither require the external injection of contrast agents, nor use ionizing radiation. Moreover, in contrast to DCE-MRI, DOI and PAI can be applied in almost all women. Especially photoacoustic imaging is promising, since it combines the high contrast of optical imaging with the good resolution of ultrasound.

One major difference between DCE-MRI and CEDM as compared to CEUS, DOI and PAI is that both DCE-MRI and CEDM not only provide information about the local vessel density, but also about the leakage of contrast agent from the vasculature to the surrounding tissue. Therefore, with those techniques crucial information on the permeability of the microvessels can be obtained. However, leakage of contrast agent out of the vasculature makes it impossible to measure true blood volume and flow and limits the differentiation between fibroadenoma and invasive carcinoma [48]. This can be one of the reasons that the specificity of DCE-MRI is rather low. CEUS, DOI and PAI, on the other hand, do image true intravascular space, because both microbubbles and hemoglobin do not leak out of the tumor vasculature. Especially with high resolution CEUS and PAI it should be possible to differentiate fibroadenoma from malignancies based on the different microvessel distributions within the two lesion types. Although DOI and PAI lack the crucial

information about the permeability of the microvessels, it is currently being investigated if dynamic optical or photoacoustic imaging can provide extra diagnostic information on the tissue's and microvessels' characteristics. This information could possibly increase the discriminating power of the techniques, without losing the advantages of imaging true intravascular contrast agent [87, 98, 99].

3.8.3 Screening

We have seen that imaging the tumor's vasculature as an adjunct to conventional imaging can provide extra information on the nature of the lesion, but it remains to be seen if those vascularization-imaging techniques can also be used as a screening modality. Currently, x-ray mammography is the method of choice for breast cancer screening since it is the only method that proved to reduce breast cancer death rates. This method is relatively easy to apply, cheap and fast, but it suffers from limitations of which one of the most important is the use of ionizing radiation. The specificity of screening x-ray mammography is too low: from the Dutch women that were referred for further diagnosis based on their first screening mammogram in 2007, only 30% had a true breast malignancy [156] and comparable numbers were found in a more recent study [12]. Moreover, x-ray mammography is a relatively uncomfortable method that requires rather large pressures to compress the breast. None of the other described imaging modalities is currently used for breast cancer screening in the conventional screening population. For obvious reasons, CEDM could not replace x-ray mammography, since it requires even more ionizing radiation and the injection of contrast agents. CEUS cannot provide a whole breast view and is too performer-dependent. Even DCE-MRI has not yet proven to be an adequate screening tool in isolation, especially because of its rather low specificity and lengthy measurement duration [157]. DCE-MRI is, on the other hand, used for screening young women with a high risk for developing breast cancer [31]. Those women cannot have an annual screening mammogram for two major reasons: 1) The overall life-time dose of x-rays would be unacceptably high, especially since those women are more susceptible for developing malignancies [31]; and 2) their denser breasts make the interpretation of the x-ray mammogram difficult, which lowers sensitivity and specificity numbers [157]. In Table 3.3, the sensitivity and specificity numbers for screening the high-risk population are shown.

The disadvantage of using DCE-MRI for annual screening in this population is that the method is time-consuming, expensive and not applicable in women who are pregnant or have metallic implants. Although more clinical data needs to be collected, DOI and PAI are potentially good methods for screening women with a high risk for breast cancer. Those methods are non-invasive, fast, can be applied in a prone position with relatively low compression and do neither require the use of ionizing radiation nor contrast agents.

It still has to be investigated if the information about the tumor's vasculature alone is specific enough to discriminate benign lesions from early signs of breast cancer. It was seen in both DCE-MRI and CEDM that specificity and sensitivity numbers significantly

improved when both the contrast enhancement and tissue morphology were used as evaluation criteria [42, 51]. In addition, the variations in optical properties of breast tissue within and between women often exceed those between benign and malignant tissue as was seen in DOI [98, 99]. Therefore, also PAI might have problems with differentiating benign tissue from early signs of breast cancer in the screening situation. However, with the future possibility of applying ultrasound transmission imaging and PAI in one system [161], the discriminating power is expected to be increased.

Table 3.3 Sensitivity and specificity numbers for screening for breast cancer in high-risk women. The only vascularization imaging method that is applied in this context is dynamic contrast enhanced magnetic resonance imaging. Its performance is compared with that of the conventional techniques: digital x-ray mammography and ultrasonography. *Specificity numbers not reported.

Technique:	Study	Number of patients	Sensitivity	Specificity
<i>x-ray(digital)</i> <i>mammography</i>	Sardanelli 2007 [158]	3,571	39.5%	*
	Kuhl 2005 [159]	529	32.6%	96.8%
	Kriege 2004 [160]	1,909	40%	95%
<i>Ultrasonography</i>	Sardanelli 2007 [158]	3,571	43.2%	*
	Kuhl 2005 [159]	529	39.5%	90.5%
<i>Dynamic Contrast</i> <i>Enhanced</i> <i>Magnetic Resonance</i> <i>Imaging</i>	Sardanelli 2007 [158]	3,571	80.7%	*
	Kuhl 2005 [159]	529	90.7%	97.2%
	Kriege 2004 [160]	1,909	71%	89.8%

3.9 Conclusions

Focusing on the tissue's vasculature is a useful adjunct to the conventional imaging techniques in the diagnosis of breast cancer. Nowadays, Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) is the only method that finds widespread clinical use. However, Diffuse Optical Imaging (DOI) and Photoacoustic Imaging (PAI) are relatively new techniques that have great potential in this field and partly overcome the disadvantages of DCE-MRI. PAI might also be useful in the annual screening of women with a high risk for breast cancer, especially when it is combined with ultrasound.

3.10 References

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4

Visualizing breast cancer using the Twente Photoacoustic Mammoscope: What do we learn from twelve new patient measurements?³

We acquired images of breast malignancies using the Twente photoacoustic mammoscope (PAM), to obtain more information about the clinical feasibility and limitations of photoacoustic mammography. Results were compared with conventional imaging and histopathology. Ten technically acceptable measurements on patients with malignancies and two measurements on patients with cysts were performed. In the reconstructed volumes of all ten malignant lesions, a confined region with high contrast with respect to the background could be seen. In all malignant cases, the PA contrast of the abnormality was higher than the contrast on x-ray mammography. The PA contrast appeared to be independent of the mammographically estimated breast density and was absent in the case of cysts. Technological improvements to the instrument and further studies on less suspicious lesions are planned to further investigate the potential of PAM.

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4.1 Introduction

Breast cancer is one of the most common forms of cancer among females and worldwide each year more than 450,000 women are diagnosed with the disease [1]. Conventional breast imaging techniques, like x-ray mammography and ultrasonography, primarily focus on morphological changes of breast tissue to discriminate benign from malignant tissue. However, sensitivity and specificity numbers for both diagnostic x-ray mammography and ultrasonography do not often exceed 85% [2]. Moreover x-ray mammography uses ionizing radiation and is not reliable in women with dense breasts and ultrasonography is strongly operator dependent [2].

Tumor angiogenesis is a critical process in tumor growth and spread. In angiogenesis, new microscopic blood vessels are formed that feed proliferating tumor cells and thereby support unregulated tumor expansion [3, 4]. Therefore, tumor vascularization is a crucial feature to be included in breast imaging [4]. While various techniques for imaging breast cancer related vascularization will be briefly discussed here, reference [5] provides a more complete overview.

One commonly used method that focuses on tumor vascularization is Dynamic Contrast Enhanced MRI (DCE-MRI). The high sensitivity of this technique for detecting breast cancer proves that vascularity can indeed provide additional information about the nature of tissue [6]. However, DCE-MRI suffers from a limited specificity, requires the injection of contrast agents and is relatively expensive [6].

The application of far-red and near-infrared (NIR) light in (non-invasively) visualizing cancer and its associated vasculature is gaining attention due to its ability to provide functional and molecular information without the use of ionizing radiation. In recent studies, it has been shown that optical imaging in the form of diffuse optical tomography (DOT) can indeed visualize breast malignancies [7], primarily because of the high absorption of hemoglobin in the NIR regime. However, DOT suffers from low spatial resolution. At a few centimeters depth, the resolution lies in the range of 3 mm and it worsens with depth [8, 9]. Several approaches are being investigated to improve the localization and resolution in DOT including using ultrasound guidance [10, 11], MRI [12] and x-ray mammography [13].

Photoacoustic imaging (PAI) also exploits the high NIR light absorption contrast between benign and malignant tissue, but provides superior resolution arising from ultrasound detection [14-17]. In photoacoustic imaging, nanoseconds pulsed laser light induces ultrasound transients due to the thermoelastic mechanism at optical absorbing sites. The pressure wave propagates to the surface where it is detected using wideband ultrasound detectors [14, 15, 18]. The amplitude of the photoacoustic signal depends on the amount of light absorbed in the object and the distance and material between the object and the detector; the time-of-flight and frequency content depend on the object's position and size, respectively. The detected signal is not the highly scattered light, but the much less

scattered ultrasound, and therefore the resolution of PAI is superior to that of pure optical techniques.

In the last years, several groups have studied the feasibility of photoacoustics in breast imaging. Special instruments with different configurations have been developed to get the optimal resolution, imaging depth and contrast in breast tissue phantoms and healthy human subjects [19-26]. Also a few clinical studies have been performed exploring the feasibility of producing high-resolution clinical photoacoustic images arising from tumor vascularization [19, 27]. Our own group developed the Twente Photoacoustic Mammoscope (PAM), to image the breast in transmission mode [22, 28]. In a first pilot study with this system in 2007, it was possible to get technically acceptable measurements on five patients with radiographically proven breast malignancies. Of those, four cases revealed a high photoacoustic contrast with respect to the background associated with tumor related vasculature [27, 28].

We have recently started an extended clinical study using PAM, as a continuation of the study performed in 2007. In the new study, we want to investigate the clinical feasibility of photoacoustic mammography in a larger group of patients with different types of breast lesions. Here we describe the results of the first phase of our study.

4.2 Materials and methods

The ethical review board of the Medisch Spectrum Twente (MST) approved the study protocol and informed consent procedure. The study has been registered in the Netherlands Trial Register (NTR) as TC 2945.

4.2.1 Patients

Patients were included from the Center for Breast Care of the Medisch Spectrum Twente hospital in Oldenzaal based on the BI-RADS® (Breast Imaging Reporting and Data System [29]) classification of their lesion. Between December 2010 and April 2011, female patients with a palpable breast lesion that was judged as being highly suspicious for malignancy (BI-RADS 5) on at least one of the conventional imaging modalities, were asked to participate to the study. Additionally, a selected number of patients exhibiting signs of cysts (BI-RADS 2) were also included in the study. Inclusion criteria were:

1. Age over 18 years and
2. The presence of a palpable BI-RADS 5 lesion or a cyst within an accessible region of the breast.

Exclusion criteria were:

1. Physical incapability to lie in a prone position for 30 minutes;
2. History of breast surgery or radiation therapy within a period of 5 years prior to this study;

3. History of breast biopsy within a period of 3 months prior to this study; and 4) incapability to give fully informed consent. All patients had to sign an informed consent form prior to the study.

4.2.2 Diagnostic procedure

All patients followed the normal diagnostic pathway at the center for breast care of the MST. A nurse practitioner performed clinical anamnesis and physical examination prior to conventional imaging. Cranio-caudal (cc) and medio-lateral-oblique (mlo) x-ray images of both breasts were made using the Lorad Selenia Full Field Digital Mammography System (Hologic, Bedford, USA). The breasts and axillas were also examined by a radiologist using a Philips IU22 ultrasound machine operating at 15 MHz (Philips Medical Systems, Best, The Netherlands). If the patient met the inclusion criteria and signed an informed consent form, she was measured using PAM in between the ultrasound examination and the ultrasound-guided core needle biopsy. Patients exhibiting signs of cysts did not get a core needle biopsy following the photoacoustic investigation. If additional imaging information was required for a proper diagnosis or treatment planning, patients were scheduled for a pre-operative dynamic contrast enhanced MRI investigation. Both high-resolution, anatomic T2 weighted images and dynamic T1 weighted images before and after gadolinium injection were obtained using a 1.5 Tesla MR system in combination with a 7 channel Sense Breast Coil (Philips Medical Systems, Best, The Netherlands). The x-ray, ultrasound and MRI images were interpreted by a breast imaging radiologist. This radiologist also estimated the lesion size on conventional imaging and the lesion depth on ultrasound imaging.

4.2.3 The Twente photoacoustic mammoscope

The Twente photoacoustic mammoscope (PAM, Figure 4.1) is built into a hospital bed on which the patient has to lie in a prone position with her breast pendant through an aperture [22, 28]. The breast is slightly compressed between a glass plate at the cranial side and the ultrasound detector array at the caudal side. The breast is illuminated through the glass plate with light from a Q-switched Nd:YAG laser (Continuum Surelite, California) at 1064 nm with 10 ns pulses at a repetition rate of 10 Hz. The illuminating beam scans a rectangular region across the breast. The photoacoustic signals that are generated are detected with a 1 MHz (130% bandwidth) unfocused 2D ultrasound detector array (Lunar Corporation, General Electric, Wisconsin) with a diameter of 80 mm. The detector's characteristics are described in detail elsewhere [28]. The photoacoustic signals are read into the PC on a dual channel digitizer (National Instruments, NI-5112, 100 MS/s, 8 bits). A Labview program (National Instruments) controls the scan stage movements and the element selection.

Information from the x-ray mammograms and ultrasound images was used to define a region of interest (ROI) on the breast. The ROI was usually chosen in the range of 40x40 mm², being a trade-off between scan area and scan time. With this size of the ROI, the total measurement time was always kept shorter than 25 minutes. Uncolored ultrasound gel

(Sonogel nr. 3010, Germany) was used for acoustic coupling between the breast and the detector. The breast was slightly compressed to ensure good contact with the detector. At the breast, the laser fluence in the 2.5 cm^2 spot was kept at approximately 25 mJ/cm^2 . For each position of the light delivery system within the ROI, the opposite detector element was activated and an average was taken over signals generated by 90 subsequent laser pulses.

Figure 4.1 *The Twente Photoacoustic Mammoscope. a) Aperture to insert breast. b) Ultrasound detector matrix. c) Glass window. d) Scanning system compartment. e) Q-switched Nd-YAG laser operated at 1064 nm with 10 ns pulses. f) Laser safety curtain which is drawn around the instrument during the measurements. g) Interface electronics between detector and computer. h) Linear stage carrying detector matrix driven by hand wheel to apply mild compression to the breast. i) laser remote control unit. j) laser power supply. Image adapted from reference [27] with permission.*

The signal processing and image reconstruction was done with Matlab (R2009b, the Mathworks Company). The offset from all individual signals was removed off-line and the signals were filtered with a low pass Butterworth filter (cut-off frequency 5 MHz) to remove high frequency noise.

The prominent breast surface signals were removed from the raw signals prior to reconstruction in order to have a more homogeneous dynamic range in the volume of interest. A modified acoustic backprojection algorithm was used to reconstruct the images. In this algorithm, the signals are back projected over spherical arcs onto their possible points of origin, taking into account the angular sensitivity of the elements [30, 31]. A homogeneous speed of sound (SOS) value of 1540 m/s was chosen for the breast. For the imaging configuration, maximum imaging depth and resolution (3.5 mm in both axial and lateral dimensions [22]) of PAM, the estimated [32] deterioration in resolution is not significant.

After reconstruction, the separate slices were Hilbert transformed and all voxel intensity values were scaled to the maximum intensity value within the total volume, giving a range of intensity values between 0 and 255.

4.2.4 Data analysis

The reconstructed PAM image volumes were analyzed for the presence of confined regions with high intensity. A subvolume was defined as that stack of transverse slices which, on visual inspection, completely included a confined high intensity region.

A threshold was chosen to be a percentage (usually 50%) of the maximum intensity value within this subvolume. The photoacoustic abnormality was subsequently defined as that region in which all voxel values exceeded this threshold. The contrast was defined as the ratio of the average intensity value within the abnormality and the average intensity value outside the abnormality. For each abnormality a maximum intensity projection (MIP) image was made. The size was defined as the maximum dimension of the abnormality measured on this MIP image.

The presence, size and shape of the photoacoustic abnormalities was compared with the lesion's appearance and size on conventional imaging as reported by specialized breast radiologists and, if applicable, with the histopathologically (H&E staining) assessed type and size of the lesion (gold standard). The radiologists estimated the depth of the lesion based on the ultrasound images. For each patient, the fibroglandular breast density was then estimated on the x-ray mammogram by a breast radiologist according to the BI-RADS breast density classification scale (Table 4.1 [29]).

Table 4.1 *BI-RADS breast density classification scale [29]*

	Percentage glandular tissue	Description
BI-RADS density 1	< 25%	The breast is almost entirely fat
BI-RADS density 2	25-50%	Scattered fibroglandular densities
BI-RADS density 3	51-75%	Heterogeneously dense breasts
BI-RADS density 4	>75%	Extremely dense breasts

The contrast of the lesion on x-ray mammography was estimated as the average intensity within an ROI in the malignancy divided by the average intensity within an ROI in the background tissue. The breast density was compared with the lesion's contrast seen on both the photoacoustic images and the x-ray mammograms. For both PAM and x-ray mammography, the contrast between the low and high density groups was tested for significant difference using a Student's two-sample t-test.

4.3 Results

In the period December 2010 - April 2011, seventeen Caucasian patients were included in the study. Three patients could not be measured either because of discomfort while lying on the bed or because their lesion was difficult to access completely in the scan region. Of the remaining 14 cases, two were technically unacceptable due to poor acoustic contact between breast and detector in the ROI. Ten of the successful measurements were on highly suspect breasts carrying one or more BI-RADS 5 lesions and two were on breasts with cysts. Table 4.2 shows an overview of the patients studied.

Table 4.2 Overview of the patients measured in phase 1 of the study*

IDC = Infiltrating Ductal Carcinoma and ILC=Infiltrating Lobular Carcinoma. The lesion size is estimated microscopically during histopathological investigation of the tissue specimen post-surgery, the lesion depth is estimated on the ultrasound (US) images as the shortest distance from the skin to the lesion.

Patient	Age (y)	BI-RADS breast density	Lesion type	Lesion size (histopathology)	Lesion depth (US)
1	78	1	Mixed IDC, ILC	30 mm	< 5 mm
4	67	4	IDC	15 mm	10 mm
5	55	3	ILC	42 mm	10 mm
6	84	3	IDC	13 mm	10 mm
7	57	1	Mixed IDC, ILC	27 mm	13 mm
9	64	2	IDC	31 mm	10 mm
10	49	4	IDC	> 30 mm**	< 5 mm
11	43	4	Cyst	28 mm**	10 mm
14	54	1	IDC	> 40 mm**	< 5 mm
15	48	4	Cyst	42 mm**	5 mm
16	71	2	IDC	33 mm	5 mm
17	68	1	IDC	27 mm	>30 mm

* Only the patient information from the twelve technical acceptable measurements is shown here.

** In patients 10-15, the lesion size was estimated radiologically, since there was no histopathological measurement available.

In the following sections, the results of three representative patient measurements are discussed.

4.3.1 Case 1 – infiltrating ductal carcinoma

In this case, a 67 year old woman (patient 4 in Table 4.2 and 4.3) had a palpable lesion in the medial upper quadrant of her right breast. The x-ray image of this lesion is shown in Figure 4.2(a). Based on the left-right asymmetry and the presence of a 20 mm lesion (white square) with a suspect calcification in this relatively dense breast tissue, the lesion was judged to be highly suspicious for malignancy (BI-RADS 5). The contrast of the lesion with respect to the fatty tissue was 2.6, while the contrast of the lesion with respect to the fibroglandular tissue was close to 1. The ultrasound image (Figure 4.2(b)) showed a 17.5 mm hypoechoic lesion (arrow) 10 mm below the skin. On ultrasound, the lesion was judged as being suspicious for malignancy (BI-RADS 4).

Transversal slices through the reconstructed photoacoustic volume at the expected lesion depth (Figure 4.2(d)) showed the presence of a high-contrast region. Based on the photoacoustic MIP image, the maximum diameter of this abnormality was 10 mm when a 50% threshold was taken to define the abnormality. Figure 4.2(e) shows a schematic of the imaging planes of the different imaging modalities that are compared throughout this paper. Note that the x-ray, MRI and PAM images are all from the transverse plane; the MRI and PAM images are always rotated to match the orientation of the cranio-caudal x-ray image.

After histopathological confirmation of the presence of an infiltrating ductal carcinoma (IDC), the patient was scheduled for a preoperative MRI. The T2 weighted MRI images showed relatively dense breast structures without cystic changes. T1 post-contrast images confirmed the presence of the malignancy medial in the right breast (white square)

with a maximum diameter of 18 mm (Figure 4.2(c)). The post-surgical histopathological assessment of the lesion revealed the presence of a 15 mm, grade 2 (on the Bloom-Richardson scale), infiltrating ductal carcinoma.

Figure 4.2 Case 1: Diagnostic images of a 15mm infiltrating ductal carcinoma in the right breast of a 67 year old woman. **a)** The cranio-caudal (cc) x-ray mammogram shows a 20 mm lesion with a calcification (white box) and is highly suspicious for malignancy. **b)** The ultrasound image shows a 17.5 mm hypoechoic lesion (arrow). **c)** The transverse view of the T1 weighted MRI after gadolinium injection confirms the presence of malignancy because of the enhancement of an 18 mm lesion (white box) in the medial upper quadrant of the right breast. This image is rotated to match the orientation of the cc x-ray view in figure (a). **d)** A transversal cross-section with a slice-thickness of 0.24 mm through the photoacoustic volume at the expected lesion location shows a confined region with high contrast with respect to the background. With the chosen threshold for abnormality definition, the contrast of the abnormality in the 3D volume is 6.4 and the maximum diameter is 10 mm. This image is rotated to match the orientation of the cc x-ray view in figure (a). **e)** The imaging planes of the different imaging modalities used in this paper. Indicated are the imaging planes for cranio-caudal x-ray mammography, transverse MRI, transverse PAM and a representative ultrasound view. Imaging planes for MRI, x-ray and PAM are comparable, but the region of interest of PAM is small compared to that of MRI and x-ray, which image the complete breast. The imaging plane of ultrasound is dependent on the position of the ultrasound probe.

4.3.1.1 Case 2 – ductal carcinoma in situ with infiltrating foci

This 64 year old woman (patient 9 in Table 4.2 and 4.3) arrived at the Center for Breast Care following a suspicious screening mammogram. A 50 mm smooth and mobile mass could be palpated in the lateral quadrant of the right breast, which was not directly suggestive for malignancy. The cranio-caudal x-ray image of this patient's right breast (Figure 4.3(a)) showed atypical and suspicious microcalcifications in the lateral upper quadrant (white square). This area was assigned as BI-RADS 5. The ultrasound images

(Figure 4.3(b)) showed a large, inhomogeneous abnormality with calcifications in this same region at 9 o'clock (white square). In contrast to the mammogram, the ultrasound image was most suggestive for a benign fibroadenoma. The 30 mm large lesion was judged as BI-RADS 3 (probably benign lesion). Close to this lesion, a comparable, but smaller, lesion could be located (not visible in this image).

The reconstructed photoacoustic volume (Figure 4.3(d-f)) showed two abnormalities, separated by less than 10 mm. The contrast and sizes of the lesions can be found in Table 4.3.

Figure 4.3 Case 2: Diagnostic images of a 31 mm infiltrating ductal carcinoma in the right breast of a 64 year old woman. **a)** The cranio-caudal mammogram of the right breast shows a large region with atypical and suspicious microcalcifications (white square). **b)** The ultrasound image shows a large inhomogeneous lobed mass (white square) with microcalcifications, which is somewhat suggestive for a benign fibroadenoma. Close to this large lesion, there is a second comparable, but smaller lesion (not visible in this image). **c)** The T1 weighted contrast enhanced MRI shows two lesions in the lateral quadrant of the right breast. The biggest lesion (white square) is visible in this image and measures 34 mm, the second, smaller lesion (14 mm) is positioned caudal to this lesion and is not visualized here. **d)** Photoacoustic imaging also shows two abnormalities, separated less than 10 mm. **e)** The upper abnormality (5 mm depth) has a contrast of 4.7 and a maximum diameter of 26 mm and can be seen in this transversal cross-section (slice thickness 0.24 mm). **f)** The smaller, lower (13 mm depth) abnormality had a contrast of 5.3 and a maximum diameter of 14 mm.

Histopathological assessment of the biopsy revealed signs of ductal carcinoma *in situ* (DCIS) with focalized IDC. A preoperative MRI revealed an extended lobed lesion in the contrast-enhanced T1-weighted images of the right breast in the lateral quadrant (Figure 4.3(c), white square). The lesion had a maximum diameter of 34 mm and showed a type 3 enhancement pattern [33] (rapid increase of contrast enhancement with fast wash-out), corresponding to the presence of an infiltrating carcinoma. Caudal to this lesion (not seen in

this image) and also in the lateral part of the breast, there was a second lobed lesion with a maximum diameter of 14 mm and also a type 3 enhancement pattern.

Post-surgical histopathological investigation of the breast, revealed the presence of a 31 mm grade 3 IDC growing in irregular fields and associated with extensive DCIS. The two lesions that could be seen on photoacoustic imaging, MRI and US, probably represented two invasive foci of this large abnormal region.

4.3.2 Case 3 – mixed infiltrating lobular and ductal carcinoma

This 78 year old woman (patient 1 in Table 4.2 and 4.3) had a palpable mass behind the nipple of her left breast. Visual inspection showed reddening and thickening of the skin close to the nipple. On palpation a 20 mm palpable mass could be felt behind this lesion. On x-ray mammography (Figure 4.4(a)), the breasts were relatively transparent, but there was a clear left-right asymmetry. Behind the nipple (green square) of the left breast, a region of dense breast tissue (blue-dashed square, lesion 1), associated with some microcalcifications, was identified; this was most likely the palpable lesion. In addition, there was a density close to the nipple (white square, lesion 2), likely the lesion observed on visual inspection. The two lesions were assigned as BI-RADS 4 and BI-RADS 5, respectively. On ultrasound (Figure 4.4(b)) only one lesion could be identified: an oval-shaped lesion in the skin just above the nipple (white square). The diameter of this lesion was 18 mm and the lesion was assigned as a BI-RADS 5 lesion. In the remainder of the breast, there were no signs for malignancy.

For photoacoustic imaging, the breast required support by a block and slight tilting to achieve good contact with the detector at the ROI. In contrast to other measurements, the surface signal was not removed before reconstruction, since the most suspicious lesion was positioned close to the breast surface. In Figure 4.4(c) and Figure 4.4(d), two transversal slices through the reconstructed photoacoustic volume are shown. Near the surface a 14 mm abnormality could be observed (Figure 4.4(c), white square). In depth, this abnormality was connected to a second abnormality (Figure 4.4(d), blue-dashed square). A third region could also be observed at this depth (Figure 4.4(d), green-dotted square). We hypothesize that the photoacoustic images show one malignant lesion with a component close to the skin (Figure 4.4(c), white square) and a deeper component (Figure 4.4(d), blue-dashed square). Please note that by x-ray mammography only two separate lesions and by ultrasonography only one superficial lesion could be observed. The macroscopic and microscopic investigation of the tissue specimen after mastectomy showed indeed one 36 mm, grade 2, mixed infiltrating ductal and lobular carcinoma (mixed IDC and ILC), which was almost attached to the skin of the nipple.

Based on its position in the image, the third region (Figure 4.4(d), green-dotted square) was thought to represent the nipple-areola complex. It is known that the nipple-areolar complex also shows contrast enhancement on MRI [34, 35]. This is probably caused by the vascular branches from variable main arteries that supply the nipple-areola complex with blood and nutrients [36]. Therefore, it is plausible that this area is also visible with

PAM. Moreover, the increased skin pigmentation of the nipple and areola might also absorb the 1064 nm light and thereby contribute to increased signals. To test this hypothesis, the measuring configuration and breast positioning as in patient 1 were repeated with a healthy volunteer. The resulting photoacoustic image (Figure 4.5) showed a region of high absorption, highly comparable to the supposed nipple in Figure 4.4(d).

Figure 4.4 Case 3: Diagnostic images of a mixed infiltrating ductal and lobular carcinoma in the left breast of a 78 year old woman. **a)** The medio-lateral-oblique x-ray mammogram shows two lesions: a lesion of dense breast tissue (blue-dashed, lesion 1) which is associated with some microcalcifications and is positioned behind the nipple (green dotted square). The second lesion, indicated by a white box, is a density close to the skin of the nipple. **b)** The ultrasound images only show one oval-shaped lesion in the skin just above the nipple (white box). **c)** Transversal cross-sections (slice thickness 0.24 mm) through the photoacoustic image volume show a very superficial 14 mm abnormality (white box), which is attached to the skin and which is in depth connected to **d)** a 15 mm abnormality (blue-dashed square) with a contrast of more than 3 with respect to the background. In this cross-section a third region with high contrast could be observed (green-dotted square), which probably represents the nipple. The circular artifacts that are visible in Figure 4.4(c and d), are inherent to the backprojection algorithm. These artifacts are more pronounced close to the surface as are these slices.

Figure 4.5 The photoacoustic image of a healthy volunteer, measured in the same configuration as patient 1. This image shows a transversal cross-section at the position of the nipple. The identified region with high-contrast is quite comparable to the region that was hypothesized to represent the nipple in case 3 (Figure 4.4d, green-dotted square).

4.3.3 Overall results

In the reconstructed volumes of all ten measurements on BI-RADS 5 lesions, a confined region with high contrast with respect to the background could be seen at the depth where the lesion was expected based on the ultrasound images. However, in some patients (patients 5, 6 and 17) this region was at the border of the scan because of slight mispositioning. Incorrect positioning was caused by the limited size of the region of interest. In those patients, the size of the abnormality on the photoacoustic mammogram could not fully be estimated. Table 4.3 provides an overview of the identified photoacoustic abnormalities with their contrast and size. For comparison, Table 4.3 also shows the contrast of the lesions on the cranio-caudal x-ray mammograms, and the histopathologically assessed size of the lesions, which is the gold standard for lesion size estimation.

Table 4.3 Measurement results from photoacoustic mammography.

Lesions as defined on the photoacoustic images are compared with x-ray mammography and histopathology, based on contrast and maximum diameter respectively.

Patient	PAM lesion visibility (positive contrast)	PAM lesion contrast	X-ray lesion contrast	PAM max. diameter (50% threshold)	Lesion size (histopathology)
1	Yes	3.2	1.6-4.7**	24 mm	30 mm
4	Yes	6.4	1 -2.6**	10 mm	15 mm
5	Yes	3.3	1.9	***	42 mm
6	Yes	6.2	1.0	***	13 mm
7	Yes	5.3	2.6	14 mm	27 mm
9	Yes, Two lesions	1) 5.3 2) 4.7	3.5	1) 14 mm 2) 26 mm	31 mm
10	Yes	4.5	1.2	13 mm	> 30 mm*
11	No	1.0	1.0	Not visible	28 mm*
14	Yes, scattered abnormality	4.0	3.8	Scattered	> 40 mm*
15	No	1.0	1.0	Not visible	42 mm*
16	Yes.	7.0	3.4	9 mm	33 mm
17	Yes	4.9	1.3	***	27 mm

*In patients 10-15, the lesion size was estimated radiologically, since there was no histopathological measurement available. **These lesions were positioned partly in fatty tissue and partly in fibroglandular tissue. Therefore, the contrast for these lesions on x-ray mammography is given with respect to both types of background. *** In these measurements the defined lesion was only partly positioned within the region of interest and the maximum diameter could not be assessed.

The two reconstructed volumes of the photoacoustic measurements on cysts (patients 11 and 15) manifested no such confined high-contrast regions which extend over multiple slices in depth. Figure 4.6(a) shows the photoacoustic imaging results of a 44 year old patient with a cyst (patient 11). On ultrasonography the cyst was estimated to be positioned at more than 10 mm in depth and to measure about 28 mm in diameter. Figure 4.6(a) shows the non-normalized MIP of the photoacoustic imaging volume in the depth range where the cyst was expected. For comparison, Figure 4.6(b) shows a non-normalized MIP from a

photoacoustic measurement on a malignancy which was positioned at comparable depth as the cyst. The confined high-contrast area which can clearly be seen in Figure 4.6(b) is absent in Figure 4.6(a). Despite the fact that both MIPs are taken over the same depth range, the absolute intensity values are lower for the measurement on the cyst than for the measurement on the malignancy.

Figure 4.6 **a)** Maximum intensity projection (MIP) in cranio-caudal direction of the photoacoustic volume of a patient with a 25 mm cyst. No confined, homogeneous, high-contrast region can be seen. The higher intensities are spread over the complete region of interest. The intensity values are not normalized in this image. **b)** For comparison, a MIP in cranio-caudal direction of the photoacoustic volume of a patient with a malignancy (patient 4, Figure 4.2) is visualized. The malignancy is located at a comparable depth as the cyst from figure a) and the MIP is taken over the same depth-range. Here a confined high-contrast region can be seen. In the cyst, the non-normalized intensity values are much lower than in the malignancy, despite the fact that the MIPS are from the same depth.

Figure 4.7 **a)** Per BI-RADS density classification scale, the average contrast of the lesions on PAM (gray) and x-ray mammography (white) is given. **b)** Here, only a differentiation between 'low' (BI-RADS density 1,2) and 'high' (BI-RADS density 3,4) breast density is made. The error bars show the plus and minus one standard deviation. When the lesion was positioned partly in fatty and partly in glandular tissue on the x-ray mammogram (patients 1 and 4), the contrast with respect to the fatty tissue was chosen, which always was the highest contrast.

The average contrast for the visible lesions on x-ray mammography and PAM is shown per BI-RADS breast density category in Figure 4.7(a). It can be seen that the contrast on PAM is fairly constant with breast density, while the contrast on x-ray mammography decreases for denser breasts. This is more pronounced when a division into two density categories is made (Figure 4.7(b)), which is the most reproducible way of grading breast density [37]. The contrast of the lesion on x-ray is statistically different ($p < 0.05$) between the high and low density breasts, while there is no statistical difference in the contrast of the lesions on PAM.

4.4 Discussion

In this study we showed that NIR photoacoustic imaging as embodied in the Twente Photoacoustic Mammoscope (PAM) visualized breast malignancies in women with a high imaging contrast, while cysts did not manifest such confined high-contrast regions. Furthermore, the PAM determined contrast seems to be independent of mammographic breast density. However, PAM determined lesion size seems to be smaller than the histologically determined tumor sizes. Here, we critically discuss the results in the context of instrumental characteristics and limitations.

4.4.1 Contrast and wavelength

Light at 1064 nm is suitable for photoacoustic breast imaging: absorption and scattering are generally lower than at other wavelengths in the optical window, so that light can penetrate deep in breast tissue. Hemoglobin, primarily in its oxygenated variant, absorbs light at 1064 nm [38] and therefore, contrast between healthy and malignant breast tissue is still expected because of the angiogenesis related increase in hemoglobin concentration.

In all ten malignancies, PAM was able to visualize a confined high-contrast region at the true lesion depth and in all cases, the contrast was higher than observed on x-ray mammography. On average, the contrast of the abnormalities on PAM was 5, while the lesions could be seen on x-ray mammography with an average contrast of less than 3. This presence of confined high-contrast regions in photoacoustic images (Figure 4.2-4.4) of malignancies, points to the visualization of the optical properties associated with carcinoma. It is most likely that we are observing, via the absorption of primarily oxyhemoglobin, the host tissue response in the form of angiogenesis [3, 4, 38, 39]. The contrast in our photoacoustic images seems not to be affected by the fibroglandular breast density (see Figure 4.7), unlike in x-ray mammography [2]. This can be explained by the expectation that neither the total hemoglobin concentration nor the oxygen saturation is significantly different between the four BI-RADS breast density scales [40].

The appearance of the rather homogeneous high-contrast regions is difficult to attribute solely to oxyhemoglobin, since it is known that the tumor vascularization is inhomogeneous and often more pronounced in the peripheral areas [41]. In addition to oxyhemoglobin at 1064 nm, other important tissue constituents also absorb at this

wavelength: water absorption is not low [42] and absorption by lipids and collagen is also not negligible [14, 38, 43]. While the fat content in cancerous tissue is decreased [44, 45], the (free) water concentration is significantly increased in invasive carcinoma compared to the surrounding normal breast tissue [45, 46]. A contribution from water absorption to the photoacoustic image intensity cannot be excluded.

Our results indicate that the contribution of water to the photoacoustic contrast is probably small with respect to the contribution of hemoglobin. First, if water was the prominent source of our photoacoustic contrast for breast carcinoma, we would also expect to see high contrast regions in the photoacoustic images of cysts which primarily consist of water. Because of the limited bandwidth of the transducer, the cysts would probably appear edge-enhanced. Though measurements on cysts have to be performed in a larger study population, the two measurements on cysts measured in the first phase of the study manifest no such features (Figure 4.6). Second, we would expect to see a decrease in photoacoustic contrast for the higher density breasts as these are associated with a significant increase in water concentration of the background tissue [40, 47]. In our results, the photoacoustic contrast seems to be independent of the breast density instead.

It is therefore likely that the high photoacoustic contrast is the consequence of the combined absorption of predominantly hemoglobin with minor contribution of water in the extracellular tissue matrix. Further research to the specific contributions of all chromophores is required for an optimal discrimination between benign and malignant breast lesions.

4.4.2 *Size and shape*

The size and shape of the abnormalities estimated from photoacoustic images, deviated from that on conventional imaging and histopathological assessment. There are several factors that can influence the abnormality's appearance.

First, deviations can be the consequence of breast positioning and the amount of compression applied, both are varying between the different imaging modalities. Second, the level of the threshold strongly influences the estimation of the lesion's extension. In Figure 4.8 it can, for example, be seen that the lesion's maximum diameter increases from 8 to 20 mm when the threshold decreases from 60% to 20%. Future evaluations in a large number of patients have to be performed to assess the optimal threshold for precise estimation of the true lesion size.

Further, the planar imaging geometry provides limited views or incomplete tomographic projections for accurate reconstruction of especially size and shape of an abnormality [48, 49]. The viewing angle is further reduced since we consciously make a trade-off between scan area and scan duration in favor of shorter scan duration.

Finally what we visualize in our images is not an absolute absorption coefficient, rather the absorbed light energy distribution. Images we obtain are thus affected with depth-dependent falloff in contrast due to optical attenuation. Keeping a constant threshold for boundary estimation in depth can lead to an underestimation of lesion size as observed. A

ground truth image contrast distribution and a minimization in the size estimation error can be achieved by using for example a forward model of light transport with iteratively corrected optical properties, that converges to match the reconstructed photoacoustic distribution [50, 51].

Figure 4.8. Transversal cross-section through the photoacoustic mammogram of patient 4 (see Figure 4.2). The lesion is defined with the help of three different thresholds, which are all expressed as a percentage of the maximum intensity value (red=60%, blue=40% and green=20%). The value of the threshold strongly influences the maximum diameter of the defined lesion. In this case, the diameter of the lesion is estimated as being 8, 12 and 20 mm for the 60, 40 and 20% threshold respectively.

4.4.3 Surface signal

Due to the relatively high fluence at the breast surface, a large photoacoustic signal from the surface is present for all signals in the ROI. Therefore, removal of the photoacoustic surface signal from the remainder of the breast signal before the reconstruction is applied, is important. However, sometimes the skin signal cannot clearly be distinguished from the interior signal, especially since the detector has limited bandwidth. Moreover, the optical absorption of the skin, and therefore the resulting photoacoustic signals, can vary between different skin types. An incorrect removal of the surface signal could introduce size and shape errors for tumors close to the skin. This problem makes photoacoustic imaging in transmission mode with a limited bandwidth detector a challenge for superficial tumors (such as in case 3). Imaging in dark-field where the light delivery is not in the direction of ultrasound detection, or imaging in multiple views, would probably overcome this problem.

4.4.4 Outlook

Photoacoustic imaging allows for the visualization of breast malignancies without the use of ionizing radiation as in x-ray mammography or external contrast agents as in contrast enhanced MRI. The images can be obtained with the patient in a comfortable prone position with only minor breast compression. This study also showed some possibilities for improvement of the Twente Photoacoustic Mammoscope in its current configuration.

The photoacoustic contrast at 1064 nm is possibly the consequence of the combined absorption of hemoglobin and water. However, also the fat content, the fat/water ratio and the oxygen saturation are known to be altered at malignant areas [46, 52]. Moreover, premalignant lesions like DCIS, and benign lesions like fibroadenoma seem to be associated with an increased blood volume fraction and water content respectively [46, 53]. Those lesions might be differentiated from invasive carcinoma based on the concentrations of other chromophores and the oxygen saturation. The use of multiwavelength strategies will facilitate discrimination between various chromophores [54], which could help in discriminating malignant lesions from premalignant and benign lesions.

In this study we mainly included Caucasian patients with lesions that were highly suspicious for malignancy on conventional imaging. Moreover, our system only allowed us to measure palpable lesions in certain regions of the breast. At last, the small region of interest we can measure within an acceptable time makes positioning difficult and can cause size and shape deviations in the reconstructed volume. Those problems can be overcome by increasing the measurement speed.

PAM can visualize breast lesions with high contrast with respect to the background, but in order to estimate the discriminative power and clinical significance of photoacoustic breast imaging, we should gradually focus on less suspicious lesions, benign lesions and normal breasts with the aforementioned technical improvements.

4.5 Conclusions

The current study demonstrates the potential value of photoacoustic imaging of breast tumors. Photoacoustic images showed the lesions with a higher contrast than with x-ray mammography, while cysts did not induce such confined photoacoustic contrast. The technique has several advantages over conventional modalities. The method neither uses ionizing radiation, nor contrast agents and seems not to be affected by the fibroglandular breast density. If the measurement speed were increased and complete 3D breast imaging were possible, the added value of photoacoustic imaging in breast diagnosis could be investigated. It is expected that even better contrasts and imaging depths would be obtained with the optimal choice of wavelengths and change of the image configuration.

4.6 References

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Appearance of breast cysts in planar geometry photoacoustic mammography using 1064 nm excitation⁴

In the search for improved imaging modalities for detection and diagnosis of breast cancer, a high negative prediction value is also important. Photoacoustic (optoacoustic) imaging is a relatively new technique that has high potential for visualizing breast malignancies, but little is known about the photoacoustic appearance of benign lesions. In this work, we investigate the visibility of benign breast cysts in forward mode photoacoustic mammography using 1064 nm light, as currently applied in the Twente photoacoustic mammoscope. Results from (Monte Carlo and k-Wave) simulations and phantom measurements were used to interpret results from patient measurements. There was strong agreement between the results from simulations, phantom and patient measurements. Depending on the absorption contrast between cyst and breast tissue, cysts were visible as either one or two confined high contrast areas representing the front and the front and the back of the cyst respectively. This ‘edge enhancement’ is most likely the consequence of the local sudden change in absorbed energy density and Grüneisen coefficients. Although the current forward-mode single wavelength photoacoustic mammoscope cannot always unambiguously discriminate cysts from malignancies, this study reveals specific features of cysts compared to malignancies which can be exploited for discrimination of the two abnormalities in future modifications of the imager.

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5.1 Introduction

Breast cysts are among the most common benign lesions in the breast [1]. These fluid-filled sacs usually develop in the ducts and acini because of an imbalance between secretion and drainage of the ducts [1, 2]. Several hypotheses regarding this imbalance exist and most of them consider either obstruction of the draining ducts and thereby retention of secretory material, or an increase in ductular-alveolar secretion [2]. Cysts are usually easily recognized using conventional breast imaging techniques. In x-ray mammography, a cyst is visualized as a circumscribed non-calcified circular density [3]. Under ultrasound investigation, anechogeneity, smooth borders and posterior echo enhancement are characteristic features for a cyst [1, 4]. The conventional clinical modalities are well capable of recognizing breast cysts, and there is no necessity for research into new methods for cyst imaging and diagnosis.

On the contrary, breast carcinoma is often ambiguously visualized using conventional imaging techniques due to their lack of sensitivity and specificity for the disease, especially in patients with dense breast tissue [5]. One of the new techniques currently under investigation for its potential in addressing some of these limitations is photoacoustic imaging (PAI), also called optoacoustic imaging [6-9]. Photoacoustic imaging combines the high contrast possible in optical imaging with the high resolution associated with ultrasound detection [10-14]. Upon illumination of the tissue with short pulses of laser light, the light is absorbed by the chromophores in the tissue. In case of malignancy, hemoglobin in the tumor vasculature is believed to be the predominant absorber [15]. The absorption of the light energy leads to the formation of a pressure wave by thermoelastic expansion. The pressure wave propagates through the tissue and can be detected with ultrasound detectors with the appropriate center frequency and bandwidth [16-19]. From the detected signals, the location of light absorption can be retrieved with ultrasound resolution, overcoming the limitation of purely optical techniques where optical scattering causes a poor resolution deeper than several mm [20]. This technique has several advantages for breast imaging besides the high contrast and good imaging resolution: it is non-invasive and does not require the use of ionizing radiation or contrast agents.

Previously, we showed that photoacoustic mammography, as applied in the Twente photoacoustic mammoscope (PAM) is able to visualize breast malignancies as high contrast regions in the reconstructed photoacoustic volume [7, 21]. However, we believe that a technique that aims at visualizing malignancies in the breast should also be able to visualize benign lesions such as fibroadenomas and cysts and to clearly differentiate them from malignancies. In the previous study [7], we also reported the imaging on two breasts with cysts. We were not able to recognize the cysts as the confined high intensity areas observed for malignancies, and we attributed this to the low absorption of the water-rich cysts compared to the hemoglobin-rich malignancies. In retrospect, it is not unlikely that instrumental issues also contributed to the photoacoustic absence of cysts. Since cysts are easy to compress and move, the possibility cannot be excluded that they shifted out of the

4x4 cm² scan area of the detector during positioning. In fact, due to the nature of cysts, they can be expected to be visible using photoacoustics at certain wavelengths, but to be different in appearance from the inhomogeneously absorbing and scattering malignancies. A limited number of publications of optical imaging of breast cysts in the far-red wavelength regions [22-26], shows a lower or higher homogeneous absorption, and scattering lower than that of the surrounding tissue. This is confirmed by the fact that the color of the cyst fluid can vary widely per subject. Colorless to brownish fluids are reported as well as transparent to slightly turbid fluid [2]. The color of the fluid is thought not to have any diagnostic importance. Moreover data about the origin and function of the mammary chromophores in the cyst fluid, which might include breakdown products of hemoglobin, cholesterol epoxides and lipofuscin complexes, is lacking [2].

Recently, we upgraded PAM to enable faster scanning in a larger FOV. With this system, the shifting of the cysts upon compression should be less of a problem, although the position estimation of a single cyst within the region of interest (ROI) and the comparison with its position in the cranio-caudal x-ray image will still be more difficult than for malignancies, especially since there are often multiple cysts in the breast. Because of this reason, we started the investigation regarding the appearance of breast cysts in PAM by using simulations: Monte Carlo simulations and k-Wave simulations were performed to couple the light-tissue interactions to the acoustic propagation. Subsequently, phantom measurements were performed to verify the results from simulations and to translate the situation to the PAM set-up. Finally, patient measurements were performed in a small patient population using the upgraded version of PAM. All results were combined to investigate if cysts can be differentiated from malignancies in a photoacoustic imaging volume using our current image configuration, since for optimal breast cancer detection, the ability to exclude benign breast lesions is of importance.

5.2 Materials and methods

5.2.1 *The Twente photoacoustic mammoscope (PAM)*

The Twente photoacoustic mammoscope (Figure 5.1) is built into a hospital bed on which the patient lies in prone position with her breast pendant through an aperture in the bed. Under the bed, the breast is slightly compressed between a glass plate for laser light illumination at the cranial side and the ultrasound detector array at the caudal side. The breast is illuminated with 1064 nm pulsed light (10 ns, 10 Hz) from a Q-switched Nd:YAG laser (Continuum Surelite, California). For patient and phantom measurements, an elliptical shaped beam with a surface of approximately 35 cm² and a maximum energy of 350 mJ is used (10 mJ/cm²). Photoacoustic signals are detected with a 1 MHz (130% bandwidth) 2D ultrasound detector array (Lunar Corporation [27, 28]) consisting of 590 PVDF elements of 2x2 mm² with an inter-element distance of 1.175 mm and -3dB directivity of 20°. The array covers an area of approximately 9x8cm² of the breast. Details of the PAM detector are described elsewhere [28].

The photoacoustic signals are read into the PC using two eight channel digitizers (National Instruments, NI PXI 5105, 60 MS/s, 12-bit). A Labview program (National Instruments) controls the scan stage movements and the element selection.

Figure 5.1 *The Twente Photoacoustic Mammoscope. a) Aperture to insert breast. b) Ultrasound detector matrix. c) Glass window. d) Scanning system compartment. e) Q-switched Nd-YAG laser operated at 1064 nm with 10 ns pulses. f) Laser safety curtain which is drawn around the instrument during the measurements. g) Interface electronics between detector and computer. h) Linear stage carrying detector matrix driven by hand wheel to apply mild compression to the breast. i) Laser remote control unit. j) Laser power supply. Image adapted from reference [21] with permission.*

The signal processing and image reconstruction was performed off-line using Matlab (R2011b, the Mathworks Company). The raw signals were filtered with a band-pass Butterworth filter (cut-off frequencies 0.2 and 1.7 MHz) to remove the low frequency trend and high frequency noise. The prominent breast surface signals were removed prior to reconstruction to expand the dynamic range for signals of interest from deeper inside. A modified acoustic backprojection algorithm [27, 29] was used to reconstruct the images, assuming homogenous speed of sound (SOS) values of 1540 m/s for the breast and 1700 m/s for the 18.6 mm protective layer of the detector. After reconstruction, the separate sagittal slices were Hilbert transformed to demodulate the signals and obtain an envelope detection. All voxel intensity values were scaled to the maximum intensity value within the total volume, giving a range of intensity values between 0 and 255.

5.2.2 Simulations

5.2.2.1 Monte Carlo

Monte Carlo simulations were performed using Montcarl 20.12 (MedPhys Software and Services [30]). A simplified model was used to mimic a breast with a cyst (see Figure 5.2a). A layer of 70 mm (the size of a relatively large breast under minimal compression in PAM) having a refractive index (N) of 1.42 [31], an absorption coefficient (μ_a) of 0.03 mm^{-1} [32] and a reduced scattering coefficient (μ_s') of 0.5 mm^{-1} , was used in the model. The μ_s' was chosen low compared to values from literature [32, 33] so that a lower number of photon injections was required to get a statistically sufficient number of photons at and beyond the depth of the cyst. The cyst was modeled as a sphere with 5 mm radius at a starting depth of 13 mm below the breast tissue. For each sphere, $N = 1.33$ and $\mu_s' = 0.00001 \text{ mm}^{-1}$,

mimicking an aqueous medium without scattering, since the μ_s' of cysts is reported and expected to be well below that of breast tissue [24]. Absorption values are reported to range from below to above the absorption of the breast tissue [23, 26], therefore with values of [0.0015; 0.015 0.030; 0.045; 0.060; 0.090] mm^{-1} , the μ_a of the cyst was varied step-wise from below to above the μ_a of the medium. All optical properties used for the simulations and experiments (see further Section 5.2.3) are summarized in Table 5.1. To model the scattering, the Henyey-Greenstein phase function was used. Optical properties are further referred to as μ_{a_c} and μ_{s_c}' for the cyst mimicking structures and μ_{a_b} and μ_{s_b}' for those mimicking the breast.

Figure 5.2 a) Simplified breast-cyst model used in the Monte Carlo simulations. b) A comparable physical phantom model used for the photoacoustic measurements (Section 5.2.3).

Table 5.1 Optical properties as used in simulations and experiments compared to literature values.

Breast and cyst optical properties at 1064 nm			
	Literature	Simulations	Phantom
<i>Breast</i>			
$\mu_a [\text{mm}^{-1}]$	0.01-0.03 [32]	0.03	0.06
$\mu_s' [\text{mm}^{-1}]$	0.06 (extrapolated from) [33] 1.1-1.3 [32]	0.5	0.5
g	0.96 [34]	0.9	0.5
N	1.4 [31]	1.42	~1.33 [35, 36]
<i>Cyst</i>			
$\mu_a [\text{mm}^{-1}]$	Varying [23, 26]	0.0015; 0.015; 0.030; 0.045; 0.060; 0.090	0.015; 0.30
$\mu_s' [\text{mm}^{-1}]$	<< breast tissue [24]	0.0001	0 [37]
g	No information available	0.9*	N.A. [37]
N	1.33 (fluid)	1.33	~1.33 (fluid)

* This value does not have any influence on the results because of the very low scattering that is applied in the simulations.

The medium was illuminated using an elliptical Gaussian beam with dimensions (FWHM) of 80 and 60 mm, approaching the dimensions of the beam used in the clinical measurements.

For each photon absorbed within the area of the detector ($0 < x < 88$ mm and $0 < y < 83$ mm) the x , y , and z coordinates of the voxel where absorption took place were stored. Each simulation was ended when 100×10^6 photons were absorbed. The spatial distribution of absorbed photons was stored in a matrix (voxel size $0.5 \times 0.5 \times 0.5$ mm³), further referred to as the ‘absorbed energy per voxel matrix’ (AE).

An extra set of simulations was performed, where the cyst was modeled as a cylinder with radius of 5 mm in y -direction and infinite length in x -direction. These simulations were required to understand any differences between the appearance of cylinders and spheres, since fluid-filled cylinders were used to mimic cysts in experiments due to ease of preparation and use.

5.2.2.2 *K-wave*

The acoustic propagation following the initial pressure distribution associated with AE was simulated using the k-Wave toolbox (version B0.5) [38] in Matlab.

Initial pressure distribution

In photoacoustic imaging the initial pressure distribution follows:

$$p_0 = E_a \Gamma, \quad (5.1)$$

with p_0 the initial pressure distribution (Pa), Γ the (dimensionless) Grüneisen coefficient and E_a the local absorbed energy density (Jm⁻³), obtained from the Monte Carlo simulations as matrix AE , and described by

$$E_a = F \mu_a, \quad (5.2)$$

where F is the optical fluence (Jm⁻²) and μ_a is the optical absorption coefficient (m⁻¹). Γ is given as:

$$\Gamma = \beta c^2 / C_p, \quad (5.3)$$

with β the isobaric volume expansion coefficient in K⁻¹, c the speed of sound in the medium (m s⁻¹) and C_p the specific heat in J/(K kg). Γ was chosen to be 0.8 for the solid tissue [39], and 0.2 for the cyst fluid, mimicking a water-like medium at body temperature [40].

Acoustic medium

The acoustic medium through which the pressure wave propagates, is assumed to have a constant speed of sound of 1540 m/s and an acoustic attenuation (AA) of 0.5 dB cm⁻¹/MHz for breast tissue and 0.0022 dB cm⁻¹/MHz for cyst fluid [41]. The properties of the acoustic medium are listed in Table 5.2. All acoustic properties were chosen for glandular breast tissue, since most cysts arise in glandular tissue.

Table 5.2 *Acoustic properties of the medium modeled in K-wave.*

	Simulations		Reference
	Background	Cyst	
Grüneisen coefficient	0.8	0.2	[39, 40]
Speed of sound	1540 m/s	1520 m/s*	[42, 43]
Density	1000 kg/m ^{3**}	1000 kg/m ^{3*}	[44]
Acoustic attenuation	0.5 dB cm ⁻¹ /MHz	0.0022 dB cm ⁻¹ /MHz	[41]

* These are values for pure water at body temperature (37 °C)

** We assume the breast density close to that of water. Breast density can vary with tissue composition, but the density is usually within 5% deviation from the water value [44, 45]

Signal detection

Signals are detected by elements mimicking the detector in PAM as described in Section 5.2.1. In the k-Wave code, the elements are specified to have a center frequency of 1 MHz and a bandwidth of 130%. Signals were processed and reconstructed as described in Section 5.2.1, however in this case, signals were filtered with a high-pass Butterworth filter only (cut-off frequency 0.3 MHz) to remove the low frequency trend in the signal.

5.2.3 Phantom measurements

5.2.3.1 Phantom preparation

The cysts were modeled as two fluid filled cylindrical cavities in a 3% agar (Sigma Aldrich) phantom (Figure 5.2b) with μ_a and μ_s as in Table 5.1. The cavities have radii of 5 and 8 mm respectively and are located at a starting depth of 15 mm.

An aqueous solution of Agar 3% by weight concentration was prepared. Black ink (Ecoline Black, Royal Talens, Apeldoorn, the Netherlands) and Intralipid 20% (Fresenius Kabi, Homburg, Germany) were added to the cooled-down solution to adjust the absorption and scattering respectively. In order to be able to measure a larger range of $\mu_{a,c}$, with the lowest available $\mu_{a,c}$ being that of water (0.015 mm⁻¹), $\mu_{a,b}$ was chosen relatively high compared to literature values (Table 5.1), For μ_s the same value as in the simulations was used (0.5 mm⁻¹), slightly lower than the value characteristic for the breast (0.6-1.2 mm⁻¹, Table 5.1). The obtained anisotropy value was less realistic ($g=0.5$) compared to breast tissue.

5.2.3.2 Phantom measurements

Measurements were performed using the Twente Photoacoustic Mammoscope as described in Section 5.2.1. Upon fixation of the phantom in the imager, the cylindrical cavities were filled with water and water-ink solutions. In the first measurement water was used with a μ_{a_c} of 0.015 mm^{-1} , which is below μ_{a_b} . In the second measurement with black ink, a μ_{a_c} of 0.3 mm^{-1} was used, far above μ_{a_b} . Signals were averaged 150 times and reconstruction was applied as described in Section 5.2.1.

5.2.4 Patient measurements

The measurement protocol and informed consent procedure were approved by the institutional review board (METC, Medisch Spectrum Twente (MST), Enschede). The study is registered in the Netherlands trial register (NTR) as TC 2945.

In the period from April to December 2012, patients at the center of breast care of the MST with one or more non-suspicious breast cysts were included in the study. Details of the measurement and imaging protocol are described in reference [7]. Photoacoustic image reconstruction was applied as described in Section 5.2.1.

In addition, cyst fluid was obtained, with permission, from patients whose non-suspicious cysts were aspirated because of physical or mental discomfort. Each fluid batch was visually inspected for color and turbidity.

5.3 Results

5.3.1 Simulations

5.3.1.1 Depth profile of absorbed energy

Figure 5.3 shows the depth profiles of the absorbed energy per voxel (AE) from the Monte Carlo simulations for three situations: Figure 5.3a for $\mu_{a\_c} = 0.0015 \text{ mm}^{-1}$ ($\mu_{a\_c} \ll \mu_{a\_b}$); Figure 5.3b for $\mu_{a\_c} = \mu_{a\_b} = 0.03 \text{ mm}^{-1}$; and Figure 5.3c for $\mu_{a\_c} = 0.090 \text{ mm}^{-1}$ ($\mu_{a\_c} \gg \mu_{a\_b}$). Grey dashed lines represent the AE profiles, while the bold lines show AE multiplied with the local Grüneisen coefficient ($\Gamma \approx 0.8$ for the breast and $\Gamma \approx 0.2$ for the cyst). The latter correction with Γ would better resemble the shape of the initial pressure distribution profile.

As expected, there is an initial exponential decay of the absorbed energy over depth. The decay rate is approximately equal to the calculated μ_{eff} (fit $\mu_{eff} = 0.20 \text{ mm}^{-1}$, calculation $\mu_{eff} = 0.22 \text{ mm}^{-1}$). Until the depth of the cyst (13 mm), the profile is the same for all three situations, except for statistical variations caused by the limited number of photons. In the case where $\mu_{a\_c} \ll \mu_{a\_b}$ (Figure 5.3a) there is a sudden drop in absorbed energy at the starting point of the cyst ($d=13 \text{ mm}$). At the end of the cyst ($d=23 \text{ mm}$) there is an increase in absorbed energy, after which the absorbed energy starts to decay exponentially again. There is hardly any difference between the profiles with and without correction for Γ .

If $\mu_{a\_c} = \mu_{a\_b}$ (Figure 5.3b), the starting point and ending point of the cyst are hardly visible when only the absorbed energy is taken into account, but can still be seen as a sudden drop and increase respectively when Γ is considered as well.

For the case where $\mu_{a_c} \gg \mu_{a_b}$ (Figure 5.3c), the situation is reversed. Here, at $d=13$, an increase in absorbed energy can be seen, while there is a slight drop in absorbed energy at the end of the cyst. After multiplication with Γ , a drop in the pressure distribution can be seen at 13 mm, while the back of the cyst becomes invisible.

Figure 5.3 Absorbed energy density and pressure versus depth. **a)** $\mu_{a_c} (0.0015 \text{ mm}^{-1}) \ll \mu_{a_b} (0.03 \text{ mm}^{-1})$, **b)** $\mu_{a_c} = \mu_{a_b} = 0.030 \text{ mm}^{-1}$ and **c)** $\mu_{a_c} = 0.09 \text{ mm}^{-1}$ ($\mu_{a_c} \gg \mu_{a_b}$). The grey dashed lines indicate the absorbed energy per voxel [a.u.] over depth [mm], while the bold lines also take the variation in local Grüneisen coefficient (Γ) into account.

5.3.1.2 Results following reconstruction of K-wave signals.

Figure 5.4 shows the reconstructed images from the simulated photoacoustic measurements for the situation $\mu_{a_c} \ll \mu_{a_b}$ (Figure 5.4 a,b,c), $\mu_{a_c} = \mu_{a_b}$ (Figure 5.4 d,e,f) or $\mu_{a_c} \gg \mu_{a_b}$ (Figure 5.4 g,f,h), following K-wave simulations including variations in Γ , SOS and AA. For all situations, the upper panel (a, d, g) shows a portion of the 3D volume, the middle panel (b, e, h) shows a slice in sagittal direction (indicated by the black dashed area in the 3D volume) and the lower panel (c, f, i) shows a profile through the center of the sphere (indicated by the white dashed line in the slices). For the latter situation both a raw signal,

as well as the Hilbert demodulated signal are shown. The grey dashed lines indicate the profile in case only AE is taken into account, i.e. in case the variations in Γ , SOS and AA are ignored.

Figure 5.4 3D volumes (a, d, g), transversal slices (b, e, h) and profiles (c, f, i) through reconstructed image volumes of K-wave simulated photoacoustic signals. The profiles (bold black) are compared to the profiles through the volume without corrections for variations in Γ and acoustic properties (grey dashed). a-c) $\mu_{a,c} \ll \mu_{a,b}$ d-f) $\mu_{a,c} = \mu_{a,b}$ and g-i) $\mu_{a,c} \gg \mu_{a,b}$.

It can be seen that in the first two situations ($\mu_{a,c} \ll \mu_{a,b}$ and $\mu_{a,c} = \mu_{a,b}$), two regions with high intensity are visible (Figure 5.4a,b,c and d,e,f). Since the locations of these regions match the starting and ending depth of the cyst, we attribute these regions as representing the front (illumination side) and back (detection side) of the cyst. In case $\mu_{a,c} = \mu_{a,b}$, the front and back of the cyst are only clearly visible if the local variations in Γ and acoustic properties are taken into account, as seen in Figure 5.4f (black lines). Thus, the absorbed energy alone is not enough to clearly visualize the cyst as could also be expected from Figure 5.3b. In case $\mu_{a,c} \gg \mu_{a,b}$ (figure 5.4 g,f,h), only one high intensity area can be observed, which originates from the front of the cyst. The back of the cyst becomes invisible after corrections for variations in Γ and acoustic properties. Based on the absorbed energy profile alone (Figure 5.3c, dashed line), one would expect the back to be visible as well, as long as enough photons are able to reach the back of the cyst.

It can be observed from the profiles (without corrections for I , SOS and AA) that the photoacoustic signal changes in shape for different transitions. The signal from the front of the cyst is reversed for the high-absorbing cyst (Figure 5.4i) as compared to the low-absorbing cyst (Figure 5.4c) and moreover the shape of the front and back signals differ for all three situations. However, these differences become less pronounced when I , SOS and AA are taken into account.

5.3.1.3 Spheres versus cylinders

We also simulated photoacoustic volumes using cylinders and spheres for the cysts. While the expected differences in the shapes of the signal coming from the sphere and the cylinder are observed, these differences are minor in the demodulated signals. This justifies the use of the easy to prepare and use liquid-filled cylinders to simulate cysts in phantom experiments as a substitute for spheres.

5.3.2 Phantom measurements

Figure 5.5 shows slices and profiles through the reconstructed photoacoustic volumes from the phantom measurements for both the low (water, $\mu_{a_c} \ll \mu_{a_b}$, Figure 5.5 b-c) and high (ink, $\mu_{a_c} \gg \mu_{a_b}$, Figure 5.5 d-e) absorbing inclusion.

Figure 5.5 Results from the photoacoustic reconstruction of the phantom measurements. **a)** Orientation of the shown signal and slices with respect to the phantom model. Slices (left, figures b, d) and profiles (right, figures c, e) through the structure of interest in the photoacoustic volume are visualized. **b-c)** The top panel shows the results for the water measurement ($\mu_{a_c} \ll \mu_{a_b}$) and **d-e)** the lower panel shows the results for the ink measurements ($\mu_{a_c} \gg \mu_{a_b}$). The black dashed lines indicate the position of the profiles.

For the low absorbing inclusion, two signals are visible at depths that match the front and the back of the inclusion as was the case for the simulations. These two signals clearly differ in shape as can also be seen, although less pronounced, in the simulations where variations Γ and acoustic properties were not taken into account (Figures 5.4 c,f,i, dashed lines). For the high absorbing inclusion, only the front of the cyst is visible. The shape of the photoacoustic signal from the front of the tube is reversed for the high absorbing fluid (Figure 5.5e) compared to the low absorbing fluid (Figure 5.5c).

5.3.3 Patients

Four cases of breasts with fluid filled cysts were imaged using PAM. In patients, $\mu_{a,b}$ and $\mu_{a,c}$ are not known. Therefore, the specific shape of the signals cannot be predicted. Moreover, the cysts are embedded in an optically and acoustically inhomogeneous medium, confounding the differentiation of signals from cysts from signals from other absorbing structures in the breast. In three of four cases, the cysts were observed as one or more high intensity areas at the expected location in the photoacoustic imaging volume. Here, we chose to present a case which is largely representative of the other cases, but with a slightly more atypical photoacoustic presentation.

This 49 year old patient discovered a palpable mass in her right breast. Upon clinical investigation, a smooth and mobile mass could be palpated at 9-10 o'clock in the right breast. X-ray mammography (Figure 5.6a) showed a sharply delineated, circular mass in the lateral upper quadrant of the right breast. The lesion was assigned as being probably benign, BI-RADS 3. Ultrasound investigation (Figure 5.6b) showed clear signs of a benign cyst at the indicated area. A 22 mm (transversal direction) hypoechoic area, with posterior enhancement and acoustic shadow caused by the refraction of the ultrasound at the edges of the cyst, was observed less than 10 mm below the skin surface. The 15 mm thick (axial direction) cyst was partly surrounded by fatty (upper side) and partly by glandular (lower side) tissue. The ultrasound image was judged to be most probably benign (BI-RADS 2).

Photoacoustic mammography shows two high intensity areas, lying almost on top of each other as is shown in Figures 5.6c and 5.6d. The depth at which the lesion can be seen, matches the expected cyst depth. The (demodulated and modulated) profiles through the lesion (Figure 5.6e) show comparable characteristics as the profiles from the simulations and phantom measurements: two photoacoustic signals are visible at depths matching the front and the back of the cysts and these two signals are reversed in sign and different in shape. The difference with simulations and phantom results, and with other cases, is that the signal from the back of the cyst appears stronger than the leading signal.

Comparable profiles could be seen in, at least part of, the cyst representing regions in two other patients. In one out of four patients, large and multiple benign cysts were present in the breast, which could not be visualized using PAM.

Five batches of cyst fluid were obtained from patients whose non-suspicious cysts were aspirated. Upon visual inspection both the color as well as the scattering was highly varying between the batches of cyst fluid from the different patients as can be seen in

Figure 5.7. Differentiation of absorption and scattering was not possible since the non-negligible scattering made the interpretation of transmission spectra difficult.

Figure 5.6 Imaging results from a 49 year old patient with a palpable lesion in her right breast. **a)** X-ray mammography shows a sharply delineated, circular mass in the lateral upper quadrant of the right breast, which is judged as being probably benign. **b)** Ultrasonography reveals the presence of a benign cyst because of a circumscribed hypoechoic lesion with posterior enhancement and acoustic refraction at the edges of the lesion **c-e)** photoacoustic imaging shows enhancement of the front and the back of the cyst, with comparable characteristics as for the phantom and patient measurements. **c)** 3D subvolume of the cyst, the black dotted square indicates the orientation of the sagittal slice of figure **d)** and **e)** profile through the center of the cyst, as indicated by the white dotted line in figure **d)**.

Cyst samples					
Sample	P1	P2	P3	P4	P5
Color	Green/brown	Yellowish	Brownish	Orange	Yellow
Turbidity	Transparent	Slightly turbid	Slightly turbid	Highly turbid	Turbid

Figure 5.7 Cyst fluid from five different patients shows the variability in turbidity and color.

5.4 Discussion

In the search for improved imaging modalities for detection and diagnosis of breast cancer, the ability to differentiate between benign cysts and malignant lesions is of great importance. Photoacoustic imaging is a relatively new imaging modality that has potential for visualizing breast malignancies, but little is known about the photoacoustic appearance of breast cysts. In this study, we investigated the visibility of benign breast cysts in forward mode photoacoustic mammography using 1064 nm light, as currently applied in clinical studies [7]. The results from (Monte Carlo and k-Wave) simulations and phantom measurements were used to interpret and understand the results from patient measurements. In this section, the results and their implications for PAM and photoacoustic mammography in general are discussed.

5.4.1 The visibility of cysts using PAM

5.4.1.1 Simulations and phantom measurements

The results from simulations and phantom measurements showed that cysts can indeed be visible using PAM as either one or two high contrast areas in the reconstructed photoacoustic volumes (Figure 5.4). Based on the depths at which the high intensity areas were observed, we attributed them to representing either the front (illumination side) or the front- and the back-side (detection side) of the cyst. From the absorbed energy profiles (Figure 5.3), we could see that these are also the locations where there is a sudden change (either a drop or an increase) in absorbed energy. Therefore, the front and back representation of the cysts are believed to be a true representation of the initial pressure distribution and not an artifact of limited detection bandwidth. This is further supported by an extra set of k-Wave simulations in which we modeled an ideal ultrasound detector, i.e. a detector for which we do not specify a center frequency and bandwidth. With the exception of signal shape variations, the simulations also show the front and back of the cyst as seen in Figure 5.8b.

In the absorbed energy profiles (Figure 5.3) it can further be seen that the variation in Grüneisen coefficient (Γ) between cysts and solid breast tissue plays an important role in the visibility of especially the back-side of the cyst. This variation causes a gradient in the initial pressure distribution upon pulsed laser illumination and thereby an observable photoacoustic signal which makes even a cyst with equal absorption as its background visible in PAM (figure 5.4 d,e,f). Moreover, for highly absorbing cysts, the front-side is less pronounced because of the decrease in Γ when going from the relatively solid breast tissue to the fluid-filled sac. In this case, the back-side is hardly visible, even if enough photons are able to reach the back-side of the cyst (Figure 5.3c). In such a situation it would be difficult to differentiate the cyst from a malignancy, using the current forward mode imaging configuration.

Including variations in Γ and acoustic properties in the simulations also makes the shape difference between signals from low and high absorbing cyst mimicking structures,

as well as from the front and the back of the cyst, less pronounced. However, these shape differences are present in the results from phantom measurements. This might indicate that the Γ of Agar is closer to water than is the Γ of biological tissue as used in the simulations, lowering its influence on the final results. Moreover, the absorption contrast used in the phantom measurements was much higher than for the simulations and this possibly overrules the effect of Γ .

Figure 5.8 Photoacoustic profile through reconstructed photoacoustic volume after K-wave simulations, for **a)** The PAM detector having 1 MHz center frequency and 130% bandwidth and **b)** an ideal transducer, which is equally sensitive for all frequencies. The same band-pass filtering is applied for both situations. Although there are some shape and amplitude difference between figures **a** and **b**, it becomes clear that also in the ‘ideal’ case a front and back enhancement can be seen.

5.4.1.2 Clinical measurements

In the clinical measurements, 3 out of 4 benign cysts were observed with at least partly the same features obtained during the simulations and phantom measurements. These features include the visibility of one or two signals representing the front or the front and back side of the cyst and the change in signal shape when comparing the signals from the front and the back of the cyst. However, we chose to present a case which is slightly atypical, while sharing the most important features with other cases (Figure 5.6). The first unusual aspect is that the back signal of the cyst appears to have higher amplitude than the front, which was not observed in phantom measurements and simulations. The explanation for this feature can be found in the ultrasound image (Figure 5.6b), where the cyst is partly surrounded by the subcutaneous fat and partly by the glandular tissue. Fat has lower μ_a than glandular tissue at 1064 nm [32], making the absorption contrast at the front of the cyst lower than at

the back of the cyst. The second aspect is the small distance between the front and the back signal of the cyst in the PA image. Based on the ultrasound image, this distance is expected to be at least 15 mm, while in the photoacoustic image the distance is less than half of this. This phenomenon was observed for the other two photoacoustically visible cysts as well. This might be the consequence of the shifting and compression of the cyst in the PAM situation, which was not modeled in simulations and was not observed in the phantom measurements because of the stiffness of Agar.

5.4.2 Phantom and simulation models

The correspondence in shape and position of the photoacoustic signals following reconstruction of the measurements and simulations showed that our models, although simplified, were sufficient to investigate the *in vivo* situation. From reported optical imaging studies [22-26], it is known that cysts are usually homogeneously absorbing, with an absorption coefficient (μ_{a_c}) varying from below to above the absorption coefficient of breast tissue (μ_{a_b}) and a scattering coefficient (μ_{s_c}) almost always below that of breast tissue (μ_{s_b}). Therefore, the optical properties of both the simulation and phantom models were chosen appropriately. However, aspiration of cyst fluid from human subjects revealed that cyst fluid can in fact be rather turbid (Figure 5.7), which was not accounted for in the simulations. Extra simulations (not shown here) with μ_{s_c} ranging from below to equal to μ_{s_b} , reveal that the scattering coefficient in this range can slightly decrease the visibility of the back of the cyst, but that the influence is minor with respect to the influence of the absorption coefficient which is believed to be much more varying between patients.

In the simulations, cysts are modeled as spherical structures, while in phantom measurements the easier to produce fluid filled cylinders are used to model the cyst. An extra set of simulations showed that this simplification is justifiable, since the difference between the sphere and cylinder signals do not change the conclusions.

Lastly, the breast was modeled as a homogenous medium, with optical and acoustic values approaching those of glandular breast tissue. The fact that cysts most commonly arise by duct obstruction in the glandular tissue, justifies this choice. A more complicated, multilayered model is not believed to improve the understandability and reliability of the results.

5.4.3 Implications for PAM and photoacoustic cyst imaging in diagnosis

Using the current forward-mode single wavelength photoacoustic mammoscope, differentiation of cysts from one or two malignancies might be problematic in some cases. The appearance of a leading and second high contrast area along the normal from illumination to detection, is not necessarily an exclusive feature for a cyst, and multifocal carcinoma could also present such an appearance. Further, in the case of highly absorbing cysts, the back of the cyst might be invisible, additionally confounding the differentiation of cysts from malignancies if the two-contrast area feature is assumed to be the signature appearance of cysts. With the forward mode single wavelength system, malignancies often

appear as single high contrast areas in the photoacoustic imaging volume. Measuring with multiple wavelengths will be of utmost importance to differentiate hemoglobin absorption associated with malignancies, from the absorption by chromophores more characteristic for cyst fluid [2]. For the optimal choice of wavelengths, the optical absorption spectra of a large set of aspirated cyst fluids should be measured.

The reported results further recommend the use of a closed detection aperture with reconstructions from multiple projections as in a tomographic imaging geometry [46, 47] with a more homogenous fluence distribution. While the inhomogeneous absorbing malignancies will be most likely visible as heterogeneous masses with absorption throughout the entire lesion, we predict that cysts will be visible as structures with a thin shell, because of the strong change in fluence at the edges of the cyst. Simulations using the CT approach should throw more light on the implications of tomographic imaging for differentiating cysts from malignancies.

Finally, a better differentiation between cysts and malignancies is expected by combining photoacoustic imaging with imaging of ultrasound properties of the medium, like acoustic attenuation imaging [47, 48].

5.5 Conclusions

Cysts can be visible in forward mode photoacoustic mammography as one or more pronounced, confined high contrast areas representing the front (illumination side), or the front and the back (detection side) of the cyst. In the current forward mode imaging configuration and with one wavelength, cysts may be mistaken for malignancies which can also present as one or multiple confined contrast areas. Performing photoacoustic imaging with multiple projections in a computed tomographic geometry will most likely permit discrimination. However, quantitative multispectral photoacoustic imaging is expected provide the most robust differentiation of cysts from malignancies.

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6

Photoacoustic image patterns of breast cancer: correlation with Magnetic Resonance Imaging and histopathology⁵

Photoacoustic mammography aims at visualizing breast cancer by exploiting the locally increased hemoglobin concentration as a consequence of angiogenic processes. High intrinsic optical contrast, combined with good ultrasound resolution and the fact that the technique does not require ionizing radiation or contrast agents, make photoacoustic mammography potentially an ideal method for visualizing breast tumors. First clinical studies using the Twente photoacoustic mammoscope (PAM), which operates in planar configuration using a single wavelength (1064 nm), showed that the technique can indeed visualize malignancies with high imaging contrast. Understanding the relation between photoacoustic image patterns and tumor vascularization is crucial for the development of future optimal photoacoustic mammography systems. In this study, an updated version of PAM is used for a first qualitative comparison between the photoacoustic lesion appearance at 1064 nm and tumor vascularization. In 14 patients, photoacoustic images obtained from a large region of interest (9x8 cm²) on the breast are compared to either Magnetic Resonance (MR) images, CD31 stained tumor slides, or both. There was good correspondence between photoacoustic and MR images with respect to lesion location, appearance and shape. Moreover, the photoacoustic lesion shape and appearance could be related to the histopathological appearance of the malignancy and often also to the pattern of vascularity as determined from CD31 stained tumor slides. These results prove once more the potential of PAM for visualizing malignancies, mainly as a consequence of the local abundance of hemoglobin in the tumor vascularity. However, results also indicate that the contribution of other chromophores to the photoacoustic lesion appearance cannot be excluded.

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6.1 Introduction

Breast cancer is the most common form of cancer and one of the major causes of cancer death among women [1, 2], accounting for about 1.4 million new cancer cases worldwide a year [1]. Early detection of breast cancer can significantly improve survival chances [2], hence all efforts to detect the disease as early and accurately as possible. Unfortunately, conventional first-line imaging modalities, like x-ray mammography and ultrasound (US) imaging, do not only suffer from non-optimal sensitivity and specificity [3, 4], but also from modality-specific limitations. X-ray mammography uses ionizing radiation, painful breast compression and shows poor performance in radio-dense breasts [5-7]. For US imaging, inter-operator dependence and poor soft tissue contrast make the technique inapplicable as stand-alone imaging modality [5, 8].

One of the hallmarks of breast cancer that is being investigated for its utility in detection and diagnosis of the disease, is tumor vascularization [9, 10]. Breast malignancies are able to set-up their own vascular network via angiogenesis: a crucial factor for tumor progression beyond 2 mm in diameter [11, 12]. The growth of new blood vessels results in a locally increased microvascular density (MVD) with dilated and tortuous microvessels [13, 14]. Visualizing this local and poorly organized abundance of microvessels can provide information about the presence of malignancy. The gold standard for measuring the end product of angiogenesis is histological estimation of the MVD after immunohistochemical staining by vessel-specific reagents such as anti-CD31, anti-CD34 or anti-Factor VIII/von Willebrandt factor [15, 16]. It has indeed been proven that the MVD is increased in malignancies with respect to healthy tissue [15]. Moreover, the MVD is known to be heterogeneous across tumor types [16, 17].

The altered MVD and vessel structure are exploited by Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI), which is currently the gold standard for imaging tumor vascularization [18]. By injection of a T1 (spin-lattice relaxation time) shortening contrast agent into the blood stream (usually a gadolinium-containing compound [19]) and imaging at various time-points before and after contrast injection, both the total amount and pattern of enhancement at specific locations, as well as the in- and outflow rates of the contrast agent can be determined. This information about the local vasculature and the leakiness of the involved vessels, makes the technique highly sensitive for visualizing malignancies [18]. The enhancement of malignancies in Magnetic Resonance (MR) images can indeed be related to an increased MVD [20, 21], but the differing enhancement behaviors of malignant and benign breast lesions cannot be explained by MVD alone [22]. Although being the gold standard for imaging tumor vascularization, DCE-MRI itself also suffers from some major limitations: the excellent sensitivity as a consequence of strong enhancement in the vasculature and its surroundings, occurs at the cost of specificity and there is a significant lesion size overestimation [23-25]. Moreover, the technique requires contrast agents and results are dependent on the hormonal status of the patient [26, 27]. Despite these limitations, MRI's high sensitivity for visualizing

malignancies proves that tumor vascularization is indeed a useful hallmark for screening and diagnosis of breast cancer.

A modality that also aims at exploiting the altered tissue composition as a consequence of angiogenic processes, is photoacoustic (PA) imaging using near infrared (NIR) light [28, 29]. This technique makes advantage of the high intrinsic contrast possible with optical imaging, but overcomes resolution problems associated with high scattering of light in tissue [28-30]. The technique does not require the use of ionizing radiation or injection of contrast agents, making it potentially an ideal technique for breast imaging. In PA mammography, the breast is illuminated by short pulses of laser light, which are absorbed by the various chromophores in tissue. Mainly because of the high hemoglobin concentration, the energy deposition is highest at the location of the malignancy. The light absorption results in a minimal temperature increase, leading, by the process of thermoelastic expansion, to a transient pressure wave which propagates through the tissue. This pressure wave can be detected by US detectors with the appropriate center frequency and bandwidth [29]. The low scattering of US compared to light gives the technique the better resolution required for diagnostic breast imaging compared to purely optical imaging techniques [28].

Over the years, PA mammography has been tested in several configurations in phantoms, small animals and even patients [31-33]. We developed the Twente Photoacoustic Mammoscope (PAM) which images the breast in transmission mode using 1064 nm light [34, 35]. First clinical studies in small patient groups [36, 37] showed the potential and limitations of PAM for visualizing malignancies. We observed that PAM was able to visualize all malignancies with high imaging contrast and that this contrast was independent of the mammographically estimated breast density [37]. However, the size of lesions in PA images was always less than the true (histopathologically defined) lesion size. Moreover, the relatively small field of view (FOV) of $4 \times 4 \text{ cm}^2$, forced us to concentrate on 1-3 cm sized palpable masses to ascertain correct positioning. This strongly reduced the variability in measured malignancies, probably leading to the fact that in almost all cases the PA intensity was relatively homogenous and most pronounced in the center of the abnormality. More heterogeneous distributions are to be expected in a more representative patient population.

We recently upgraded PAM to enable faster imaging within a larger region of interest. This strong increase in image size allows us to include a higher variety of lesions and to get more acoustic information from the lesion of interest, most likely leading to better size and shape reconstruction. The upgraded system gives us the opportunity to investigate a fundamental question in the research to PA mammography: what is the relation between the photoacoustic lesion appearance at 1064 nm and tumor vascularization? The answer to this question can help us to further define the potential of PA mammography in the breast care model and to develop future versions of PAM. The first step to get information on the

relation between the PA lesion appearance and tumor vascularity is to compare the results from the upgraded PAM with MR images and histopathology slides.

6.2 Materials and methods

6.2.1 Diagnostic trajectory

Clinical measurements were performed at the Center for Breast Care of Medisch Spectrum Twente (MST) in Oldenzaal. The study protocol and informed consent procedure were approved by the hospital's review board. The study has been registered in the Netherlands Trial Register (NTR) as TC 2945. In the period between April 2012 and April 2013, adult female patients with suspicious breasts following clinical examination and conventional imaging, were asked for their participation in the study.

The normal diagnostic pathway comprised the triple assessment: clinical investigation and anamnesis; followed by conventional imaging using x-ray mammography and US imaging; and ending with core needle biopsy. A patient was included in the PAM study if a suspect lesion was visible in images from one or both modalities. PAM measurements (see Section 6.2.2) were performed before the biopsy procedure. The x-ray and US images were interpreted by a breast imaging radiologist and graded according to the BI-RADS classification scale [38]. Decisions on further treatment or follow-up were based on the integrated information from the triple assessment.

6.2.2 PAM measurements

Some earlier published details of the Twente Photoacoustic Mammoscope (PAM) [34, 35] and the study procedure [37] will be concisely repeated here. During the PAM investigation, the patient lies in prone position on the bed (Figure 6.1) with her breast pendant through an aperture. The breast is slightly compressed between a glass plate for laser illumination from the cranial side and the ultrasound detector array from the caudal side. After positioning, a digital photograph of the breast against the detector is being made. By using the photograph of the breast in cranio-caudal (CC) position in combination with the detector position, the CC PA and x-ray images can be merged retrospectively.

The breast is illuminated with pulsed (10 ns, 10 Hz) laser light at 1064 nm, from a Q-switched Nd:YAG laser (Continuum Surelite, California, USA). The size of the beam is approximately 35 cm² and the beam is kept at a fixed position throughout the measurement. At the breast surface, the energy per pulse does not exceed 350 mJ, giving a radiant exposure of 10 mJ/cm², well below the Maximum Permissible Exposure (MPE) of 1064 nm on the skin (100 mJ/cm²) [39]. In case the breast does not cover the complete detector, the remainder of the detector is protected from light falling directly onto it by a protective sheet. PA signals are detected by a 1 MHz (130% bandwidth) 2D ultrasound detector array consisting of 588 elements in a roughly circular layout with diameter of approximately 85 mm [35].

Figure 6.1 *The Twente Photoacoustic Mammoscope. a) Aperture to insert breast. b) Ultrasound detector matrix. c) Glass window. d) Scanning system compartment. e) Q-switched Nd-YAG laser operated at 1064 nm with 10 ns pulses. f) Laser safety curtain which is drawn around the instrument during the measurements. g) Interface electronics between detector and computer. h) Linear stage carrying detector matrix driven by hand wheel to apply mild compression to the breast. i) Laser remote control unit. j) Laser power supply. Image adapted from reference [40] with permission.*

Signals from ten elements, spread over the detector area, are acquired simultaneously using two eight channel digitizers (National Instruments, NIPXI 5105, 60 MS/s, 12-bit) controlled by a Labview program (National Instruments). With 10 averages per acquisition, the measurement duration is 10 minutes for the complete detector, covering an area on the breast of approximately $9 \times 8 \text{ cm}^2$.

Signal processing, image reconstruction and analysis were performed using Matlab (R2011b, the Mathworks Company). Prior to reconstruction, the high signal from the breast surface was removed to have a more homogenous dynamic range in the PA volume. Signals were filtered with a band-pass Butterworth filter (cut-off frequencies 0.2 MHz and 1.7 MHz) to remove the low frequency trend and high frequency noise. Reconstruction was performed using an acoustic backprojection algorithm, assuming a homogenous speed of sound of 1540 m/s. The inaccuracies arising from the use of this homogenous speed of sound are estimated to be minor with respect to the system's resolution (approximately 3 mm in both axial and lateral direction [35, 41]). After reconstruction, the separate sagittal slices of the volume were Hilbert transformed and all voxel intensity values were normalized to the maximum intensity value within the total volume and scaled to obtain intensity values between 0 and 255.

The PA imaged volume was examined for the presence of high-intensity areas or other abnormally structured lesions. A sub-volume was defined such as to include the maximum depth extension of the lesion in the volume. The maximum intensity projection (MIP) of the sub-volume was overlaid on the CC x-ray mammogram using the position information obtained from the digital photograph. In this way, the lesion position in the PA image could be compared to the lesion position in the CC x-ray images. In case of good co-location of the lesions, the lesion in the PA image was assumed to represent the abnormality under diagnostic investigation. The lesions were classified according to their appearance on the

MIP (see Section 6.3.1). The size of the PA lesion was estimated via a manually drawn contour around the lesion on the MIP, using information from conventional imaging.

6.2.3 *MRI protocol and analysis*

A dynamic contrast enhanced MRI (DCE-MRI) investigation after triple assessment was recommended in case of high breast density, inconclusive diagnosis or the need for preoperative assessment of lesion extent, especially in lobular breast cancer. High-resolution, anatomic T2 (spin-spin relaxation time) weighted images, and dynamic T1 (spin-lattice relaxation time) weighted images before and after gadolinium injection (usually 1 pre and 3-4 post-contrast images divided over a time-scale of at maximum 9 minutes after contrast injection) were acquired using a 1.5 or 3 Tesla MR system in combination with a dedicated 7 channel Sense Breast Coil (Philips Medical Systems, Best, The Netherlands). In pre-menopausal patients, the MRI was planned in the 2nd week of the menstrual cycle when the enhancement of the (benign) glandular tissue is known to be least pronounced [42].

MR images were interpreted by a specialized breast radiologist with extensive MR experience. The T2 weighted images were first examined for morphological changes and architectural distortion of the breast tissue. Following subtraction of the pre-contrast images, the T1 weighted post-contrast images were examined for the presence of abnormally enhancing areas. In case it was doubtful if the enhancing area indicated a malignant or benign lesion, the dynamic enhancement curve was further examined. The tortuous and dilated microvessels cause a rapid inflow and therefore rapid enhancement, but also a rapid outflow by extravasation of the contrast agent. By this rapid outflow, the enhancement curve of malignancies is different from that of hypervascularized benign abnormalities such as fibroadenomas [26]. The radiologist usually described the lesions as abnormally enhancing areas with or without evident mass contribution (space occupation and distortion). Further information concerned the shape, the borders, the heterogeneity, the presence of ring enhancement, the presence of spiculae and the presence of other foci of the lesion elsewhere in the breasts.

PA and MR images were compared for the presence, size and appearance of abnormal areas. The pendant position of the breast during MRI made it possible to retrospectively compare the lesion position with the information obtained from the digital photograph. However, since the amount of compression varied from heavily (x-ray mammography) via slight (PAM) to none (MRI), the tissue was deformed differently during all investigations and one-to-one comparisons could not be made.

6.2.4 *Histopathological assessment*

Excised tissue was examined at the Laboratory for Pathology East Netherlands (LabPON). The orientation of the breast or lump was marked by the surgeon, after which it was fixated in formalin 4% for at least 24 h and cut in approximately 5 mm thick sagittal slides. If the tumor was associated with microcalcifications, x-ray specimen images of the tissue slides

were acquired. For further investigation, slides were taken from the tumor and its surroundings and embedded in paraffin blocks. From these, various 2 μm slides were cut and stained with Hematoxylin and Eosin (H&E). These slides were reviewed by a pathologist who reported on the status of the surgical resection margins; the histologic type (according to the WHO classification [43]), the histologic grade (according to the Bloom-Richardson scale [44]); the hormone receptor status (ER, PR and Her2Neu); the presence of *in situ* components; and the complete size of the invasive (and/or non-invasive) part of the lesion.

In a subset of patients for which the lesion in PA images could be related with certainty to the abnormality visible in conventional images, an extended histopathology protocol was performed. The breast or lump was sliced in transversal direction in order to obtain slices in the direction from which most information is available from the PA images as a consequence of the forward mode imaging. In addition to the stains applied for normal clinical diagnosis, one or more slides of the malignancies were stained for vascularity using anti-CD31 (further referred to as CD31), according to standard procedures visualized with DAB (diaminobenzidine) antistaining which gives a brownish color to the antibody [45]. This antibody is mainly located at the borders between endothelial cells and can thereby give information about the density and pattern of vascularity present [46]. If possible, this CD31 stained histopathology slide contained a complete cross-section of the malignancy. However, if the lesion was too big for this, and whole-mount (4x4 cm^2) sections could not be obtained, multiple slides were cut and stained for vascularity. These slides together either composed an entire cross-section of the tumor or represented areas spread over the entire lesion, including center and border areas. The CD31 stained slides and the H&E stained slides were digitized using the Hamamatsu Nanozoomer 2.0-HT slide scanner (Hamamatsu, Japan). The pattern and amount of microvasculature as well as the size, shape, type and grade of the lesion were qualitatively compared to the appearance patterns on PAM and MRI.

6.3 Results

6.3.1 General results

In the period between April 2012 and April 2013, technically acceptable measurements on 29 patients having one or more breast malignancies were performed. In 28 patients, the lesion(s) could be identified with great certainty in the PA imaging volume. In contrast to the previously reported clinical data [37], lesions not only presented as homogenous masses, but now various types of lesion appearance could be described. These patterns are reminiscent of enhancement types generally reported for DCE-MRI [47, 48] and the same descriptions as in MR nomenclature have been adopted here:

1. Mass-like or confined appearance: a confined region of high intensity, being either homogenous or heterogeneous in intensity and regular or irregular in shape;

2. Ring appearance: regions of high intensity, completely or partially surrounding an area with lower intensity;
3. Non-mass or scattered appearance: scattered foci of high intensity within an extended region.

From each appearance type, one or more representative cases were selected for the extended pathology protocol. In total, 6 cases were included for the vascular (CD31) staining of which for 3 MR images were available. Of these 6 patients, two followed the intended protocol and four were investigated retrospectively, which means that their lesion was sliced in sagittal (normal histopathology protocol) instead of transversal direction. The remainder of the protocol was the same for all six patients. For 8 additional patients not included in the extended histopathology protocol, MR images were available in addition to conventional and PA images.

Table 6.1 describes the lesion characteristics of the total number of 14 patients included in this study.

Table 6.1 *Lesion characteristics for PAM, MRI and histopathology*

Case (Sect. 6.3.3) and Figure	P	Type of malignancy	PAM appearance category	MRI appearance category	Extended histopathology appearance	Size PAM (mm)	Size MRI (mm)	Size pathology (mm)
1, Fig 6.2a-b, 6.3	38	IDC Grade 3	Mass	Mass with ring enhancement	Vascularity heterogeneously spread, but higher density at border	19	22	19
Fig 6.2c-d	55	IDC, Grade 2	Mass	Mass	Vascularity spread	28	50	34
	70	IDC, Grade 3	Mass	Mass	Vascularity heterogeneously spread, but higher density at border	21	30	19
2, Fig 6.4	47	IDC Grade 2	Ring		Vascularity confined to border	24		24
3, Fig 6.5	39	IDC Grade 3	Non-mass		Vascularity most pronounced in non-cohesively organized fields	41		63
4, Fig 6.6	37	IDC Grade 2	Atypical ring		Vascularity heterogeneously spread	31		28

Table 6.1 Lesion characteristics for PAM, MRI and histopathology – continuation

Case (Sect. 6.3.3) and Figure	P	Type of malignancy	PAM appearance category	MRI appearance category	Extended histopathology appearance	Size PAM (mm)	Size MRI (mm)	Size pathology (mm)
	40	ILC ¹	Non-mass	Diffuse non-mass with mass component		45	N.A. ³	N.A. ¹
	49	LCIS (+ ILC) ²	Non-mass	Largely non-mass		54	50	~40
Fig 6.2 e-f	52	IDC ¹	Non-mass	Mass		53	55	N.A. ¹
	56	IDC Grade 3	Mass	Mass		11	32	22
	58	ILC Grade 2	Mass	Mass		19	55	22
	61	IDC ¹	Mass	Mass		21	20	N.A. ¹
	62	IDC Grade 2	Mass	Mass		16	19	18
	67	IDC ¹	Mass	Mass		16	15	13

IDC=Infiltrating Ductal Carcinoma; ILC=Infiltrating Lobular Carcinoma; LCIS = Lobular Carcinoma in Situ; N.A.=Not Applicable

¹These patients underwent neoadjuvant chemotherapy instead of surgery. Therefore histopathology data is limited to the information available from the biopsy specimen.

²In this patient, only LCIS was found in the post-mastectomy specimen. However, the presence of lymph node metastases proved that there should have been an invasive component associated with the disease. For the histopathological size (last column), here the LCIS extension is given. For the other 13 patients, the size of the invasive component is given.

³For this patient the size could not be estimated on MR images, because of its large extension in the breast and the non-mass rather than mass appearance.

6.3.2 Comparison between PA and MR images

Figure 6.2 shows average intensity projections (AIPs) in CC direction of MR (left) and PA (right) data. The area from which the PA image is obtained is indicated in the MR image by a white dashed box. The first two cases (Figure 6.2a-b and 6.2c-d) are representative for the patients with lesion sizes up to 35 mm. On visual inspection there seems to be excellent correspondence between the lesion location in PA and MR images for all but one patient presenting with such lesions. There was also reasonable to good correspondence in lesion shape and general appearance as can especially be seen in Figure 6.2a-b, but also in Figure 6.2c-d and in Table 6.2. The resemblance between PA and MR images was minor (P49, P52) to poor (P40) for the three patients with larger lesions (see Figure 6.2e-f and Table 6.1 and 6.2), which all presented as non-mass appearance in the PA images. In these patients, the lesion size was estimated to be >40 mm on conventional imaging, probably too large to have an appropriate PA reconstruction of the complete malignancy and its surroundings.

Figure 6.2 Comparison between MRI (left) and PAM (right) images for three patients. In all cases a cranio-caudal average intensity projection (AIP) of the MRI and PAM detected lesion is given. Left: AIP MRI, the white-dashed boxes indicate the area from which the PA images are obtained. Right: PAM AIP. **a-b)** Patient 38, having an IDC, grade 3 of 19 mm. **c-d)** Patient 55, having an IDC, grade 2 of 34 mm. **e-f)** Patient 52, having an IDC (unknown grade) of more than 50 mm.

Table 6.2 shows the comparison between PAM and MRI detected lesions. In one patient (P56), the breast was severely tilted during the PAM measurement, making it difficult to correlate the PA positioning to the x-ray and MR positioning. However, in this case the

position of the lesion in the PA volume perfectly matched with the position where the mass was expected based on palpation and ultrasound imaging.

The estimated size of the lesion was significantly larger in MR images than in PA images for a number of cases. This was mostly the consequence of the visibility of spiculae in the MR images, which were not visible in PA images. In those patients (P55, P58, P70), the estimated lesion size from PA images was closer to the histopathologically defined lesion size (see Table 6.1).

Table 6.2 PAM and MRI representation of the identified lesions

P	Figure	PAM-MRI co- localization	PAM description	MRI description	PAM size (mm)	MRI size (mm)
38	6.2 a-b, 6.3	Good	Heterogeneous mass	Mass with ring enhancement; enhancement most pronounced at border and surrounding several hypodense fields.	19	22
40*		Reasonable	Non-mass; area with abnormal appearance	Diffuse non-mass with mass component in lateral upper quadrant; architectural distortion with malignant enhancement	45	N.A.
49		Good	Non-mass; area with multiple enhancing foci	Non-mass, with possible mass contribution	54	50
52	6.2 e-f	Good	Non-mass; area with abnormal appearance	Strongly heterogeneous mass, vascularity most pronounced at border	53	55
55	6.2 c-d	Good	Irregularly shaped mass	Irregularly shaped mass	28	50
56		Poor-reasonable	Spherical mass	Spherical mass	11	32
58		Reasonable-good	Star-shaped mass	Star-shaped mass	19	55
61		Good	Irregularly shaped mass	Irregularly shaped mass	21	20
62		Good	Spherical mass	Spherical mass	16	19
67		Reasonable-good	Oval mass	Oval mass	16	15
70		Good	Lobed mass	Oval mass	21	30

* In this patient the size of the lesion could not be assessed, because it occupied a large part of the entire breast and was largely associated with diffuse non-mass appearance in MRI.

6.3.3 Comparison between PA appearance and histopathology

In general, the size and shape of the lesions in PA images showed similarities with the histopathologically derived size and shape of the lesions. Further, there was good

correspondence between the pattern of microvasculature obtained from the CD31 stained tumor slides and the PA appearance in 5 out of 6 of the investigated cases.

- For all three cases of mass appearance in PA images (P38, P55, P70), the microvasculature was spread over the entire lesion (see further Section 6.3.3.1).
- For the two cases of ring appearance in PA images (P37, P47), the tumor nests were confined at the lesion border, surrounding a fibrotic core. Only in one patient the expected higher vascularity could be observed at the lesion border (see further Sections 6.3.3.2 and 6.3.3.4).
- In the case of non-mass appearance investigated here (P39), the microvasculature was most pronounced in fields of tumorous cells which were non-cohesively organized over the entire lesion (see further Section 6.3.3.3).

In the following sections, 4 specific cases with different PA appearance types are discussed with detailed comparison with MR images (if available) and histopathology.

6.3.3.1 Case 1- Mass appearance for IDC

This 44 year old Caucasian patient (p38) came to the Center for Breast Care for annual x-ray mammograms and clinical investigation following a history of infiltrating ductal carcinoma (IDC) in the left breast. No abnormalities were observed on the x-ray mammograms of both breasts. However, the presence of a palpable mass in the right breast required a localized US investigation. Figure 6.3a shows the x-ray mammogram of this breast (no abnormalities, BI-RADS 1) and Figure 6.3b shows the results for US imaging, which revealed the presence of an unsharply delineated, irregular, hypoechoic mass of 22 mm which was highly suspect for malignancy (BI-RADS 5). Histopathological investigation of the tissue specimen after core needle biopsy, revealed the presence of IDC. Because of the discrepancy between x-ray and US, a preoperative DCE-MRI was being performed. MRI did not reveal any other malignancies, but could clearly identify the malignancy of interest as can be seen in Figure 6.3c. This average intensity projection of the MRI volume shows a heterogeneous intensity distribution at the expected lesion location, which was described as a mass of 22 mm with ring enhancement by the radiologist. Moreover, the MR images show substantial enhancement of the surrounding (benign) parenchymal tissue.

Figure 6.3d shows the PA data as the MIP of the of the lesion in CC direction (the AIP of the same data is visualized in Figure 6.2b). The PAM ROI is indicated by a white-dashed box in the x-ray and MR images (Figures 6.3a and 6.3c respectively). The lesion measures 19 mm in the CC MIP and is defined as ‘mass’ because of the confined region of high intensity in the PA volume. Figure 6.3e shows a 3D representation of the region of the lesion. The ring enhancement of MRI cannot be observed in the PA image volume. Further, in the PA images the glandular tissue is much less pronounced than in the MR images. For the remainder, there is good correspondence with respect to location, size and shape of the lesion comparing the PA and MR images.

Figure 6.3 44 year old patient with infiltrating ductal carcinoma (P38). **a)** The cranio-caudal x-ray mammogram of the right breast does not show any abnormalities and is judged as BI-RADS 1. The white dashed area indicates the region of the PA image. **b)** A localized ultrasonic investigation of the palpable mass, reveals the presence of a 22 mm, hypoechoic, unsharply delineated and highly suspect mass. **c)** The average intensity projection of the MRI volume reveals the presence of a 22 mm mass with some ring-enhancement. The white-dashed area indicates the region of the PA image. **d)** A cranio-caudal maximum intensity projection of the PA volume shows a confined high intensity area, which is defined as mass appearance. The images show great resemblance with the MR images in size, shape and localization of the high intensity area. Differences lie in the more pronounced heterogeneity and in the (falsely) enhancing benign glandular tissue in MRI. **e)** The PA mass appearance is more prominent in the 3D representation of the lesion. The origin (0,0) of this subvolume does not correspond to the origin of the entire volume (Figure d). **f)** Post-surgical histopathological investigation reveals the presence of a 19 mm IDC, grade 3. **g)** The CD31 stained tumor slide shows the vascularity to be mostly heterogeneously distributed over the entire tumor. **h)** This is further highlighted by the four selected areas at the margin and in the center of the tumor.

The H&E stained slide (Figure 6.3f), which was used for normal histopathological diagnosis, indicated the presence of a 19 mm IDC, grade 3. Further staining revealed the

triple negative hormonal status (ER-, PR- and Her2Neu-) of the lesion. In the CD31 slide (Figure 6.3g and details in Figure 6.3h), the brownish color indicates the presence of CD31 and therefore of the endothelial cells which line the vessels. The brownish stain is spread over the entire malignancy. Both in the center (red and blue bold insets) as well as at the border (black and green dashed insets) of the lesion, fields with higher or lower vascularity are observed, which most likely explains the heterogeneity observed in the MR images. Although sometimes the vascularity seems to be more pronounced at the lesion border, the vascularity is spread over the entire tumor and a true ring appearance, as described by the radiologist in the MRI report, cannot be recognized from this particular tumor slide.

Also in the other two patients with mass-appearance in the PA images, the vascularity was spread over the entire lesion. Moreover, these two lesions also presented as mass in the T1 weighted MRI post-contrast subtraction images.

6.3.3.2 Case 2 - Ring appearance for IDC

This 69 year old Caucasian patient (P47) presented with a highly suspicious palpable mass in her left breast. Figure 6.4a shows the CC x-ray image of the left breast, which revealed a highly suspect lesion central in the breast by the presence of a 24 mm, partly unsharply delineated, inhomogeneous mass associated with skin thickening. The US investigation (Figure 6.4b), showed a hypoechoic, partly unsharply delineated, 29 mm inhomogeneous mass suspect for malignancy. In this case, the CC MIP of the PA volume (Figure 6.4c) shows high intensity areas which surround an area with low intensity. The high intensity regions appear to match the border of the suspicious mass in the CC x-ray image (see also the area from which the PA data are obtained, indicated by the white dashed box in Figure 6.4a). This lesion appearance is therefore defined as ‘ring appearance’. The ring appearance is more prominent in a 3D representation of the lesion (Figure 6.4d). The ring measures 24 mm in diameter on the MIP.

The H&E stained slice through the center of the malignancy (Figure 6.4e) revealed the presence of a 24 mm IDC of grade 2. This staining already shows differences between the border and the center of the lesion: a cell-rich (darker purple) border and a cell-poor, but connective tissue rich (pink) core. This specific appearance is the consequence of central fibrosis in the tumor. Figure 6.4f shows the CD31 stained slide of part of the tumor, including both central and margin areas. Although the brownish color and therefore the vascularity is not extremely pronounced, it appears to be confined to the tumor border (Figure 6.4f-g black and green dashed insets) and it is most pronounced at the interface between the tumor and normal tissue. In the center, fields without vascularity are observed (Figure 6.4f-g, red and blue bold insets). Therefore this appearance can be described as ‘fields of vascularity surrounding an avascular area’. This is in strong correspondence with the PA appearance, where a ring appearance was observed which was described as ‘areas of high intensity surrounding an area with low intensity’.

Figure 6.4 69 year old patient with IDC (P47). **a)** The CC x-ray mammogram of the left breast shows a highly suspect mass central in the breast, which is inhomogeneous, partly unsharply delineated and measures about 24 mm in diameter. The white dashed box indicates the area on which the PAM measurement is being performed. **b)** A localized ultrasonic investigation of the palpable mass revealed the presence of a highly suspect hypoechoic, inhomogeneous mass, which is partly unsharply delineated and measures 29 mm. **c)** At the PA MIP, the lesion presents as fields of high intensity which surround an area without intensity. These higher intensity fields seem to be located at the border of the malignancy. **d)** The ring structure is more pronounced in a 3D representation of the lesion. The origin (0,0) of this subvolume does not correspond to the origin of the entire volume (Figure c). **e)** The whole mount H&E stained slide through the tumor reveals the presence of an IDC of 24 mm in diameter, which is associated with central fibrosis. **f)** The CD31 stained tumor slide shows relatively low vascularity, which is most pronounced at the border of the malignancy. In the center, a large area without vascularity can be observed. **g)** Details of the border (green and black dashed insets) and center (red and blue bold insets) areas of the malignancy highlight the vascularity which is most pronounced at the border of the malignancy.

6.3.3.3 Case 3 - Non-mass appearance for IDC

This Caucasian patient (P39) came to the center for breast care following a suspicious screening mammogram of the right breast. A palpable mass was present above the nipple, which seemed to be associated with skin and nipple retraction on clinical investigation. Figure 6.5a shows the CC x-ray mammogram of the right breast, in which a large architectural distortion central in the breast and nipple retraction can be observed. The abnormal area measured more than 5 cm and was suggestive for the presence of a large malignancy. The US investigation (Figure 6.5b) revealed the presence of a large, irregularly shaped hypoechoic mass with hyperechoic border, which was partly invading into the subcutis. Further, widened ducts were observed, which was indicative for the presence of *in situ* components. The total abnormal area measured at least 5 cm and was judged as BI-RADS 5 (highly suspicious for malignancy).

Figure 6.5c shows results from PA imaging as a MIP in CC direction. Although it is difficult to truly link the PA appearance to the appearance on conventional imaging, it can be seen that this area is markedly different from the surrounding supposedly healthy tissue and the appearance of healthy tissue that is recognized from other measurements. The lesion cannot be attributed to one confined high intensity area, but is mainly composed of several moderate intensity regions of which the largest has a vessel or duct-like elongated shape. This appearance is therefore defined as ‘non-mass’ or ‘scattered abnormality’. The abnormal moderate to high intensity regions are indicated by white contours for clarification. The total abnormal area is shown as a 3D volume in Figure 6.5d. This region measures 41 mm on the MIP of the subvolume.

Figure 6.5e shows the results for H&E staining as used for conventional histopathological diagnosis. Because of the size of the lesion, multiple sections spread over the malignancy were investigated and no full view of the malignancy was obtained. Moreover, the extended histopathology protocol was performed retrospectively, after slicing in sagittal direction. Therefore, a one-to-one comparison between histopathology and PA images could not be made. Histopathological investigation revealed the presence of a 63 mm IDC, grade 3, which was associated with *in situ* components inside the process. The malignancy seemed to grow in a pre-existential duct with some nodular fields. The abnormal cells were mainly organized in non-cohesive dissociative infiltrating fields within an area of dense sclerotic fibrous tissue. Figure 6.5f shows the CD31 stained tumor slide of the same area as presented in Figure 6.5e. The overall rather poor amount of microvasculature is mainly confined to the cell-rich fields and less prominent, but still present in the sclerotic stroma (see also Figure 6.5g, for some details). This matches the appearance on PA images, where several non-organized moderate to high intensity regions could be observed rather than one confined high contrast area.

Figure 6.5 67 year old patient with IDC(P39). **a)** The CC x-ray mammogram of the right breast shows a 5 cm architectural distortion associated with nipple retraction. The area was suspicious for malignancy, especially since these specific features were absent in the right breast. The white dashed box indicates the area on which the PAM measurement is being performed. **b)** A localized ultrasonic investigation of the suspect area revealed the presence of a large, irregular shaped hypoechoic mass with hyperechoic border which was partly invading into the subcutis. The total abnormal area measured at least 5 cm and was judged as BI-RADS 5 (highly suspicious for malignancy). **c)** At the MIP of the PA volume, the lesion presents as separate fields of moderate intensity in an area which is different from the remainder of the ROI. This 41 mm abnormal area is defined as non-mass appearance. **d)** A 3D volume of the suspicious area shows the difficulty in appointing such a lesion. The origin (0,0) of this subvolume does not correspond to the origin of the entire volume (Figure c). **e)** The H&E stained slice through part of the tumor (sagittal direction), confirms the presence of IDC, grade 3. The tumor cells are mainly organized in non-cohesive fields within a dense fibrous stroma. The total malignancy measured 63 mm. **f)** The CD31 stained tumor slide shows relatively low vascularity, which is mostly confined in the tumor nests and less prominent in the sclerotic stroma **g)** This is also confirmed by a detailed look at the various parts of the malignancy.

6.3.3.4 Case 4 – Atypical ring appearance for IDC

Figure 6.6 Example of atypical ring appearance in PA images, observed in a 61 year old patient having IDC (P37). **a)** CC x-ray image of the right breast shows a dense structure in the lateral upper quadrant, which is associated with skin changes. **b)** The ultrasound image is highly suspect for malignancy because of the presence of a large hypoechoic, unsharply delineated and irregularly shaped mass. The lesion shows a hyperechoic border and is invading into the skin. **c)** The PAM MIP image highlights the nipple and further shows a ring-like area of high intensity foci around the lesion under investigation. **d)** The atypical ring appearance is more prominent in a 3D representation of the lesion. The origin (0,0) of this subvolume does not correspond to the origin of the entire volume (Figure c). **e)** The ring appearance can also be observed in the H&E stained histopathology slides of the tumor post-surgery. This (28 mm) IDC of grade 2 shows its tumor cells mainly in solid fields at the lesion border, surrounding a more fibrous core. **f)** The ring appearance is not prominent in the CD31 stained tumor slide, where the vascularity seems to be spread over the entire lesion. Visually, the CD31 staining seems the least present at the lesion border and most pronounced in the area in between the tumor nests at the inner side of the border, as is further highlighted in Figure g.

This 61 year old Caucasian patient presented with a palpable mass in her right breast. The lesion appeared as a highly dense region on CC x-ray mammography (Figure 6.6a) and was causing skin deformation. In US images (Figure 6.6b) the lesion presented as an irregularly shaped, hypoechoic mass with hyperechoic border, which was invading into the skin. The x-ray mammography and ultrasonography findings made the lesion highly suspicious for malignancy. In the PA images (Figure 6.6c-d), the presence of the nipple [37] can be observed. Further, multiple moderate to high intensity scattered fields are observed, which appear at the border of the malignancy after co-location with x-ray mammography (see also the white-dashed box in Figure 6.6a, which indicates the area from which PA information is obtained). The appearance can neither be fully described as ring appearance nor as non-mass appearance, but seems to belong to an intermediate category. The presence of the high intensity foci at the border of the malignancy, led to the PA description of atypical ring appearance.

H&E staining of the tissue specimen post-surgery (Figure 6.6e), revealed the presence of IDC, grade 2, also showing differences between border and center areas: the tumor cells are mainly organized in solid fields at the lesion border and surround a fibrous core. This matches with the H&E appearance of case 2 (ring appearance), however in this case the vascularity (CD31 stained slides in Figure 6f-g) is present throughout the entire lesion and not confined to the tumor border. It seems to be least pronounced inside the cell-rich border fields and most pronounced in the areas in between such fields.

6.4 Discussion

6.4.1 General findings

Photoacoustic imaging has been shown earlier to be able to visualize malignancies with high imaging contrast [37, 40]. In this study, we conducted first investigations into the pathophysiological basis for PA lesion appearance and contrast at 1064 nm, specifically examining the contribution of tumor vascularization. Despite its limitations for use in daily clinical practice, the straightforwardness of PAM and the perfect clinical setting in which it is embedded, allowed us to make a first detailed comparison between PA mammography, MRI and histopathology in a small patient population. This has given us first insights into the relation between PA lesion appearance and tumor vascularization. The most important findings of this study are:

1. There is excellent correspondence between PA and MR images based on localization of the malignancy, and a good to reasonably good correspondence for the shape and appearance of the malignancy.
2. The PA lesion shape and appearance correlates well with the histopathological lesion appearance.
3. The PA lesion appearance often correlates well with the histopathologically assessed vascularity pattern. When not the case, the appearance is most likely also modulated by the important contribution of chromophores other than hemoglobin.

The results lead to important discussion points that can help to assess the future potential of PA mammography and to eventually develop a system both sensitive and specific for visualizing malignancies.

6.4.2 *PA lesion appearance*

In general, three main types of lesion appearance were observed in this study: ‘mass or confined’, ‘non-mass or scattered’ and ‘ring’. These specific lesion appearances are reminiscent of enhancement types described in MRI literature: mass enhancement, non-mass enhancement, focal enhancement and mass enhancement with ring enhancement [47, 48].

Although it is tempting to relate the correspondence between the lesion appearances in PAM and MRI to the underlying pattern of microvasculature, MRI and histopathology literature do not provide the proper information to truly make this connection. Except for the rim-enhancement [21, 22, 49], little is reported about the microvascular pattern and/or underlying pathophysiology causing the specific appearance patterns in MRI. Moreover, there does not seem to be a clear relation between the lesion’s appearance type and the tumor type, since almost all patients investigated here presented with an infiltrating ductal carcinoma (IDC) of grade 2 or 3, while the PA, MRI and histopathology appearances were all highly varying across patients. In this study, per appearance category one or more lesions were selected for a first qualitative comparison between PAM, MRI and histopathology.

6.4.3 *PA appearance versus MRI*

In eleven patients, the correspondence between PA and MR images was investigated. On visual inspection, there was good correspondence between PA and MR images with respect to lesion location and to lesser extent with respect to lesion shape and appearance. However, the lesion of one of the patients (case 1, P38) was described as showing ring enhancement on MRI, which was not observed in the PA images and was neither described explicitly by the pathologist as visible in the H&E or CD31 stained tumor slides. A further look at this case is crucial for understanding the PA and MR imaging results in relation to tumor vascularization. Two key factors might play a role:

- First, for certain regions of the CD31 stained tumor slide (Figure 6.3g-h) the vascularity seems to be more pronounced at the tumor margin. The presence of highly vascular fields in the center of the malignancy (Figure 6.3h, red contour) in addition to lower vascularized areas (Figure 6.3h, blue contour) led to the description of a heterogeneous vascularity spread over the entire malignancy rather than ring vascularization. The inhomogeneous light distribution and relatively poor resolution of PAM, make it difficult to differentiate the fields of high vascularity from the 2-3 mm sized lower vascularized areas, leading to the rather homogenous PA lesion appearance. The heterogeneity is more pronounced in the MR images and it is not

unlikely that the specific vascular distribution can lead to a more ring-like appearance in certain MRI slides.

- Second, it is known from MRI literature that there are two types of ring enhancement [49]. The first type is visible early after contrast injection and is thought to be associated with a higher vascular density at the lesion periphery compared to its center. The second type is visible relatively late after contrast injection at the time the tortuous and dilated microvessels start to lose their contrast agent. This type is thought to be associated with inflammation and/or fibrosis outside the malignancy, which prevents the outflow of contrast agents in the outer microvessels compared to the central ones. Therefore, this type of rim-enhancement will not necessarily be observed in PA images. In fact, the ring enhancement in the MR images of this specific patient seems to be most pronounced at later time series, beyond 4.5 minutes post contrast injection. Possibly, the strong desmoplastic fibrotic stroma reaction described by the pathologist is preventing the outflow of contrast medium at certain locations of the malignancy.

For the patients with non-mass appearance in the PA images that were also investigated using MRI, the comparison between PA and MR images was more difficult. Technological limitations of PAM leading to a small FOV compared to the lesion sizes of >40 mm, and therefore to problems in an accurate representation of the lesion and its surroundings, are expected to play a prominent role in this.

6.4.4 *PA appearance versus CD31 stained tumor slides*

For all patients, the general lesion appearance, shape and size on PA images could be related to the histopathological appearance, shape and size of the malignancy. The CD31 stained tumor slides enabled comparison between PA images and the general vascular appearance. The latter was described as vascularity spread over the entire malignancy; vascularity confined to the tumor border; or vascularity non-cohesively organized in fields. Based on these descriptions, we could observe good correspondence between the PA appearance and the (micro)vascular appearance in all but one patient:

- Mass enhancement (3 patients) in PA images could be linked to a microvasculature pattern that was spread over the entire malignancy.
- For PAM's non-mass appearance (1 patient), the vascularity was mainly concentrated in the non-cohesively organized tumor nests and less present in the tumor stroma. This can also explain the non-negligible size deviation for PAM compared to histopathology in this case: the fields of vascularity do not constitute the entire malignancy.
- In two patients from this study, ring appearance was observed in the PA images. In both cases, H&E stained tumor slides, showed a region of tumor cells at the border of the malignancy, surrounding a fibrotic core in which less tumor cells and much more collagen fibers and fibroblasts could be observed. Based on the results from the other 4 patients and based on MRI literature [22, 50], it was expected that this ring

appearance could also be related to a higher microvascular density at the lesion margin compared to its center. However, this was only the case in one of the two patients, whereas in the other patient the vascularity was spread over the entire malignancy.

The findings indicate the sensitivity of PAM at 1064 nm for hemoglobin in the tumor vasculature. The fact that we observed an atypical ring appearance in PA images, despite the lack of a clear ring in the vascularity stained tumor slides in one patient (case 4, P37) can have several causes. First, in this particular patient, the lesion was very close to and partly even invading into the skin layers as seen both in Figures 6.6a and 6.6b, and in the H&E slide (Figure 6.6e) where part of the skin in relation to the malignancy is visualized. We cannot exclude that in some regions the PA signals from skin and malignancy were merged, resulting in partial removal of the malignancy signal in the pre-reconstruction processing described in Section 6.2.2. This can artificially create a non-existing ring appearance for the lesion, but would not explain the correspondence between the PA appearance and the histopathological appearance as seen in the H&E stained tumor slides. Second, high CD31 enhancement is found in a band just inside the cell-rich area. It is possible that we are in fact visualizing this inner ring of high vascularity, rather than the outer cell-rich, but poorly vascularized ring. This would also explain the atypical ring appearance, with the high intensity areas localized in scattered foci. However, if this was the case, this would lead to size underestimation in case of PAM, while in fact the PAM estimated size was slightly bigger than the histopathologically measured sized. A third explanation concerns the organization and function of the vessels. Figure 6.6f-g shows a number of CD31 stained structures deep in the fibrous core, which do not seem to be organized into functional vessels. It is plausible that these new vessels are dysfunctional, and that the higher amount of CD31 staining in the fibrous core is not necessarily related to higher hemoglobin concentrations.

In addition to these possible explanations that still take the tumor vascularity as primary contrast source, we cannot exclude the important contribution of chromophores other than hemoglobin to the PA contrast (see further Section 6.4.5).

No quantitative information was derived from the limited number of vascularity stained tumor slices investigated in this study. Selected, μm thick, slices from the tumor were investigated histopathologically, which could not exactly be related to specific, 0.23 mm thick, slides from the PA imaging volume. This possibly contributes to the observed differences between histopathology and PA images. The CD31 staining was chosen based on the pronounced staining compared to CD34 and Factor VIII/von Willebrandt factor in the first patient investigated in this study. Although the CD31 staining is known to be correlated to the MVD and microvessel distribution [15], it is not always recognized as the most reliable vascular staining [51]. Therefore, for future research in a larger patient population, a more thorough search for the most reliable vascular staining should be made.

Furthermore, not all lesions could be fully processed according to protocol, which made that some malignancies were sliced in sagittal rather than transversal direction.

6.4.5 *Contribution of different chromophores and suggestions for future research*

In most patients investigated histopathologically during this study, the vascularity was most confined to the cell-rich area of the malignancy. Therefore one can argue that the PA appearance cannot solely be linked to the tumor vascularity but that there might also be a relation with the cell density or the local presence of other 1064 nm absorbing chromophores. From optical spectroscopy and Magnetic Resonance Spectroscopy (MRS) studies it is known that the water content is significantly higher in malignant tissue compared to the surrounding healthy glandular or fatty tissue [52-55]. Moreover, with the loss of differentiation, the tissue diversity and structure change, which results in the wide range of (free) water content across patients [53]. It is especially this free (unbound) water concentration which is thought to be different across the different (necrotic, fibrotic, cell-rich) areas in the malignancy, because of changes in the extracellular matrix composition [53]. In addition to the water content, the lipid content is also known to be altered in malignant tissue compared to normal tissue [52, 54]: where the lipids are in general thought to be decreased in tumor tissue compared to the surrounding [52, 54]; necrotic cores can be very lipid rich instead [55]. The last important NIR chromophore that can vary in concentration within a tumor is collagen, as we also have seen in several cases presented here: the fibrous stroma is expected to contain much more collagen than the cell-rich tumor nests.

Because of the high inter- and inpatient variation in breast cancer chromophore composition [56, 57], the contribution of each of these chromophores to the PA lesion appearance cannot be predicted and spectroscopic PA measurements in combination with histopathological assessment are crucial for the complete understanding of the PA lesion appearance. However, such spectroscopic measurements will not fully answer all questions, since in addition to the absorption differences, scattering properties are also known to be related to the cell density and the collagen/lipid ratio [56, 58] and it can be expected that there are also variations in the Grüneisen coefficient, which relates the light absorption to the initially generated pressure. All these aspects together can cause local changes to the absorbed energy and generated pressure wave that can contribute to the PA lesion appearance. In addition to spectroscopic measurements, simulations on light behavior and acoustic wave propagation, e.g. using Monte Carlo and k-Wave [59], should therefore be applied to shed more light on the PA lesion appearance.

The strong correspondence between PA and MR images, both in general and in individual cases, and the correspondence between PA images and CD31 stained tumor slides in 5 out of 6 patients, indicates the sensitivity of PAM at 1064 nm for visualizing tumor vascularity. In combination with the fact that PAM can visualize almost all malignancies with high contrast, PA breast imaging is believed to be a highly sensitive method for detection of breast cancer. However, the specificity of the current system using

a single wavelength is expected to be limited because of the aforementioned difficulty in discriminating the contribution of hemoglobin from that of heterogeneously present water, fat and collagen to the PA appearance. The suggested spectroscopic measurements and simulations can help in the optimal choice of wavelengths for future more specific PA imaging systems. Furthermore, if the light is more homogeneously distributed throughout the medium and fluence corrections are applied, more spatial information about the malignancy can be obtained. The more complete information will not only increase the diagnostic power of the technique but will also allow for better comparison between MRI and vascularity-stained tumor slides in a research setting.

6.5 Conclusions

Photoacoustic imaging at 1064 nm can visualize breast malignancies with high imaging contrast. The strong relation with Magnetic Resonance Imaging and histopathology, indicates the high potential for visualizing malignancies in the breast. The PA contrast at 1064 nm seems to be largely related to the tumor vasculature, because of the strong correlation between PA and MR lesion localization and appearance and because of the relation between PA appearance and vascular distribution in 5 out of 6 patients. However, the results also indicate that a contribution of other chromophores to the PA lesion appearance cannot be excluded. Assessing the contribution of each of these chromophores is crucial for the future development of a PA imaging system that is both sensitive as well as specific for the visualization of malignancies.

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7

The state of the art in photoacoustic breast imaging: results from 31 measurements on malignancies and validation to conventional imaging, patient demographics and lesion characteristics⁶

Photoacoustic mammography is potentially an ideal technique for imaging the breast. Although prototype imaging systems have been applied in clinical feasibility studies, the amount of patient data remains limited. In this chapter, an overview of the latest clinical measurements using the Twente Photoacoustic Mammoscope (PAM) is given. Photoacoustic lesion visibility and appearance are compared to conventional imaging findings, lesion characteristics and patient demographics. In addition to furthering our knowledge of the performance of the method, we expect this work to guide the research and development of photoacoustic breast imaging. Thirty-one patients with one or more breast malignancies participated in the breast imaging study using PAM, which operates in forward mode using 1064 nm light in combination with a 590 element, planar, 1 MHz ultrasound detector array. For all patients, data from conventional imaging (x-ray mammography and ultrasonography) and histopathology was available. In total, 32 of 33 malignancies could be visualized with high imaging contrast and good co-localization with conventional imaging. The contrast of the detected malignancies was independent of the mammographically estimated breast density and the size estimation was reasonably good with an average 28% deviation from the gold standard (histology). All these aspects indicate the high potential of photoacoustic mammography in future breast care. However, the presence of high contrast areas outside the malignant region is suggestive for low specificity of the current imaging system. Statistical analyses did not reveal any further relation between PAM results and patient demographics and lesion characteristics. However, the results do indicate that it is useful to investigate the relation between lesion contrast and tumor grade, and the appearance of ILC with respect to IDC in future studies.

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7.1 Introduction

With breast cancer the most common cause of cancer and one of the major causes of cancer death among women [1, 2], there is a continuous search for improved methods for non-invasively imaging the breast and its abnormalities. Such methods either focus on developments in existing techniques [3-5] or investigate new approaches [6-8], not only for improvements in performance, such as sensitivity and/or specificity, but also for improvements in wide-spread applicability, patient comfort, personalized care and safety [9].

One of the techniques currently under investigation for its potential is photoacoustic (PA) imaging [10, 11]. PA mammography combines high intrinsic optical contrast of tumors with ultrasound resolution in detection, does not use ionizing radiation or contrast agents and can be used without breast compression, making it potentially an excellent technique for visualizing breast abnormalities [12-14]. In PA mammography, the breast is illuminated with short pulses of near infrared (NIR) laser light. This light is absorbed by endogenous chromophores such as hemoglobin in the tissue. The absorbed energy leads, via the process of thermoelastic expansion, to a transient pressure wave that propagates through the tissue. The pressure wave can be detected with ultrasound detectors with the center frequency and bandwidth appropriate for the specific application [10, 11]. Since ultrasound undergoes low scattering and attenuation, the resolution problems of pure optical imaging techniques due to high light scattering in tissue, can largely be avoided [11, 12, 15]. The most prominent NIR absorber at malignant sites is hemoglobin, whose concentration can be twice as high at malignant sites compared to healthy tissue [16, 17]. Moreover, it is known from optical spectroscopy and magnetic resonance spectroscopy that also the oxygen saturation and the concentrations of fat, water and collagen, all to some extent absorbing in the NIR regime, are altered at the location of malignancies [18-20]. This gives NIR PA imaging potentially the opportunity to be sensitive for visualizing malignancies, and to be specific by the use of spectroscopy methods [21].

Over the years, prototype PA mammography systems have been investigated in early clinical feasibility studies. However, the amount of clinical data available remains limited [13, 14, 22-24]. We developed the Twente Photoacoustic Mammoscope or PAM [25, 26], which can image a region of interest (ROI) in the breast in forward mode using 1064 nm light excitation in combination with a 2D detector array comprising 590 elements. We showed that PAM can visualize breast cancer with high imaging contrast [23, 24] and that this contrast appears to be independent of the mammographically estimated breast density. However, these first studies were performed in small patient populations, and due to technical constraints, the measurements were performed in a small ROI (40x40 mm² with measuring time of 25 min). The small ROI led to positioning difficulties, which constrained us to measure on a selection of palpable masses measuring between 1 and 3 cm in size [23]. Although these first measurements showed the potential of photoacoustic mammography for the visualization of malignancies, the small study population, poor variability in

included lesions and small ROI measurements, made it impossible to relate the photoacoustic lesion visibility and appearance to patient demographics and lesion characteristics. This information is required for further improvements to the performance of the technique.

In this work, we use an upgraded clinical system, which enables us to measure a larger field of view ($90 \times 80 \text{ mm}^2$ in scan duration of 10 minutes). This provides the opportunity to investigate a wider variety of tumors and to obtain better resolution than earlier. The standardized large FOV measurements allow for better comparisons with x-ray mammography and MRI. We present and critically discuss all clinical data from malignancies obtained with the improved version of PAM. In addition to assessing the visibility of the lesion, the PA lesion features are compared to lesion and patient characteristics in order to further our knowledge of the performance and potential of the method, but above all to guide the research and developments towards future optimal breast imaging systems.

7.2 Materials and methods

7.2.1 Clinical setting

Clinical measurements were performed at the center for breast care of Medisch Spectrum Twente (MST) in Oldenzaal, the Netherlands. The study protocol and informed consent procedure were approved by the hospital's review board. The study has been registered in the Netherlands Trial Register (NTR) as TC 2945.

From April 2012 to May 2013, adult female patients that came to the center for breast care for conventional imaging of their breasts were asked for participation in the study. The focus was mainly on patients with suspect lesions following clinical investigation and conventional imaging and included:

1. Patients with 'self-discovered' symptoms who were referred by the general practitioner;
2. Patients referred from the national screening program following suspect screening x-ray mammograms;
3. Patients with suspicious findings during regular surveillance following a history of breast cancer;
4. A limited number of patients with benign breast lesions.

All patients followed the normal diagnostic pathway: after clinical investigation and anamnesis, conventional imaging was performed. For x-ray imaging, cranio-caudal (CC) and medio-lateral-oblique (MLO) images of both breasts were made using the Lorad Selenia Full Field Digital Mammography System (Hologic, Bedford, USA). Next, both breasts and axillae were investigated using the Philips iU22 ultrasound machine operating at 15 MHz (Philips Medical Systems, Best, The Netherlands).

The suspect breast of a consenting patient was imaged using PAM (see *Section 7.2.2*) before the core needle biopsy procedure. In cases when conventional imaging was not

conclusive, a dynamic contrast enhanced MRI (DCE-MRI) was performed a few days to weeks post-biopsy. High-resolution anatomic T2 weighted images and dynamic T1 weighted images before and after gadolinium injection were acquired using a 1.5 or 3 Tesla MR system in combination with a 7 channel Sense Breast Coil (Philips Medical Systems, Best, The Netherlands).

The x-ray, US and MR images were interpreted by a breast imaging radiologist. For all modalities, the radiologist graded the suspiciousness of the lesion according to the BI-RADS classification scale (Breast Imaging Reporting And Data System [27]), described the lesion appearance and location in the breast and estimated the lesion size. Moreover, two radiologists independently graded the mammographic density of the breast using the BI-RADS density scale in the CC x-ray mammograms [27, 28]. This four grade scale reflects the percentage of glandular tissue present in the breast. In case of markedly different opinions, a third radiologist could be solicited for an unbiased grading.

In the CC x-ray mammogram, a random area inside the malignancy and a random area outside, but adjacent to, the malignancy were chosen. The average intensity in both areas ($\langle I_m \rangle$ and $\langle I_b \rangle$ respectively) was used to calculate the contrast C as $\langle I_m \rangle / \langle I_b \rangle$. To reduce subjectivity in the choice of the specific areas, this procedure was repeated ten times and the average outcome was used to obtain C_{xray} for the specific lesion. In case the lesion was occult on x-ray mammography, the contrast was defined as being 1.

$$C_{xray} = \frac{1}{10} \sum_i \left(\frac{\langle I_{m,i} \rangle}{\langle I_{b,i} \rangle} \right) \quad (7.1)$$

7.2.2 The Twente Photoacoustic Mammoscope

Earlier published details of the instrument [25] as well as the study procedure [23] will be concisely repeated here. During the PAM investigation, the patient lies in prone position on the bed (Figure 7.1) with her breast pendant through an aperture. The breast is immobilized by slight compression between a glass plate for laser illumination from the cranial side and the ultrasound detector array from the caudal side. After positioning, a photograph is made of the breast against the detector and the CC thickness of the breast under compression is measured using a caliper. By using the photograph of the breast in combination with the detector position, the CC x-ray images and PAM images can be merged retrospectively to provide an approximation of the co-localization of the identified lesions (see Figure 7.2).

The breast is illuminated from the cranial side with pulsed (10 ns, 10 Hz) laser light at 1064 nm from a Q-switched Nd:YAG laser (Continuum Surelite, California, USA). The size of the beam is approximately 35 cm² and the beam is kept at a fixed position throughout the measurement. At the breast surface, the energy per pulse does not exceed 350 mJ, giving rise to radiant exposure of 10 mJ/cm², which is well below the Maximum Permissible Exposure (MPE) on the skin (100 mJ/cm²) for the specifications of the laser

light applied [29]. In case the breast does not cover the complete detector, the remainder of the detector is protected from light falling directly onto it by a protective sheet. PA signals in the breast are detected at the caudal side of the breast by a 1 MHz (130% bandwidth) 2D ultrasound detector array (Lunar Corporation, General Electric, Wisconsin, USA) consisting of 588 elements in an almost circular configuration. Details of the detector are given elsewhere [25, 26]. Sequences of ten elements, spread over the entire detector area, are activated at a time. For all 588 elements, the average of 100 signals are read into the PC using two 8 channel digitizers (National Instruments, NI PXI 5105, 60 MS/s, 12-bit) controlled by a Labview program (National Instruments, Texas, USA). This leads to total measurement duration of 10 minutes for the complete detector covering an area on the breast of approximately $9 \times 8 \text{ cm}^2$.

Figure 7.1 *The Twente Photoacoustic Mammoscope. a) Aperture to insert breast. b) Ultrasound detector matrix. c) Glass window. d) Scanning system compartment. e) Q-switched Nd-YAG laser operated at 1064 nm with 10 ns pulses. f) Laser safety curtain which is drawn around the instrument during the measurements. g) Interface electronics between detector and computer. h) Linear stage carrying detector matrix driven by hand wheel to apply mild compression to the breast. i) Laser remote control unit. j) Laser power supply. Image adapted from reference [24] with permission.*

7.2.3 Image reconstruction and analyses

Reconstruction and analyses were performed using Matlab (R2011b, the Mathworks Company). Prior to reconstruction, the large signal from the breast surface was removed to have a more homogenous dynamic range in the PA volume. Signals were filtered with a band-pass Butterworth filter (cut-off frequencies 0.2 MHz and 1.7 MHz) to remove the low frequency trend and high frequency noise. The PA volume was reconstructed from these processed signals using an acoustic backprojection algorithm, assuming a homogenous speed of sound of 1540 m/s in the medium. The inaccuracies that arise because of the assumption of this homogenous speed of sound are estimated to be minor with respect to the system's resolution (approximately 3 mm in both axial and lateral direction [25, 26]). After reconstruction, the separate profiles through the volume were demodulated and all voxel intensity values were scaled to the maximum intensity value within the total volume, giving a range between 0 and 255.

The PA volume was investigated for the presence of high-intensity areas. When high intensity PA pixels were organized in a way reminiscent of lesion appearances from experiences in conventional imaging modalities, these structures were referred to as PA lesions. A subvolume was defined such as to include the depth (cranio-caudal) extension of the lesion. The maximum intensity projection (MIP) of the subvolume was overlaid on the x-ray mammogram by using the position information obtained from the digital photograph (Figure 7.2).

Figure 7.2 **a)** A digital photograph of the breast in the Photoacoustic Mammoscope was obtained after positioning of the lesion in front of the detector. **b)** By overlapping the contours of the breasts on the digital photograph and the CC x-ray mammogram, **c)** the PA and x-ray images could be merged retrospectively. This merging can give an approximation of the position of the lesion, however, since the compression is different between the different imaging modalities and different tissue types will not deform equally, a real one-to-one comparison is not possible. Some of the overlaid PA images have an aspect ratio different from 1, because they are deformed to fit the x-ray image.

In this way, lesion positions in the CC PA images could be compared to lesion positions in the CC x-ray images. In case of good co-localization, the lesion on PA images was assumed to represent the suspicious abnormality. The lesions were classified according to their appearance on the MIP of the subvolume as either showing ‘mass (confined) appearance’, ‘non-mass (scattered) appearance’ or ‘ring appearance’ (for further details, see Section 7.3.1).

The depth of the lesion was calculated from the z (depth) position of the lesions’ maximum intensity inside the PA volume and the measured breast thickness. A contour was manually drawn around the lesion on the MIP of the subvolume, using the information available from conventional imaging. This contour was used to measure the maximum dimension of the lesion in the CC projection and to calculate the average intensity inside the lesion $\langle I_l \rangle$ in this same MIP. Ten random areas outside but close to the lesion were chosen. For each area, the average intensity was calculated to obtain $\langle I_{b,i} \rangle$ and to calculate the contrast C_i of the lesion as $C_i = \langle I_l \rangle / \langle I_{b,i} \rangle$. To reduce subjectivity in the choice of the specific background area, the average of the ten calculated contrasts was used to obtain C_{pam} .

$$C_{pam} = \frac{1}{10} \sum_i \left(\langle I_l \rangle / \langle I_{b,i} \rangle \right) \quad (7.2)$$

The estimated size of the lesion in PA images was compared to the true lesion size defined histopathologically. For each modality (PAM, Xray, US, MRI) The percentage (absolute) size deviation from the gold standard (histopathology) was calculated as:

$$\left(|Size_{Histo} - Size_{Mod}| / Size_{Histo} \right) \times 100\% (7.3)$$

in which $Size_{Mod}$ is the measured maximum diameter of the lesion on images from the specific modality and $Size_{Histo}$ is the maximum diameter of the lesion measured by the pathologist in the post-surgical specimen. If lesions were not visible, but correctly positioned in the measurement area, the size was defined to be 0 mm for that specific modality. Further, for all imaging modalities (x-ray, US, MRI and PAM) the correlation coefficient between the measured lesion sizes and the true lesion sizes was calculated.

Per BI-RADS breast density group, $\langle C_{pam} \rangle$ and $\langle C_{xray} \rangle$ were calculated and compared to have an indication of the influence of breast density on the relative performances of PAM and x-ray mammography. Both for PA and x-ray lesions, the density groups were tested for significant differences in their mean contrast by using a (one-sided) two-sample Student's t-test with a significance level of 5%.

7.2.4 Further used clinical information

Following core needle biopsy, all tissue specimens were investigated for the presence and type of malignancy at the Laboratory for Pathology East Netherlands (LabPON). Following surgery (mastectomy or breast conserving therapy), pathology was performed to determine the status of the resection margins, the type (according to WHO criteria) and size of the lesion, the grade of the lesion on the Bloom-Richardson scale [30], the presence of *in situ* components outside the lesion and the hormonal status of the tumor. All this information, except the status of the resection margins, was compared to the PA lesion visibility and appearance.

At the end of the PA measurement, patients were asked to fill a short questionnaire regarding their physical condition as well as their experience during the measurement. The PA lesion contrast can thus be compared to age, Body Mass Index (BMI) and menopausal status of the patient. Pre-menopausal patients were asked to fill the week of the cycle. From MRI it is known that results can be influenced by the hormonal status of the patient [31] and since MRI and photoacoustic mammography are believed to largely rely on the same contrast mechanism [32, 33], namely vascularization, this factor should be investigated here as well. Further information from the questionnaire concerned the preference of the patient for either x-ray mammography or photoacoustic mammography.

7.3 Results

From April 2012 to May 2013, 43 patients volunteered in the PAM study. From these, 2 patients were not imaged because of incapability to climb on/lay on the bed for 10 minutes.

For 2 other patients it appeared retrospectively (with information from the digital images, x-ray mammograms, MR and US images) that the lesion was not favorably positioned in front of the detector. Of the remaining 39 technically acceptable patient measurements, 5 were on cysts; 2 were on fibroadenoma; 1 was on a chronic active inflammation; and 31 were on malignancies. Here, the results for the measurements on malignancies will be presented. The number of fibroadenoma measured was too low and the two lesions were too much different in appearance and size (~10 mm versus >60 mm) on conventional imaging to analyze and compare their PA appearances. The results from cyst measurements will be described elsewhere [34].

7.3.1 General PA results

In 30 patients, the malignancies could be visualized with high certainty. This was confirmed by a high degree of co-localization between the lesions in x-ray and PA images. In the few cases where there was no good co-localization, the shift of the lesion in PA images compared to the expected location from the CC x-ray image, could be related to an intended or unintended tilting of the breast during the PA investigation (see further Figure s7.2e-f in the Supplementary Information). In one patient (P60), the lesion could not be visualized at the expected position in the PA images. Based on data from x-ray mammography and MRI, mispositioning could neither be excluded nor fully be ascertained.

7.3.1.1 PA lesion characteristics

Table 7.1 summarizes the results from PA imaging in comparison to the lesion type and size. The types of the malignancies (25 infiltrating ductal carcinoma (IDC); 4 infiltrating lobular carcinoma (ILC) and/or lobular carcinoma in situ (LCIS); and 2 other/non-defined) measured in this study represent those of the normal breast cancer population [35-37]. Further patient characteristics are specified in Table 7.3 in Section 7.3.1.3.

Table 7.1 Results from photoacoustic imaging

P ¹	Fig. Nr. ²	Lesion type	Lesion size ³ (mm)	PA Lesion appearance	PA depth (mm)	PA Contrast	PA size (mm)	Co-localization x-ray / MRI
32		IDC	12	Mass	20-25	2.6	16	Good
35	7.5a-c	IDC	26	Mass	5-10	2.7	18	Good
36		IDC (3x)	6, 6, 20	Mass (3x)	<5, 5-10, <5	4.2, 3.1, 3.3	8, 8, 11	Good
37	7.s2d	IDC	28	Ring	5-10	2.2	31	Good
38	7.2a-c	IDC	19	Mass	<5	5.1	19	Good
39		IDC	63	Non-mass	<5	3.6	41	Reasonable
40		ILC	un	Non-mass	5-10	3.7	45	Poor ⁴
42		IDC	un	Mass	5-10	3.9	28	Good
43		IDC	15	Mass	20-25	2.9	16	Good
44	7.s1b	IDC	25	Mass	<5	3.7	38	Not defined
45	7.s2b	Adenotype	un	Non-mass	5-10	3.2	61	Reasonable ⁴
47	7.5d-f	IDC	24	Ring	<5	5.5	24	Good

Table 7.1 Results from photoacoustic imaging – continuation

p ¹	Fig. Nr. ²	Lesion type	Lesion size ³ (mm)	PA Lesion appearance	PA depth (mm)	PA Contrast	PA size (mm)	Co-localization x-ray / MRI
49 ⁵	7.s1a	LCIS	40	Non-mass	5-10	3.9	54	Good
52		IDC	un	Non-mass	<5	2.7	53	Good
53	7.s1d	IDC	un	Ring	<5	3.3	46	Good
54		IDC	22	Mass	20-25	6.4	18	Reasonable ⁶
55	7.s1c	IDC	34	Mass	<5	2.8	29	Good
56	7.s2e-f	IDC	22	Mass	15-20	3.4	11	Reasonable-Poor ⁶
58	7.5j-l	ILC	22	Mass	20-25	2.3	19	Good
59		IDC	28	Non-mass ⁷	20-25	3.5	29	Reasonable
60		ILC	22	Not visible	n.a.	1.0 ⁸	0	n.a.
61	7.s2c	IDC	un	Mass, atypical	5-10	4.3	21	Good
62		IDC	18	Mass	<5	2.6	15	Good
63		IDC	un	Mass	<5	7.0	13	Poor ⁶
65		IDC	18	Mass	25-30	2.6	13	Good
66	7.5g-i	MC	15	Mass	<5	4.0	15	Good
67		IDC	13	Mass	5-10	5.3	16	Good
68	7.s2a	IDC	14	Mass	15-20	2.9	10	Good
70	7.6a-m	IDC	19	Mass	<5	3.0	23	Good
72		IDC	un	Non-mass ⁷	5-10	2.8	44	Good
73		IDC	25	Multifocal mass	<5	2.9	42	Reasonable ⁶

P=patient number; IDC=infiltrating ductal carcinoma; ILC=infiltrating lobular carcinoma; LCIS=lobular carcinoma in situ; MC=mucinous carcinoma; n.a. =not applicable, un=unknown. ¹ Internal numbering system; ² Figures 7.s1 and 7.s2 can be found in the supplementary information; ³ The size of the lesion is estimated from histopathology post-surgery. If patients did not undergo surgery in MST, the size is indicated as 'unknown' (un); ⁴ The size of the lesion and/or the high breast density made a comparison between PA and x-ray lesion localization difficult; ⁵ An adenotype metastasis was found in one of the lymph nodes, however, the post-mastectomy specimen only revealed LCIS in the breast. The metastasis proves that there should have been an invasive component; ⁶ For these patients, the non-perfect co-localization could be attributed to the significant breast tilting during the PA measurement; ⁷ In these patients, the mass was localized in the areas around/outside the lesion as observed by comparison with conventional imaging; ⁸ The lesions with a contrast of '1' for PAM were not visible in the PA imaging volume.

The 32 detected lesions were classified according to their appearance as:

- 'Mass or confined appearance', defined as a confined region of high intensity, which can be either regular or irregular in shape and homogenous or heterogeneous in intensity.
- 'Non-mass or scattered appearance', defined as an area which is completely different from the surrounding healthy tissue by the presence of multiple moderate to high intensity foci.

- ‘Ring appearance’, defined as one or multiple regions of high intensity enclosing an area with low intensity.

Most lesions were of the mass-type, as in the first phase of the clinical study [23]. However, the lesions are much less smooth and show more heterogeneity in the current study.

Although the only lesion which was not visible in the PA images was of the less common ILC type, the variation in PA appearance cannot directly be related to the tumor type: there is large variation within the group of lesions of the same type (see for example Figure 7.5c and 7.5f), while lesions of completely different type can have quite similar appearance (Figure 7.5c and 7.5l). Moreover, the number of non-IDC lesions (5) is too low with respect to the IDC group to make a reliable comparison between the different lesion types.

Representative PA images can be found in Figures 7.5 and 7.6 in Section 7.3.2 and in the supplementary information as indicated in the second column of Table 7.1.

7.3.1.2 PA lesion characteristics in relation to conventional imaging findings

In Table 7.2 the PA lesion characteristics are compared to the lesion characteristics in x-ray mammography, US and MR images. Three lesions (P36_3, P38, P44) were occult in x-ray mammography images, of which one was also not observed in US images (P36_3). All these lesions could be identified with high imaging contrast in the PA images (for an example see Figure 7.s1b in the supplementary information). The position of the lesion was verified by the position information from palpation and US imaging. For patient P36, at the time of the PAM investigation only the existence of two lesions was known, however, three lesions were observed in the PA images. The post-surgical histopathology report showed the existence of a third IDC at a location which co-localized with the third lesion in the PA images.

Table 7.2 Results from conventional imaging

P ¹	BI-RADS Density	x-ray contrast	PA contrast	Size X-ray (mm) (% deviation) ²	Size US (mm) (% deviation) ²	Size MRI (mm) (% deviation) ²	Size PA (mm) (% deviation)
32	1	2.4	2.6	12 (0%)	11 (-8%)		16 (+33%)
35	1	2.3	2.7	24 (-8%)	25 (-4%)		18 (-31%)
36	2	3.1, 2.0, 1.0 ³	4.2, 3.1, 3.3	6, 10, 0 (0, +66, -100%)	6, 8, 0 (0, +33, -100%)		8, 8, 11 (+33, +33, -45%)
37	3	2.2	2.2	35 (+25%)	36 (+29%)		31 (+11%)
38	3	1.0	5.1	0 (-100%)	21 (+11%)	22 (+16%)	19 (0%)
39	2	5.6	3.6	50 (-21%)	50 (-21%)		41 (-35%)
40 ⁴	4	np ⁵	3.7	np	np	np	45
42 ⁴	2	1.5	3.9	30	np		28
43	1	2.8	2.9	15 (0%)	15 (0%)		16 (+7%)
44	2	1.0	3.7	0 (-100%)	30 (+20%)		38 (+52%)
45 ⁴	3	np ⁵	3.2	np	np		61

Table 7.2 Results from conventional imaging – continuation

p ¹	BI- RADS Density	x-ray contrast	PA contrast	Size X-ray (mm) (% deviation) ²	Size US (mm) (% deviation) ²	Size MRI (mm) (% deviation) ²	Size PA (mm) (% deviation)
47	2	6.6	5.5	25 (+4%)	29 (+21%)		24 (0%)
49 ⁶	2	np ⁵	3.9	15	0	20 -50	54
52 ⁴	3	1.3	2.7	60	42	70	53
53 ⁴	2	6.6	3.3	65	50		46
54	1	2.0	6.4	18 (-18%)	13 (-41%)		18 (-18%)
55	1	6.8	2.8	20 (-41%)	23 (-32%)	50 (+47%)	29 (-15%)
56	3	1.3	3.4	np ³	34 (+54%)	32 (+45%)	11(-50%)
58	1	1.8	2.3	20 (-9%)	25 (+14%)	55 (+150%)	19 (-14%)
59	2	2.3	3.5	35 (+25%)	30 (+7%)		29(+4%)
60	1	3.5	1.0	np ⁵	25 (+14%)	67 (+204%)	0 (-100%)
61 ⁴	1	6.4	4.3	16	10	20	21
62	3	1.4	2.6	20 (+11%)	20 (+11%)	19 (+6%)	15(-17%)
63 ⁴	1	2.7	7.0	22	21		13
65	2	1.0 ⁷	2.6	14 (-22%)	18 (0%)		13 (-28%)
66	2	3.0	4.0	20 (+33%)	16 (+7%)		15 (0%)
67	1	3.2	5.3	18 (+38%)	13 (0%)	15 (+15%)	16 (+23%)
68	3	2.3	2.9	15 (+7%)	15 (+7%)		10 (-29%)
70	2	2.0	3.0	25 (+32%)	20 (+5%)	30 (+58%)	23 (+21%)
72 ⁴	2	4.3	2.8	35	36	50	44
73	2	3.4	2.9	49 (+96%)	26 (+4%)	35 (+40%)	42 (+68%)

P= patient number; un=unknown; np=not possible, n.a.=not applicable

¹ Internal numbering system; ² The percentage size deviation is calculated according to Formula 3. For lesions which were correctly positioned, but invisible, the lesion size was defined as 0 mm (100% size deviation); ³

Lesions with a contrast of '1' for either x-ray mammography or PA imaging, were occult on the specific images;

⁴These patients did not go for surgery at MST, mostly because of the treatment by neoadjuvant chemotherapy.

Therefore post-surgical histopathological sizes could not be obtained; ⁵In these patients, the contrast and/or size of the lesion could not fully be estimated on conventional imaging, because of the appearance of architectural distortion rather than mass; ⁶In this patient, an adenotype metastasis was found in one of the lymph nodes,

however, the post-mastectomy specimen only revealed LCIS in this breast. The metastasis proves that there should have been an invasive component. The size given here is the one of the in situ component. These are not taken into account in the estimation of the average size over- or underestimation for the different imaging modalities. ⁷The lesion was visible in x-ray mammography as a radio-opaque abnormality of approximately 14 mm, associated with microcalcifications, hence the contrast was approaching '1'.

The lesion of P49 was associated with calcifications and a mass component on x-ray mammography, but could not be located using US imaging. In PA images, a suspect non-mass appearance was seen in the expected breast region (Figure 7.s1a in the supplementary information), for which the location and appearance matched with the location and appearance of the suspect lesion in the MR images. The core needle biopsy of this lesion

revealed LCIS without clear invasive components. Lymph node biopsy showed metastasis of invasive (lobular or ductal) carcinoma, proving the existence of an invasive component. Post-surgery, this invasive component could not be located in the mastectomy specimen. Since this was the only patient with LCIS measured in this study, it is difficult to relate the PA appearance to either invasive or *in situ* components.

Figure 7.3 shows the breast density versus contrast for both x-ray mammography and PA imaging. The final result of the density assessment (Table 7.2, column 2) by the three radiologists is used to define the density groups for this analysis (this will be further referred to as ‘complete assessment’). Here, a two-scale classification is given (low: BI-RADS density 1, 2 and high: BI-RADS density 3, 4), because this is proven to be more reliable and reproducible than the four scale classification [28]. Both for the individual assessment of the two radiologists as well as for the complete assessment, there is no significant difference in average PA contrast between the high and low density groups, while the contrast of x-ray mammography is significantly lower for the high density group as compared to the low density group. Examples of lesions that are visible with high imaging contrast in highly dense breasts can be found in Figure 7.2 (P38, see also reference [33]) and in the supplementary information.

Figure 7.3 PA contrast versus breast density. There is no significant difference in PA contrast (grey, cross) between the low and high density breasts. There is a significant drop (*) in contrast for x-ray mammography (white, sphere) comparing the high density to the low density groups.

In this study, the average size deviations for x-ray mammography, ultrasonography and MRI were: 34%, 18% and 65% respectively. The average size deviation for PAM was 28%. For the PA detected lesions, there was a positive correlation coefficient of 0.74 ($p < 0.0001$) between the PA size and the histopathologically defined size. This value was slightly lower than the correlation coefficients between the ultrasound and histopathology sizes and the x-ray and histopathology sizes (0.88 $p < 0.0001$ and 0.79, $p < 0.0001$ respectively) for the

visible lesions. There was no significant correlation between the MRI and histopathology measured sizes (0.59, $P>0.05$).

7.3.1.3 PA appearance versus patient and lesion characteristics

Table 7.3 consolidates the patient and lesion characteristics for the 31 measured patients. No significant differences can be observed in PA contrast for the different hormonal receptor status groups. Figure 7.4 shows the average PA and X-ray contrast for the three different tumor grades. There is no significant difference in PA contrast between any of the different groups.

The size deviation from the gold standard could not be related to the depth of the lesion, the hormonal receptor status, or the presence of DCIS components outside the invasive malignancy.

Table 7.3 Patient and lesion characteristics.

p ¹	Age (y)	BMI	Hormonal status (week)	Detection	Type and grade of malignancy	DCIS (yes/no)	Receptor status (Er, Pr, Her2Neu)	Size of lesion ² (mm)
32	54	28.7	Post	Screening	IDC, grade 1	No	++-	12
35	79	35.6	Post	Self-detected	IDC, grade 1	No	++-	26
36 (3x)	56	27.5	Post	Screening(2x), post-surgery (1x)	IDC, grade 1 (3x)	Yes	++- (3x)	6, 6, 20
37	61	25.9	Post	Self-detected	IDC, grade 2	No	+++	28
38	44	26.0	Pre (3)	Annual surveillance	IDC, grade 3	Yes	---	19
39	67	33.2	Post	Screening	IDC, grade 3	No	++-	63
40	47	26.2	Pre (2)	Self-detected	ILC	n.a.	+++	un ^{3,4}
42	91	20.0	Post	Self-detected	IDC	un	++-	~30 ²
43	83	25.0	Post	Self-detected	IDC, grade 2	Yes	++-	15
44	55	24.5	Post	Annual surveillance	IDC, grade 3	Yes	--+	25
45 ⁵	51	un	Post	Screening	Adenotype	un	un	un ^{3,4}
47	69	22.5	Post	Self-detected	IDC, grade 2	No	++-	24
49	64	29.0	Post	Screening	LCIS	n.a.	+--	> 40
52	44	un	un	Self-detected	IDC	un	++un	> 60 ³
53	72	21.3	Post	Self-detected	IDC	un	+--	> 60 ³
54	70	30.1	Post	Screening	IDC, grade 1	No	++-	22
55	63	24.8	Post	Self-detected	IDC, grade 2	No	+--	34
56	62	26.9	Post;	Self-detected	IDC, grade 3	No	+++	22
58	65	27.7	Post	Screening	ILC, grade 2	n.a.	++-	22
59	79	27.1	Post	Self-detected	IDC, grade 3	No	++-	28
60	66	23.1	Post	Self-detected (pain)	ILC, grade 1	n.a.	++-	22
61	74	41.4	Post	Screening (lymph node)	IDC	un	++-	22 ³
62	32	18.6	Pre (1)	Self-detected	IDC, grade 2	No	++-	18

Table 7.3 Patient and lesion characteristics – continuation

p ¹	Age (y)	BMI	Hormonal status (week)	Detection	Type and grade of malignancy	DCIS (yes/no)	Receptor status (Er, Pr, Her2Neu)	Size of lesion ² (mm)
63	79	24.1	Post	Accidental finding	IDC	Un	++-	22 ³
65	78	un	Post	Self-detected (retracted nipple)	IDC, grade 1	Yes	--+	18
66	83	26.6	Post	Self-detected	MC, grade 2	Yes	++-	15
67	73	23.8	Post	Self-detected	IDC, grade 2	Yes	++-	13
68	63	25.5	Post	Screening	IDC, grade 2	Yes	++-	14
70	63	29.8	Post	Accidental finding	IDC, grade 3	No	++-	19
72	61	24.3	Post	Self-detected	IDC	un	++-	35 ³
73	50	28.2	Post	Self-detected	IDC, grade 3	No	++-	25 + 5 ⁶

P=patient number; BMI=body mass index; DCIS=ductal carcinoma in situ; IDC=infiltrating ductal carcinoma; ILC=infiltrating lobular carcinoma; LCIS=lobular carcinoma in situ; MC=mucinous carcinoma; un=unknown; n.a.=not applicable; ¹ Internal numbering system; ²The size of the lesion is estimated from histopathology post-surgery or conventional imaging; ³These patients did not undergo surgery in MST, therefore the lesion size, grade and presence of DCIS are not available. If possible the lesion size (last column) was estimated from conventional imaging; ⁴In these patients, the size of the lesion could not be estimated from conventional imaging, because of the large extension in the breast and the appearance of architectural distortion rather than mass; ⁵In this patient the biopsy of the lesion was inconclusive, but biopsies lymph node biopsies showed metastases from an adenotype carcinoma. The patient left to a different hospital for further diagnosis and treatment and was lost for follow-up; ⁶In this patient, in addition to the 25 mm abnormality, a second, 5 mm focus of the same type of malignancy was found in the histopathology specimen. There were no invasive or in situ components present in between these lesions.

Figure 7.4 Tumor grade versus contrast on PA images. There is no significant difference between the groups.

Further, the average PA contrast was compared to the patients' BMI. Neither a trend nor a significant difference can be observed for the different BMI categories. This was also the case for the PA contrast versus the patient's age. In this study, only three pre-menopausal patients were measured, which were all in a different week of their menstrual cycle. The influence of the hormonal status on the PA results could therefore not be investigated.

7.3.2 Specific representative PAM results

Figure 7.5 shows the comparison between x-ray mammography and PA imaging for 4 representative patients in various overlays and projections.

More patient results, amongst others from patients with higher density breasts or atypical lesion appearance in conventional imaging, can be found in Figures 7.s1 and 7.s2 in the supplementary information and other results will be published elsewhere [33].

Figure 7.5 Four representative photoacoustic imaging results. Left: Left CC x-ray mammogram; Middle: Left CC x-ray mammogram with MIP of the PAM identified lesion overlaid; Right: detailed 3D representation of the lesion of interest. **a-c)** This infiltrating ductal carcinoma (IDC, grade 1) of 26 mm in a 79 year old patient, presents as a high contrast mass appearance of 18 mm in the PA volume. The size underestimation is most likely the consequence of the poor positioning of the lesion at the edge of the detector. **d-f)** This infiltrating ductal carcinoma (IDC, grade 2) of 24 mm in a 69 year old patient, presents as a ring (diameter 24 mm) in the PA volume. The information from cranial to caudal is very limited as can be observed in the 3D representation in figure f. The ring appearance is hypothesized to be partly the consequence of a higher vascular density at the lesions border as compared to its center [33]. Since x-ray mammography relies on a different contrast mechanism, this ring appearance does not necessarily appear in the x-ray images. For Figures g-i, see next page.

Figure 7.5 (continuation) g-i) This mucinous carcinoma (MC, grade 2) of 15 mm detected in a 83 year old patient, presents as lobed mass of 15 mm in the PA images. In the MIP of the PA volume (Figure h,) an elongated structure seems to be connected to the lesion, which is not that prominent in the x-ray images (Figure g). A detailed look at the 3D volume reveals that the lobed and elongated structure are in fact separated and that the latter most-likely represents a blood vessel. This can, however, not be verified, because of the lack of MR images for this patient. **j-l)** This infiltrating lobular carcinoma (ILC, grade 2) of 22 mm in a 65 year old patient, presents as an irregularly shaped mass of 19 mm in PA images. The location of the lesion in the PA volume can be perfectly co-localized with the location of the lesion on the x-ray mammogram. Even at depths of more than 20 mm, the lesion can be visualized with good contrast and a rather reliable (although slightly underestimated) size.

Figure 7.6 shows a more detailed look at the PA imaging results for a 63 year old patient having an IDC (P70). This case is chosen to further explain the strengths and limitations of the current planar photoacoustic mammography set-up that operates at a single wavelength. The patient noticed changes to a known palpable mass in her right breast, which was previously diagnosed as being most likely a benign lipoma. In x-ray mammography, the lesion in her right breast was unchanged, however, a highly suspect, focalized, 25 mm abnormality was detected in the lateral upper quadrant of the left breast (Figure 7.6a), which was absent in previous mammograms. A localized US investigation (Figure 7.6b) revealed the presence of an approximately 20 mm sized hypoechoic lesion, with hyperechoic border, invading into the subcutis (<5 mm below the outer skin surface) and which was highly suspect for malignancy. After the PA measurement and the US guided

biopsy procedure, which revealed the presence of an IDC, a pre-operative MRI was being performed. A slice through the T1 weighted post-subtraction data-set at the level of the malignancy can be seen in Figure 7.6c. MRI visualizes the malignancy as an approximately 30 mm enhancing region associated with small spiculae. Histopathological investigation of the lumpectomy specimen revealed the presence of a 19 mm IDC, grade 3, which was not associated with *in situ* components.

Figure 7.6 63 year old patient (P70) with IDC. **a)** The x-ray mammogram of the left breast shows the presence of a highly suspect lesion of about 25 mm. The white dashed box indicates the area on which the PAM measurement is being performed. **b)** A localized US investigation of this palpable mass further increases the suspiciousness by the presence of a 20 mm hypoechoic mass with a hyperechoic border, which is close to the skin surface. **c)** A pre-operative MRI (T1-weighted, post-subtraction) image shows the lesion as an approximately 30 mm large enhancing structure with small spiculae. **d)** A maximum intensity projection (MIP) at the depth where the lesion was expected reveals the presence of a lobed, 23 mm mass-like structure in the PA image (red-dashed box), which most-likely represents the malignancy of interest as indicated by the good co-localization with x-ray mammography (see white dashed box in Figure 7.6a) and MRI. However, artifacts are also pronounced (black boxes) this superficial in the PA volume. **e-h)** A detailed look at the lesion (red dashed box Figure 7.6d) at various depths give more information about the shape of the lesion. For Figures **i-m**, see next page.

Figure 7.6 (continuation) i) A MIP between 22 and 27 mm depth reveals the presence of more high-contrast areas which cannot with certainty be related to known structures in the breast. According to the appearance and location, the yellow-dashed box might represent a cyst, while the orange box might show an artifact of measuring close to the curved border of the breast. j-m) Slices at different depths provide more details about these high contrast areas.

PA imaging results of the lesion are given in Figure 7.6d-h. The PA ROI is indicated in the x-ray mammogram by the white dashed area. At the depth and the location where the lesion was expected, a 23 mm lobed high intensity mass-like lesion could be observed, having a contrast of 3. Figure 7.6d, shows the maximum intensity projection of this lesion, with the lesion location indicated by the red dashed box. As observed in the ultrasound images (Figure 7.6b), the lesion was present rather close to the skin and the distance between the skin and the lesion was further decreased by the slight compression applied during the PAM investigation. Therefore, the lesion was positioned within the first few mm of the reconstructed PA volume. In this depth range, significant reconstruction artifacts can arise because of the manual removal of the skin surface. These artifacts are clearly visible in this superficial MIP and indicated by the black bold boxes. Figure 7.6e-h, shows a detailed view at different depths through the lesion. Although there is limited information in depth (from cranial to caudal), the multilobed nature of the lesion and the high imaging contrast can be clearly seen in these slices.

Figure 7.6i-m show a MIP (Figure 7.6i) and slices (Figure 7.6j-m) 20 mm deeper in the PA volume. This depth is beyond the depth where the malignancy was expected. However, two high contrast areas can be observed. The one at the far left of the image (indicated by the yellow-dashed box) might represent a cyst, according to its appearance, by two overlying high contrast areas (see also slice 7.6k and 7.6l) [34]. However a cyst was not reported at this location in the x-ray mammogram or MR images. The area (orange double, lines) at the far right of the image might be the consequence of imaging very close to the border of the breast; the curvature of the breast can cause high-skin signals even deep in the PA volume. Such high contrast areas, which were outside the region where the

malignancy was expected and which could not always be attributed to known structures in the breast, were present in approximately 1/3rd of the patients investigated in this study.

7.3.3 Patient experience

Thirty patients filled out the short questionnaire following the PAM investigation. Of those, 11 reported to prefer PAM far above x-ray mammography; 14 preferred PAM above x-ray mammography; for 3 the burden of x-ray mammography was equal to that of PAM; and 2 indicated to prefer x-ray mammography far above PAM. However, it must be noted here that patients were well aware of the fact that this system was a prototype and that it would not be applied in its current configuration in daily clinical practice outside a research setting.

7.4 Discussion

We reported here on the imaging results of clinical photoacoustic breast imaging. The results of 31 measurements on patients having one or more breast malignancies, obtained with a PA mammoscope which operates in planar configuration using 1064 nm light (PAM [25, 26]), were presented and compared with patient demographics, lesion characteristics and results from conventional imaging. The most important observations are:

1. PAM can visualize breast malignancies with high imaging contrast;
2. The contrast of the PA lesions is independent of the mammographically estimated breast density, while there is a significant drop in lesion contrast in x-ray mammography for high density breasts compared to low density breasts;
3. The PA lesion appearance and contrast could not be related to the lesion type, grade and hormonal status;
4. In a non-negligible number of patients, high contrast areas could also be observed at areas in the PA volume which could not be related to the location of the (proven) malignancy.

In the following sections, these four most important findings are discussed in more detail and possibilities for future improvements are given.

7.4.1 Visualizing breast malignancies

In agreement with results from previous clinical studies [23, 24], this study proved that PA imaging as currently applied in our prototype planar mode imaging system using 1064 nm, can visualize breast malignancies with high imaging contrast. In some cases even malignancies that were occult on one of the conventional imaging modalities could be clearly visualized with reliable shape and size. This resulted in a visualization rate of 32 (of 33) malignancies in 31 patients in this study, while only one malignancy was occult in PA images.

The missed malignancy (P60) was an infiltrating lobular carcinoma (ILC) which presented as architectural distortion in x-ray images and as a suspicious hypo-echoic area in US images. It is known that there are morphological differences between IDC (the most

common measured malignancy in this study) and ILC, making the visualization of ILC in conventional imaging sometimes troublesome [36-38] and possibly also causing difficulties in PA visualization of ILC. However, two cases of ILC (P40 and P58) were visualized using PAM. Further, ILC is often associated with increased tumor vascularization as a consequence of angiogenic processes [39, 40], which caused the lesion of P60 to be visible as enhancing mass in MR images. In fact, MRI has proven to be the most useful imaging modality in the pre-operative investigation of ILC [36, 41]. If the PA contrast is indeed mainly the consequence of the increased tumor vascularization, one would expect this lesion type to be visible in the PA image as well. A further comparison between the digital photograph of the breast during the PAM investigation and the x-ray and MR images cannot exclude the possibility of mispositioning of the lesion in front of the detector. In any case, it is recommended to perform more patient measurements on ILC.

A few aspects of the general results of the 31 visualized malignancies require further discussion. First, although previously all lesions were observed as homogenous and rather smooth masses, in the current study we see larger variations in PA lesion appearance. This higher variation can largely be explained by the higher variation in included patients and lesions: where we previously focused on small palpable masses, in this study also non-palpable lesions, lesions with appearances of architectural distortion in x-ray images and very large (>60 mm) and very small (<10 mm) lesions were measured. Moreover, the increase in illuminating beam size and FOV, leads to a more reliable reconstruction of the true lesion appearance. This might also explain the problems we still observe for the reconstruction of very large (>40 mm) lesions (for an example see P45, Figure 7.s2b in the supplementary information).

Second, in this study the PAM-estimated lesion sizes were both larger and smaller than the histopathologically defined lesion sizes, while previously the lesion size was always underestimated in the PA images [23]. The previous size underestimation was also most likely the consequence of the limited FOV with respect to the lesion size, a problem that is largely avoided in the current clinical study. Moreover, in the previous study, the lesions were defined by -6dB thresholds, while in this work a manual discrimination of abnormal with respect to normal tissue was used. The latter is closer to the method that radiologists use for conventional imaging and leads to an average size deviation for PAM with respect to histopathology of 28% compared to size deviations of 34%, 18% and 65% for x-ray mammography, ultrasonography and MRI respectively. Therefore, PAM performs reasonably well compared to the other imaging modalities. However, there are still non-negligible under- and overestimations in lesion size observed, despite the fact that the lesions are defined with prior knowledge from conventional imaging. This size deviation is partly the consequence of the slight compression applied in the PAM set-up, causing the breast and lesions to deform. Moreover, in histopathological investigations (the gold standard) the largest dimension of the malignancy is measured, regardless the orientation of the malignancy, while in PA images the largest dimension on a cranio-caudal projection is

taken. A more thorough analysis into the different chromophores that are contributing to the photoacoustic lesion contrast is further required to truly relate the photoacoustically defined size and appearance to the histopathologically defined size and appearance.

The last observation concerns the depth of the detected lesions in the PA volume. While even high intensity regions at depths beyond 25 mm can be visualized, most lesions seemed to be located very superficial. The large number of lesions that are positioned above 5 mm in depth is not realistic for the normal breast cancer population [42, 43]. This can be explained by several aspects. First, there was a slight bias towards more superficial lesions in this study. The aim of this study was not to investigate the maximum imaging depth, but rather to investigate the PA appearance of malignancies. Since, the superficial lesions were often easier to position in front of the detector, the patient group with superficial lesions might be overrepresented in this study. Second, the compression applied in PA imaging decreased the depth from the cranial surface to the lesion, primarily because of the fact that the skin and subcutaneous fat are often easier to compress than the stiff lesions. Finally, in some cases the breast was tilted to position the lesion favorably in front of the detector, leading to a shift of the lesion towards the cranial part of the imaging volume.

7.4.2 Contrast versus density

As in our previous study [23], this work indicates that the PA contrast is independent of the mammographically estimated breast density. In the present study, the breast density was estimated in 31 cases by 2 (or 3) radiologists, which is an improvement from the 1 observer in our 2012 study (10 patients). The 3rd radiologist's judgement was used in 11 (of 31) cases and mostly occurred within and not between the low (BI-RADS density 1,2) or high (BI-RADS density 3,4) groups. Therefore, we used a two-grade scale to investigate the contrast dependence on breast density. Again, no significant differences can be observed in average PA contrast for the different density groups, while there is a significant drop in x-ray contrast for the high density breasts compared to the low density breasts. Although the number of patients per density group is still limited, this repeated finding indicates that PA contrast at 1064 nm is largely independent on the mammographically estimated breast density. This is most likely the consequence of the independence of the total blood volume and oxygen saturation on mammographic breast density [44]. In future multi-wavelength studies, the contrast-density dependence should be investigated per wavelength since the water, fat and collagen contents of high density breasts are known to be different from that of lower density breasts [44].

7.4.3 Contrast and appearance versus tumor type and grade

A number of lesion and patient characteristics was investigated for its relation with PA appearance, PA contrast and PA size. In the investigated population, the number of non-IDC was too low with respect to the number of IDC to compare the PA lesion appearances for the different tumor types. The same was the case for the number of pre-menopausal patients with respect to post-menopausal patients. No significant differences were found for

the PA contrast versus BMI, PA contrast versus age, PA contrast versus hormone receptor status, PA contrast versus lesion depth or size deviation versus either lesion depth or the presence of *in situ* components.

The positive correlation between MVD and tumor grade in IDC [39, 45], led to the expectation of an increasing PA contrast with increasing tumor grade. This expectation makes it tempting to observe a subtle trend of increasing PA contrast with increasing tumor grade in Figure 7.4. However, the differences in PA contrast between neither of the different tumor grades are significant. The main problem of the analyses performed here, is the small number of patients per group, especially in cases where a lot of groups were compared, like for BMI or hormone receptor status. The presented results indicate that in future studies with larger numbers of patients, it is useful to investigate the appearance of ILC with respect to IDC as well as the dependence of PA contrast on tumor grade. Moreover, the results further strengthen the hypothesis that PA imaging performs equally well in various population groups (age, BMI, breast density).

7.4.4 Sensitivity versus non-specificity

The main conclusion of this work should be put into perspective. Using a priori information about the lesion location in the breast, for almost all patients abnormal or high intensity regions could be located and related to the presence of malignancy with great certainty. However, in a non-negligible number of patients high contrast or abnormally structured areas were also observed at different locations in the PA volume, as illustrated in Figure 7.5g-i and 7.6. In some cases, these high contrast areas could be related to structures in the breast, like blood vessels, cysts or the nipple. For this, the lesion shape information as well as the information from the digital photograph and conventional imaging was used. However, there are also patients in which, without prior knowledge about either the presence or the position of the malignancy, differentiating malignant from other high intensities areas would have been very difficult using the current imaging system. Although we cannot give numbers for sensitivity and specificity for PAM due to the current study design, this observation indicates that currently the specificity of PAM is limited. This was to be expected by the use of a single wavelength. Although the used wavelength, 1064 nm, is believed to be mostly absorbed by hemoglobin [10], other chromophores are also known to absorb this wavelength [10]. Moreover, hemoglobin is not a sole indicator of malignancy, but is also present in larger and smaller blood vessels in normal breast tissue. Therefore, in future PA imaging systems, better specificity can be achieved by the optimal choice of wavelengths. A wavelength combination that can for example differentiate the contribution of oxy-hemoglobin from that of deoxy-hemoglobin or the contribution of hemoglobin from that of water, can be very useful in this, since both the oxygen saturation as well as the water content are known to be altered at malignant sites [20, 46, 47]. The high visualization rate seems to indicate very good sensitivity of PA imaging for the detection of malignancies. However, true sensitivity and specificity numbers can only be determined in a blind study using an imaging system that can visualize the complete breast.

7.5 Conclusions

Photoacoustic mammography can visualize breast malignancies with high imaging contrast using the Twente Photoacoustic Mammoscope, operating in planar mode and using one wavelength. All detected lesions could be co-localized with great certainty with the lesions on conventional imaging. The size estimation was comparable to conventional imaging techniques with an average 28% deviation from the gold standard. From a comfort point of view, by far most patients strongly preferred the PAM investigation above conventional x-ray mammography.

The contrast at 1064 nm was independent of the radiological breast density. All in all, the study confirms the high potential of PA mammography in breast care. However, the appearance of high contrast areas outside the malignant region indicates that the specificity of the implementation with the use of a solitary wavelength is not optimal. The results further indicate that in future studies it is useful to investigate the appearance of ILC with respect to IDC as well as the dependence of PA contrast on tumor grade in a larger number of patients.

7.6 References

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Supplementary information

Representative PAM results (9 patients) compared with x-ray mammography

Figure 7.s1 Representative results from photoacoustic mammography (1). For all patients, the cranio-caudal (CC) x-ray mammogram is compared with the combined x-ray/PA image. For the latter, the maximum intensity projection (MIP) of the lesion visible in the PA volume is overlaid on the x-ray image using positioning information from the digital photograph. **a)** P49; a 64 year old patient with suspicious microcalcifications and architectural distortion in the lateral upper quadrant of the left breast. This suspicious area is indicated by the red dashed box in the x-ray mammogram. Photoacoustic mammography showed focalized regions of high intensity in the suspicious area. This area was different from the surrounding, supposedly healthy tissue. The abnormal area measured 54 mm on the MIP of the PA volume. There was good correspondence with respect to size and appearance with MRI (not shown here). Post-surgical investigation of the breast revealed the presence of extensive (>40 mm) lobular carcinoma in situ (LCIS), while no invasive components could be found. However, the presence of adenotype metastases in an axillary lymph node indicated that invasive components should have been present as well. **b)** P44; This 55 year old patient came to the center for breast care for annual checks following a history of breast cancer in the right breast. Although both breasts did not reveal any abnormalities on x-ray mammography, clinical investigation and a localized US investigation revealed the presence of a highly suspect lesion medial in the left breast, which turned out to be an infiltrating ductal carcinoma (IDC, grade 3) of 25 mm. In the PA volume, the lesion presented as a multilobed mass of 38 mm. Although the lesion was visible with high imaging contrast at the location of the palpable mass, there was significant size overestimation as compared to the histopathologically assessed lesion size and it can be questioned if the 'tail' of the high intensity area (towards the left of the ROI), truly belonged to the lesion or if it was associated to

another structure or artifact. **c)** P55; This 63 year old patient presented with a palpable mass at the center for breast care. The lesion was highly suspicious on x-ray mammography by the presence of an irregularly shaped, unsharply delineated 20 mm mass. In PA images, the lesion was clearly visible as an highly irregular, high contrast, 29 mm mass, which could be perfectly co-localized with the lesion on x-ray mammography. Histopathological assessment of the tissue specimen post-surgery revealed the presence of a 34 mm IDC, grade 2. **d)** P53; This 72 year old patient presented with a large palpable mass in the left breast, which measured more than 60 mm on the x-ray mammogram and turned out to be an IDC. In PA images, multiple regions of high intensity were observed around a region without intensity. These high intensity areas could be co-localized with the border region of the malignancy and therefore this appearance was defined as a ring appearance of 46 mm. The patient went for neoadjuvant chemotherapy and therefore no further histopathological information is available.

Figure 7.s2 Representative results from photoacoustic mammography (2). For all patients, the CC x-ray mammogram is compared with the combined x-ray/PA image. For the latter, the maximum intensity projection of the lesion visible in the PA volume is overlaid on the x-ray image using positioning information from the digital photograph. **a)** P68; This 63 year old patient came to the center for breast care following a suspicious screening mammogram. The red-dashed box indicates a low contrast atypical, suspicious area far lateral in the breast which was further associated with subtle microcalcifications. Despite the relatively large depth of the lesion in the PA imaging volume and the non-favorable position close to the lateral border of the breast, the lesion presented as high-contrast mass of 10 mm. Histopathological investigation proved the presence of a 14 mm IDC, grade 2. **b)** P45; This 51 year old patient presented with a suspicious screening mammogram, showing highly suspicious microcalcifications in the complete lateral quadrant of the right breast. Although

the biopsy of the suspicious area was not conclusive, a metastatic lymph node was found, indicating the presence of invasive components. No further histopathological information is available, since this patient went to a different hospital for further diagnosis and treatment and she was lost for follow-up. PA images showed a so-called non-mass appearance by the presence of multiple moderate to high-contrast lesions in the suspicious area. Those are emphasized by the white circles and most likely represent the malignant part of the abnormality. However, in such a patient, where the lesion occupies a large part of the breast, only comparison with the supposedly healthy contralateral breast can prove the abnormality of the PA detected lesions. **c)** P61; This 74 year old patient presented with a very large lymph node, which post-biopsy proved to contain metastases of adenotype carcinoma. MRI (not shown here) and X-ray mammography revealed the presence of an architectural distortion and a density close to the nipple of the right breast, which was absent in the left breast. Biopsy revealed the presence of IDC. In PA images, a high-contrast area which was defined as a mass of 21 mm consisting of multiple components, could be seen. The shape was highly comparable to the lesion shape on MRI. The lesion could be co-localized with the density on x-ray mammography and is believed to represent the IDC. **d)** P37; This 61 year old patient came to the center for breast care because of a self-detected palpable mass in the lateral upper quadrant of her right breast. The lesion was visible as a mass in the dense breast tissue and prominent skin changes were visible. In PA images, multiple moderate to high contrast structures could be seen, of which the first most-likely represented the nipple based on its appearance [23] and location. At the location where the lesion was expected, a ring of high contrast structures could be seen. Therefore, this lesion was defined as ring appearance of 31 mm. The lesion was diagnosed to be an IDC, grade 2, of 28 mm in size. **e)** P56; This 56 year old patient presented with a palpable mass at the center for breast care. The breast was rather dense and the lesion itself, present at 12 o'clock close to the nipple, could not be properly delineated in the cc x-ray images. The lesion was clearly visible on US imaging and histopathological investigation of the biopsy specimen proved the presence of IDC. PA images revealed a high contrast mass which was smaller (11 mm) than expected and not at the expected location in the breast (closer to the chest wall and deeper in the volume), when PA and x-ray images were compared **f)** The explanation for this can be found in the digital photograph. Because of the traction of the lesion inside the breast, the breast had the tendency to tilt and the nipple was turned towards the glass plate during the PA investigation. It is plausible that this made the lesion, which was positioned just above the nipple, to move upwards and backwards in the ROI. In x-ray mammography, the breast was not tilted and therefore, overlaying x-ray and PAM images is not useful in this case. In fact, the positioning was being performed based on the position of the palpable mass (pointing of the finger in the right photograph), which seems to nicely correlate with the position of the PAM-detected lesion. The palpable mass turned out to be a 22 mm IDC of grade 3.

8

Conclusions and recommendations

Tumor vascularization is an important hallmark of breast cancer, providing opportunities for screening and diagnosis of the disease. Photoacoustic (PA) mammography is theoretically an ideal technique for breast imaging, because it combines the high optical absorption contrast of hemoglobin with ultrasound resolution in detection, without the use of ionizing radiation or contrast agents. In order to assess the true potential of PA mammography and to guide the developments towards future equipment, clinical studies were performed using the Twente Photoacoustic Mammoscope (PAM), further referred to as PAMI. The results from measurements on 73 patients with symptomatic breasts, as well as the results from simulations on light-tissue interactions, were described in the previous seven chapters of this thesis. In this chapter, the conclusions are summarized and discussed, leading to recommendations for future research and future equipment.

8.1 Conclusions

In the following section, the most important conclusions from this work are given. In addition, the chapter sections that support the conclusions are repeated.

1. PA mammography can visualize malignancies with high imaging contrast.

In **Chapter 4**, the PA imaging results of twelve technically acceptable patient measurements were described. Measurements were performed in a small region of interest (ROI) of $4 \times 4 \text{ cm}^2$.

- *“In the period December 2010 - April 2011, seventeen Caucasian patients were included in the study. Three patients could not be measured either because of discomfort while lying on the bed or because their lesion was difficult to access completely in the scan region. Of the remaining 14 cases, two were technically unacceptable due to poor acoustic contact between breast and detector in the ROI. Ten of the successful measurements were on highly suspect breasts carrying one or more BI-RADS 5 lesions and two were on breasts with cysts.”*
- *“In the reconstructed volumes of all ten measurements on BI-RADS 5 lesions, a confined region with high contrast with respect to the background could be seen at the depth where the lesion was expected based on the ultrasound images.”*

In **Chapter 7**, the state of the art of photoacoustic imaging of breast malignancies is given by comparing PA images of 31 breasts with malignancies to conventional imaging findings, patient demographics and lesion characteristics. Results were obtained with an updated version of PAM, allowing measurements in a $9 \times 8 \text{ cm}^2$ ROI.

- *“From April 2012 to May 2013, 43 patients volunteered in the PAM study. From these, 2 patients were not imaged because of incapability to climb on/lay on the bed for 10 minutes. For 2 other patients it appeared retrospectively (with information from the digital images, x-ray mammograms, MR and US images) that the lesion was not favorably positioned in front of the detector. Of the remaining 39 technically acceptable patient measurements, 5 were on cysts; 2 were on fibroadenoma; 1 was on a chronic active inflammation; and 31 were on malignancies.”*
- *“In 30 patients, the malignancies could be visualized with high certainty. This was confirmed by a high degree of co-localization between the lesions in x-ray and PA images.”*

2. The specific PA lesion appearance is largely, but not solely, the consequence of the increase in tumor vascularization at malignant sites.

In order to get first insights in the relation between photoacoustic lesion appearance and tumor vascularization, in **Chapter 6** PA imaging results are compared to the gold standard for imaging tumor vascularization: magnetic resonance imaging (MRI). In a limited number of patients, PA results are further compared to vascularity stained histopathology slides (using anti-CD31 immunostaining).

- *“In general, three main types of lesion appearance were observed in this study: ‘mass or confined’, ‘non-mass or scattered’ and ‘ring’. These specific lesion appearances are reminiscent of enhancement types described in MRI literature namely mass enhancement, non-mass enhancement, focal enhancement and mass enhancement with ring enhancement.”*
- *“In eleven patients, the correspondence between PA and MR images was investigated. On visual inspection, there was good correspondence between PA and MR images with respect to lesion location and to lesser extent with respect to lesion shape and appearance.”*
- *“In general, the size and shape of the lesion in PA images showed similarities with the histopathologically derived size and shape of the lesions. Further, there was good correspondence between the pattern of microvasculature obtained from the CD31 stained slices and the PA appearance in 5 out of 6 of the investigated cases.”*

3. The PA lesion contrast is independent of the mammographically estimated breast density.

For the ten successful measurements on malignancies described in **Chapter 4**, the radiologist was asked to estimate the mammographic breast density on the cranio-caudal (CC) x-ray mammograms. The effect of breast density on lesion contrast was investigated for x-ray mammography and PA mammography. The first analyses revealed, as expected, a significant decrease in x-ray lesion contrast for breasts with high fibroglandular density compared to breasts with low density, while there was no significant difference in lesion contrast for the two density groups in PA images (**Figure 4.7**). However, results were obtained in a very small patient group and the density assessment is known to be poorly reproducible. Therefore, the analyses were repeated for the 31 patients described in **Chapter 7 (Figure 7.3)**. In this case, the breast density was independently graded by two radiologists and in case of disagreement a third radiologist was solicited.

- *“The final result of the density assessment by the three radiologists is used to define the density groups for this analysis (this will be further referred to as ‘complete assessment’). Here, a two-scale classification is given (low: BI-RADS density 1, 2 and high: BI-RADS density 3, 4), because this is proven to be more reliable and reproducible than the four scale classification. Both for the individual assessment of the two radiologists as well as for the complete assessment, there is no significant difference in average PA contrast between the high and low density groups, while the contrast of x-ray mammography is significantly lower for the high density group as compared to the low density group.”*

4. Using PAM, benign cysts cannot unambiguously be discriminated from malignancies.

In the search for improved imaging modalities for detection and diagnosis of breast cancer, the ability to differentiate between benign cysts and malignant lesions is of great importance. In **Chapter 5**, the appearance of breast cysts in planar geometry photoacoustic mammography at 1064 nm is investigated using simulations, phantom and patient measurements.

- *“Cysts can be visible in forward mode photoacoustic mammography as one or more pronounced, confined high contrast areas representing the front (illumination side), or the front and the back (detection side) of the cyst. In the current forward mode imaging configuration and with one wavelength, cysts may be mistaken for malignancies which can also present as one or multiple confined contrast areas.”*

The findings prove the potential of PA mammography for the visualization of malignancies. However, some critical remarks regarding this work and the conclusions should be made before an outlook on the usefulness of PA mammography in a clinical setting can be given. In the following sections, this critical discussion is used to give recommendations for future research (Section 8.2) and future equipment (Section 8.3).

8.2 Remarks and recommendations for future research

8.2.1 PA mammography can visualize malignancies with high imaging contrast

In this study, the focus was mainly on lesions that were highly suspicious for malignancy. The study design can raise some questions since we started investigating the abnormal breast before knowing the appearance of healthy breasts. The choice for the focus on malignancies is partly necessitated by the limitations of the prototype imaging system: PAM1 works with a limited field of view, making it impossible to scan the complete breast. Focusing on highly suspect and palpable lesions makes it possible to investigate, in one view, a known part of the breast with both healthy and abnormal tissue.

However, more than necessity, the focus on abnormalities was primarily a conscious choice. A set of clinical data is available from the highly suspect lesions: x-ray mammography, ultrasonography and often even MRI data can be combined with information from histopathology. This provides the opportunity to compare the PA appearance to gold standard(s) (**Chapters 4-7**), to make statements about the visibility of the malignancy (**Chapters 4, 7**), the accuracy of lesion representation (**Chapter 6, 7**) and to learn how malignancies look like (**Chapters 4, 6, 7**). For the non-malignant breast, conventional imaging is usually absent or limited to screening x-ray mammography. The presence of, for example, blood vessels, cysts, inflammation, scars, undetected fibroadenoma or even undetected malignancies would make the evaluation of PA images highly troublesome if one would not first learn to recognize malignancies.

In **Chapters 4 and 7** we have seen that almost all known malignancies could be visualized with high imaging contrast. Hereby, the potential of PA mammography for the *visualization* of known malignancies has been proven. Although this was the first and most

important step to take, to truly say something about the potential of PA mammography for use in clinical practice, one should investigate if (unknown) malignancies can be *detected* and *differentiated* from benign lesions. First investigations to the PA appearance of benign cysts (**Chapter 5**) showed that the current imaging system cannot always discriminate cysts from malignancies. Moreover, after increasing the measurement field of view, regularly high contrast abnormalities were seen in the PA mammograms (**Chapter 7, Figure 7.5 and 7.6**) that could not always be associated to known structures in the breast. Therefore it is expected that the specificity of the current imaging system is non-perfect and that modifications to the system are required to optimize its diagnostic accuracy. Those recommended modifications are further discussed in Section 8.3.

Figure 8.1 Research and development of photoacoustic mammography over time. The dashed box indicates the current status (December 2013). The time has come to investigate healthy breasts and benign lesions in addition to malignancies. All information should be used to further improve PA mammography equipment. Only a blind study in the screening population can determine the potential of PA mammography for use in a screening setting.

The clinical studies using future versions of PAM should gradually move towards the investigation of unknown breasts. A recommended approach (Figure 8.1) is to continue the investigation of highly suspect lesions, but to extend the study by investigating the healthy contralateral breast as well. In this way, the photoacoustic appearance of a specific healthy breast can also be compared to conventional images. Moreover, by comparison of the healthy and diseased breast of one patient, effects of tissue and skin composition on the light and sound propagation and therefore on the PA results, can largely be cancelled. It is assumed that the two breasts are rather comparable in tissue composition. Investigating the

same abnormal breast at different time-points before treatment is required to define the reproducibility of the technique. In addition, the PA appearance of benign lesions such as fibroadenoma, cysts and papilloma should be investigated. In the final step, all knowledge should be combined in a blind study, preferably linked to the screening program, where PA mammograms are investigated without prior knowledge of the presence of malignancy. During these clinical studies, all acquired information should be used to improve the PA mammography equipment.

8.2.2 *The PA lesion appearance is largely the consequence of tumor vascularization*

In **Chapter 6**, we compared PA images to MR images and vascularity stained histopathology slides in order to investigate the relation between tumor vascularization and PA lesion appearance. The origin of the PA lesion appearance cannot only help us to further define the potential for visualizing malignancies, but also to determine if the technique is theoretically able to differentiate malignant lesions from benign lesions and to optimize future systems to make this differentiation possible.

The correspondence between PA images and individual MR images and CD31 stained tumor slides, shows that the PA lesion appearance at 1064 nm is indeed largely, but not solely, dependent on the tumor vascularization. Variations in lesion appearance can be observed in individual PA images, which are also visible in MR images and as variations in the vascular pattern in the CD31 stained tumor slides. It is recommended to apply more quantitative analysis of MR data in future studies. For example, by using the temporal data of contrast enhancement, MR maps of pathophysiological features, such as vascular permeability and extracellular fraction, can be compared to the PA appearance [1, 2]. Although the PA contrast as a consequence of increased hemoglobin concentrations is not likely to depend on the vascular permeability, it might well be that the areas with dysfunctional vessels are differently perfused and therefore lead to an altered PA appearance pattern.

The study and literature investigations further indicate that hemoglobin in the tumor vasculature is most-likely not the sole contributor to the photoacoustic lesion appearance, and that the contribution of fat, water and collagen should further be investigated. This finding in combination with the fact that various benign lesions are also associated with an increase in vascular density makes the use of a single wavelength a-specific.

In order to get a feeling for the theoretical contribution of the different chromophores to the PA contrast, Table 8.1 shows concentrations of hemoglobin, fat, water and collagen for healthy breast tissue as well as for malignancies, together with their absorption properties at 1064 nm. All data is derived from literature. Since *in vivo* optical data is highly variable throughout literature and partly dependent on the measurement method, the concentrations are as much as possible obtained from a single study that provides the most complete chromophore information [3]. For all chromophores, the contribution to the total optical absorption is calculated. It can be seen that at 1064 nm especially the increase in water concentration at malignant sites leads to a non-negligible increase in absorption,

while the effect of collagen can be neglected. Based on other values from literature [4, 5], the hemoglobin contribution is lower than expected, as is the total absorption coefficient by summation of the most prominent contributors. A possible explanation for this is the fact that the diffuse optical spectroscopy (DOS) measurements have a poor resolution, smearing out the hemoglobin concentration in the microvessels over a large volume. This will underestimate the hemoglobin contrast. For the simulations in **Chapter 2**, the hemoglobin concentrations derived from the more localized Differential Pathlength Spectroscopy (DPS) measurements are used [6], which are an order of magnitude higher (see blue values in Table 8.1) than the values from [3].

Table 8.1 Influence of different chromophores to the optical absorption at 1064 nm

	Absorption properties		Normal		Malignant	
	e ($M^{-1} mm^{-1}$)	μ_a ($10^{-3} mm^{-1}$)	Concentration	Contribution to μ_a ($10^{-3} mm^{-1}$)	Concentration	Contribution to μ_a ($10^{-3} mm^{-1}$)
HbT		--	18 μM [3] 5%, 116 μM^2 [6]	2.7 (1) 22.3	25 μM [3] 11%, 256 μM^2 [6]	3.7 (5) 36.5
SO ₂		--	68% [3] 93% ² [6]		69 % [3] 65% ² [6]	
Hb	12 ¹ [7]		6 μM 8 μM	0.2 0.2	8 μM 90 μM	0.2 2.5
HbO ₂	88.9 ¹ [7]		12 μM 108 μM	2.5 22.1	17 μM 166 μM	3.5 34.0
H ₂ O		14.8 [8]	19% [3]	2.8 (2)	26% [3]	3.8 (6)
Lipid		5.0 [9]	66% [3]	3.3 (3)	59% [3]	3.0 (7)
Collagen		4.4 [9]	17% [10]	0.7 (4)	18% [10]	0.8 (8)
Total (HbT+H ₂ O + lipid + collagen)				9.5 (1+2+3+4)		11.3 (5+6+7+8)

¹ This Molar extinction coefficient is for 1042 nm

² This data is used for the simulations in Chapter 2.

It is recommended to further investigate the contribution of the various chromophores to the PA lesion contrast and to define the ultimate wavelength combination for future equipment. A possible approach is to perform optical spectroscopy measurements using a technique called (multi-diameter) single fiber reflectance spectroscopy (SFR) [11, 12] in which a single fiber is used to emit as well as detect light. The back-reflected light is fit to empirical models in order to estimate the wavelength dependent absorption and scattering values with high spatial resolution. Moreover, the local concentrations of expected chromophores (hemoglobin, fat, water, collagen, beta-carotene) can be derived from the reflectance

spectra. Since these measurements can be combined with the conventional ultrasound guided biopsy procedure, information from healthy and diseased tissue can be obtained in a semi-invasive manner. First feasibility measurements using this technique have been performed in 7 patients joining the photoacoustic mammography study. The performed measurements showed that it is possible to obtain *in vivo* spectra from healthy tissues and different locations in the malignancy (see Figure 8.2 for an example), however the number of successful measurements was too small to draw quantitative conclusions from the data.

Figure 8.2 SFR results P47 (see Chapter 6 and 7 for more information on this patient). **a)** The x-ray mammogram of the left breast, shows a highly suspect abnormality central in the breast, which is associated with skin thickening. **b)** The PA image is overlaid on the x-ray image. Regions of high intensity are located at the border of the malignancy and surround an area with low intensity. This type of appearance is referred to as ‘ring appearance’. **c-d)** The ultrasound guided biopsy procedure (top panel) was combined with Single Fiber Reflectance (SFR) measurements (lower panel). Here the optical reflection spectra from the tumor margin (**c**) and tumor core (**d**) are shown. There are clear differences in the spectra from the two regions. The reflection from the core of the tumor is larger than from the margin. This corresponds with the PA image (**b**), where the intensities at the margin of the tumor were higher (more optical absorption and thus less reflection). Moreover, the steeper slope for the core measurement, seems to indicate alterations in scattering properties for the different tumor regions. However, the number of patients measured in this feasibility study was too low to optimize the fitting procedures and obtain quantitative information.

In general, trends that could be observed comparing malignant to normal tissue were: a decrease in saturation, an increase in water content and a decrease in fat content. Using the

information from the feasibility study, the measurement procedure needs to be improved in order to increase the success rate of the measurements. Subsequently, larger numbers of patient data are required to optimize the fitting parameters and estimate *in vivo* chromophore concentrations.

The current disagreement in literature on chromophore concentrations, indicate the importance of reliable and localized measurements. The obtained data cannot only provide information on the best wavelength combination for future PA imaging systems, but can also be used to give patient specific information about the tissue composition in relation to the PA lesion appearance.

8.2.3 *The PA lesion contrast is independent of the mammographically estimated breast density*

In **Chapters 4 and 7**, the PA contrast is compared to the mammographically estimated breast density. Both for the small field of view measurements (**Chapter 4**, 10 patients) as for the large field of view measurements (**Chapter 7**, 31 patients), there was no significant difference in PA lesion contrast between high and low density breasts. For x-ray mammography, there was an expected significant decrease in lesion contrast for the higher density breasts. This poor performance of x-ray mammography in (young) women with dense breast tissue is one of the reasons that in most countries breast cancer screening starts at the age of 50 years [13]: younger (pre-menopausal) patients usually have more parenchymal tissue and thus higher radiographic breast density (see **Section 1.2.1, Chapter 1**). However, the breast cancer incidence is also high in the age group from 40-50 years [14], as can also be seen in Figure 1.3 in **Chapter 1**. Moreover, high breast density is associated with higher incidence and mortality of breast cancer [13]. Another problem is the high-risk or genetically predisposed population in which annual check-ups are recommended from the age of 30 years [15]. X-ray mammography is not the method of choice because of various reasons: in addition to the poor performance in those young women because of the dense glandular tissue, the life-time radiation dose would become too high, especially since young and dense breasts are more susceptible for developing radiation malignancies [16]. Currently, the method of choice is either ultrasonography with its known limitations as poor soft tissue contrast and user dependency; or MRI with its low specificity, dependence on the hormonal status of the patient and dependence on contrast agents. A technique like PA mammography, which does not use ionizing radiation or contrast agents and meantime performs well in (young) women with dense breasts, is needed to solve this problem. The first studies showing the independence of PAM's performance on breast density are promising, but more clinical data is needed to further prove this independence and to determine the technique's sensitivity and specificity in different age groups.

Further, if the technique would be used in young women, it is important to know the dependence of the imaging results on the menstrual phase/hormonal status of the patient, which is known to influence MR imaging results [16]. In the current study, most patients

were post-menopausal and this relation could therefore not be investigated. For future studies more pre-menopausal patients in different stages of their menstrual cycle should be included in order to test the dependence of PA results on mammographic breast density, age and menstrual phase.

8.2.4 *Benign cysts cannot unambiguously be discriminated from malignancies using PAM1*

The expected limited specificity of the current imaging system has previously been mentioned. In **Chapter 5** we showed, using simulations, phantom and patient measurements, that cysts can be visible using PAM1, but that they cannot always unambiguously be discriminated from malignancies. A technique that cannot differentiate cysts from malignancies is inapplicable as screening modality, since cysts are highly common [17]. The later recommended (Section 8.3.5) addition of US imaging to future PA systems, can overcome this problem, since US is the method of choice for differentiating cystic from solid masses [17].

However, the study also showed that cysts do have a signature PA appearance, which can make them differentiable from malignancies with a modified image configuration and multiwavelength approach. More patient measurements are required to further clarify this signature appearance. Simulations using a different image geometry should be applied to investigate the PA appearance for future PAM systems. Although we have observed that the intra-patient variations in color and turbidity of the cyst fluid can be large, we strongly recommend spectroscopic measurements of cyst fluid in order to define the best wavelength combination for the differentiation of cysts from malignancies.

The approach that has been followed in the cyst study, namely simulations on light-tissue interactions and sound propagation, followed by phantom and patient measurements, could also be very useful for other types of abnormalities such as fibroadenoma. For this, the general composition of the abnormality of interest should first be known, either from available literature or from the aforementioned *in vivo* optical (SFR) measurements. This approach can then provide information on the origin and consequences of the (in)visibility of specific types of lesions and could help to make the future imaging systems more specific.

8.3 Recommendations for future equipment and their application in PAM2

As has become clear from the previous discussion, PAM1 suffers from limitations which make the system inapplicable for use in daily clinical practice. To name a few: the system cannot image the complete breast; uses a single wavelength; applies breast compression; uses inhomogeneous light distribution and cannot image regions close to the chest-wall. Most of these limitations were well-known at the beginning of this study, still we decided to use the system for clinical studies. In fact, despite or maybe because of its limitations,

PAM1 works in a straightforward manner and can provide a lot of information about the combination photoacoustic imaging and breast tissue. The forward mode and single wavelength approach made it for example relatively easy to simulate and understand light-tissue interactions and sound propagation in the cyst study (**Chapter 5**); and the planar geometry made comparisons with x-ray mammography, MRI and histopathology possible (**Chapters 6 and 7**). Now the time has come to move forward and to develop a system that can be used for detection of breast cancer. In the previous discussion and throughout this thesis, recommendations for such a PA mammography system have been given and Figure 8.1 schematizes how the interplay between research and development should finally lead to a perfect mammography system. This futuristic system will further be referred to as ‘PAM perfect (PAMP)’’. In this section and in Table 8.3, the most important requirements for PAMP are given and compared to their application in PAM1 and the recently developed PAM2 (Figure 8.3).

8.3.1 Patient position and patient comfort

One of the limitations of x-ray mammography is the lack of patient comfort. The upright position and especially the heavy breast compression, make the procedure, although very fast, uncomfortable and painful. In PAM1 and PAM2, the patient is lying in prone position on a bed. Despite the fact that in PAM1 minor breast compression is required for ultrasound coupling, almost all 73 measured patients indicated to prefer PAM1 (far) above x-ray mammography. Still, these patient measurements also showed limitations with respect to patient comfort for PAM1 specifically and the prone position in general.

Figure 8.3 The breast in PAM2. The patient is lying in prone position and the breast is pendant in an imaging tank (dashed area). Ultrasound detector arrays are positioned either at one side or completely around the breast. The light is delivered from the bottom and via fibers that point to locations at the chest wall. The complete imaging tank can rotate to allow for tomographic imaging.

First, the bed was too flat and too stiff, which often led to a sore back or painful skin, especially for longer measurement duration. Moreover, the flat configuration made it difficult to assess regions of the breast close to the chest wall and axilla. These areas are of importance, since they are often poorly covered by conventional imaging modalities, and worth for a large proportion of the false negative interval cancers [18]. In PAM2 a more

natural curvature to the bed should increase comfort and meantime allow the breast to be more pendant in order to make a larger part of the breast visible. A soft top layer on the bed is used to further optimize comfort and avoid sharp edges.

Second, the prone position made the measurement of a number of patients impossible. Patients in wheel chairs, with back or shoulder problems, or obese patients, could not lay on the bed without having problems and also in pregnant patients, the prone position could cause problems for imaging and comfort. These problems will also arise in PAM2, while the independence of PAM's performance on breast density and the lack of ionizing radiation make PA mammography potentially an ideal imaging system for pregnant women. Moreover, the age of the screening population (50-75) is the age group where back and shoulder problems and obesity are not uncommon. It is recommended to investigate the possibility of using other than prone positions for PAMp. For example, from experience during the measurements and feedback from the patients, a sitting position might be considered. This position is better durable than standing for most people and applicable in (most) physically disabled patients as well. However, this technique will cause problems for the recommended tomographic approach (Section 8.3.2) and acoustic coupling. A sitting position in which the patient slightly bends forward (further referred to as semi-sitting, Figure 8.4) to have the breast more pendant, can partly overcome these problems, but will cause new comfort problems. As will be further discussed in Section 8.4, the design choices will strongly depend on the specific application for PAMp and cooperation between photoacoustic imaging specialists and ergonomics and industrial design specialists is recommended for design of a system which is as comfortable as possible.

Figure 8.4 For PAMp a semi-sitting position might be considered.

Finally, besides patient comfort, also the comfort of the health employee should be considered. For PAM1, for which the height of the bed cannot be changed, positioning the patient is an uncomfortable task: the employee has to look and work at a height of 20-100 cm. This would lead to physical problems if applied in a screening setting, where dozens of patients are measured a day. This problem is partly avoided in PAM2, for which the height of the bed can be altered. The aforementioned (semi-)sitting position can further improve employee ergonomics.

8.3.2 Image configuration

For use as screening modality, the complete breast should be visualized in a standardized manner. Standardized full-breast imaging will make post-imaging review of the data possible and will allow for inter- and inpatient comparisons. These requirements cannot be fulfilled with handheld systems, where the focus is on real-time imaging and reviewing. Also the forward mode configuration, as applied in PAM1, can no longer be applied since breast compression is preferably avoided and light should be as homogeneously distributed in the medium as possible. Therefore, a tomographic imaging approach is recommended, in which ultrasound detector elements surround the breast and the light illumination is such that the complete breast is illuminated. By this, 3D information from the breast can be obtained. However, the combination between the sitting position and tomography might cause design challenges.

In PAM2 a first attempt to fulfill the requirement of complete breast imaging is made by the design of a rotating imaging tank in which the breast is pendant and in which the ultrasound detectors are arranged in vertical arrays around the breast (see Figure 8.3). The light is delivered via broad beam bottom (ventral side) illumination in addition with fiber illumination around the breast. In the progress from small field of view measurements (**Chapter 4**) to large field of view measurements (**Chapter 5-7**) using PAM1, advantages of having a broad illumination beam have been observed with respect to penetration depth and fluence homogeneity at superficial areas. The fiber illumination is used to further cover the complete breast: by slightly directing the fibers upwards, areas close to the chest-wall can be illuminated as well. Clinical measurements with PAM2 should teach us if this illumination is sufficient to cover the complete breast. A possible problem of the bottom illumination can arise by illumination of the nipple. This highly vascularized (and colored) area can lead to high intensity areas in the photoacoustic mammograms as seen in **Chapter 4 (Figure 4.4)**. However, this area can also be the origin of malignancies and it is important to ascertain that the nipple signals do not overrule the signals from possible malignancies. For this, the signature appearance of the nipple should be investigated and compared to the signature appearance of malignancies.

8.3.3 Light specifications

Chapters 4-7 all end with the recommendation to use multiple wavelengths. It is expected that the addition of multiple wavelengths will increase the specificity of photoacoustic imaging, since it makes it possible to differentiate the contribution of the different chromophores to the PA signal by means of multispectral analyses.

The presented clinical data show the sensitivity of PAM at 1064 nm for tumor vascularization, but using this single wavelength there is a non-negligible contribution of, amongst others, water to the PA contrast of malignancies (see Table 8.1). Therefore, it is recommended to add lower wavelengths for which the water absorption is much less pronounced. Information that might further be useful in diagnostic imaging is that of blood oxygen saturation, especially in the estimation of therapy response [19]. Therefore, it is

recommended to measure at least at two wavelengths at the two sides of the isosbestic points of the oxy- and deoxy hemoglobin curves (around 800 nm). To fulfill these requirements, in PAM2 the choice has been made for a laser that can pulse interleaved at 1064 nm and 755 nm. These wavelengths can both be delivered to the breast with sufficient energy for deep PA imaging. Table 8.2 is an updated version of Table 8.1, which provides more information on the contribution of each of the most important chromophores to the photoacoustic contrast at 755 nm and 1064 nm. The difference from normal to malignant tissue is indicated by arrows. Indeed, the water contribution is much less pronounced for 755 nm, while the total absorption (increase) is much more affected by the deoxygenated hemoglobin at 755 nm compared to 1064 nm.

Table 8.2 Chromophore contribution to the PA contrast at 755 and 1064 nm.

	Absorption properties				Normal			Malignant		
	755 nm		1064 nm		conc.	contribution to μ_a		conc.	contribution to μ_a	
	e	μ_a	e	μ_a		755 nm	1064 nm		755 nm	1064 nm
	(M ⁻¹ mm ⁻¹)	(10 ⁻³ mm ⁻¹)	(M ⁻¹ mm ⁻¹)	(10 ⁻³ mm ⁻¹)	(μM)	(10 ⁻³ mm ⁻¹)	(10 ⁻³ mm ⁻¹)	(μM)	(10 ⁻³ mm ⁻¹)	(10 ⁻³ mm ⁻¹)
HbT [3]	--		--		18	4.0	2.7	25	5.5	3.7
SO ₂ [3]	--		--		68%			69%	↑	↑
Hb	167.8 [7]		12 ¹ [7]		6	2.2	0.2	8	3.0	0.2
HbO ₂	64.1 [7]		88.9 ¹ [7]		12	1.8	2.5	17.1	2.5	3.5
H ₂ O [3]		2.7 [8]		14.8 [8]	19%	0.5	2.8	26%	0.7	3.8
Lipid [3]		1.0 [9]		5.0 [9]	66%	0.7	3.3	59%	0.6	3.0
Collagen [10]		3.6 [9]		4.4 [9]	17%	0.6	0.7	18%	0.6	0.8
Total (HbT+ H ₂ O + lipid + collagen)						5.8	9.5		7.4	11.3
									↑	↑

¹ This extinction coefficient is for 1042 nm

Clinical studies using PAM2 and the recommended research to the *in vivo* optical properties should teach us if more than two wavelengths are required for further increase of sensitivity and specificity of the technique. By addition of more and more wavelengths, part of simplicity of the technique in application and interpretation is lost. The required number of wavelengths will further depend on the specific use of the system (see Section 8.4).

8.3.4 Detector requirements

The center frequency of the transducer as used in PAM1 (1 MHz, 130% bandwidth) has proven to be useful for visualization of breast cancer. However, improved detector sensitivity and signal-to-noise ratio are required in order to improve the system's diagnostic sensitivity and imaging speed. The specifications of the recently designed ultrasound detector for PAM2 that fulfills these requirements are further explained in [20, 21]. The size of the elements, the thickness of the element layers and the detector material (PZT) are chosen to obtain a center frequency approaching 1 MHz (80% bandwidth) and meanwhile optimize the sensitivity of the elements [21], while the directivity is further increased by use of acoustic lenses [22]. Phantom and patient measurements using PAM2 will further teach us if these detectors fulfill the demands. It is expected that at least the bandwidth of these detectors needs improvement in order to be sensitive for large and small structures in the breast.

8.3.5 Multimodality

PA mammography as currently applied in PAM1 and PAM2 largely relies on the increased tumor vascularization as a consequence of angiogenic processes. This approach has proven to be successful by the high sensitivity of MRI for breast cancer visualization (**Chapter 3**). However, in MRI the T1 weighted functional data of contrast enhancement of the tumor vasculature is combined with anatomical data of the T2 weighted images. Also in contrast enhanced digital mammography (CEDM) and contrast enhanced ultrasound (CEUS), the contrast information is seen as addition to the anatomical images. PA imaging does not provide this anatomical data, while it is expected that such information will be of importance to detect small differences in the normal structure of the breast.

For PA imaging, the anatomical information can be relatively easy obtained by adding conventional ultrasound imaging (transmission and detection). This is currently not yet being applied in PAM2 by active ultrasound transmission. However, structural information in this system can be obtained by using the PERPACT (passive element enriched photoacoustic computed tomography) method [23] that makes it possible to measure speed of sound (SOS) and acoustic attenuation (AA) without the addition of active ultrasound transmitters and without any additional measurements to the conventional photoacoustic imaging protocol. Using a small-scale tomographic imager, the method was evaluated with phantoms and biological specimen. The PERPACT method made it possible to obtain information on the composition of the phantoms that could not be obtained by PA imaging alone. Moreover, the retrieved SOS maps will allow for improvements in reconstruction of PA images [23].

8.3.6 Measurement time

It is clear that the current imaging speed of PAM1 is unacceptable for use in daily clinically practice. The question arises what imaging speed is acceptable. The answer to this question depends on where PA mammography will be applied: screening or diagnosis. The high

number of patients to be measured each day in the screening program requires a very high imaging speed. In x-ray mammographic screening, usually two views per breast are made and the total procedure time is in the order of minutes. For a good estimation of the maximum time allowed for breast screening, one should make a cost-analysis, including the number of patients to be scanned and the number of systems and staff available. A first rough estimation is that the procedure time per breast should not exceed 3 minutes. Preferably, the two breasts are measured simultaneously to double the throughput. However this will strongly affect the cost-price of the equipment and will induce a high burden on the data acquisition requirements. If the system is to be used in a diagnostic setting, a somewhat longer measurement time is allowed. The procedure time for an ultrasound investigation (without biopsy) is approximately 15 minutes and a DCE-MRI investigation takes roughly 30 minutes to image both breasts. However, the latter is often considered to be too long.

Table 8.3 *Specifications of PAM1, PAM2 and PAMp*

	<i>Patient position</i>	<i>Image configuration</i>	<i>Wavelengths</i>	<i>Detection</i>	<i>Time</i>	<i>Multimodality</i>
PAM1	Prone	Forward mode, Limited FOV	1064 nm	1 MHz, 130% bw	25 min, 4x4 cm ² 10 min, 9x8 cm ²	None
PAM2	Prone	Tomographic, Complete breast	755nm, 1064 nm	1 MHz, 80% bw	tbd	PA+ Speed of Sound (SOS) + Acoustic Attenuation (AA)
PAMp	Tbd/ semi-sitting	Tomographic, Complete breast + chest wall	1064 nm, <800 nm, + tbd ¹	1 MHz, 130% bw	<3 minutes complete breast	PA + Ultrasound imaging

tbd=to be determined; bw=bandwidth

8.4 A final note

In this chapter, recommendations for future research and future equipment have been given from the starting point that PA mammography has proven its potential for the visualization of malignancies. In my experience, further improvements to the system and clinical studies can lead to a technique that is useful in both the diagnosis and screening setting, but also in specialized situations such as screening of young women with dense breast. In order to exploit this potential, the ultimate role for PAMp should be defined, since the exact requirements depend on the discipline in which the system will be used. To name a few:

- The speed of the system needs to be higher for screening equipment than for diagnosis equipment.
- If PAM's role is to be highly sensitive and to have a high negative prediction value in a screening setting, two wavelengths might be sufficient. In the presented studies even one wavelength was enough to visualize almost all malignancies. The second

wavelength can be chosen such to differentiate the most common benign abnormalities (cysts, fibroadenoma) from malignancies. Each addition of more wavelengths will make the technique complicated and unwanted slow. However, for diagnostic purposes, more specific information of the tissue is required and as many wavelengths as the number of chromophores to investigate are required.

- Where for screening the complete breast needs to be imaged, for diagnosis or preoperative assessment a more detailed and focused look together with the possibility of PA guided biopsy, might be desirable.

In my opinion, a lack of differentiation between screening and diagnosis requirements, might have been the cause that all new imaging technologies that have been developed in the last years, failed to change the clinic. For example breast thermography, breast specific gamma imaging, contrast enhanced digital mammography, contrast enhanced ultrasound and ultrasound elastography, all showed reasonably to highly promising results, but currently, none of them is widespread used in daily clinical practice. Therefore, the design choices for PAMp should strongly depend on the answer to the question in which discipline (screening, diagnosis or specific situations) the technique will be used.

Thinking about the potential application of PA imaging at the end of a thesis, after four years of clinical measurements and many more years of development, might give the uncomfortable feeling that we are working and thinking in the wrong direction. Do we have a ‘technology in search for a problem’, rather than a ‘problem in search for a technology’? Shouldn’t we have thought about this question long before starting developments of PAM? Although this is a logical thought, there are two arguments that explain the chosen approach.

First, both in screening and diagnosis, there are clear breast imaging problems awaiting solutions. Exploratory (unfocused) studies were required to find out if PA imaging can visualize breast cancer and if so what its strengths and pitfalls are. These studies provided information to further specify the problems, but also to narrow the focus for future research. For example, the fact that PAM could visualize almost all malignancies with high imaging contrast and that this contrast was independent of the mammographically estimated breast density (**Chapter 4 and 7**), suggests that both screening in general and more specific screening of young women with dense breasts, could be potential applications. Hence the recommendations (Sections 8.2.1. and 8.2.3.) to investigate (using the recently developed PAM2) the PA appearance of healthy and diseased breasts and the influence of age, menstrual status and breast density on the imaging results. On the other hand, the multiwavelength studies, the studies to the appearance of benign lesions and the measurements of local optical properties are recommended to further assess the potential in a diagnosis setting. The outcomes of these studies can then be used to further narrow the focus and make the appropriate design choices for PAMp.

Second, it can be questioned if starting from the technology rather than from the problem is truly the wrong approach. What these four years in BMPI and at the center for

breast care taught me, is that both ways of thinking are correct and necessary to develop new technologies that will be applied in a clinical setting. Where ‘the physician’ can raise a problem (breast imaging techniques are not good enough), he can never find the solution without knowledge of new technologies and their pros and cons. On the other hand, the ‘technician’ can have a beautiful technique (photoacoustic imaging), but can never fully exploit its advantages before finding a problem to apply it on. I had the privilege to work in (between) the two disciplines and to observe both ways of problem solving. However, I also noted and experienced the danger of not understanding and not involving the other discipline, leading to solutions to non-existing problems or leading to solutions that induced new problems. From this experience a final recommendation is: extend the cooperation between university and hospital and keep communicating. Only then the true problem might find a solution. Only then the beauty of the technique can fully be exploited. Only then a technique can be developed that fulfills the needs.

8.5 References

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Summary

In this thesis, different aspects of photoacoustic (PA) imaging of suspicious breast lesions are described. The problem of conventional breast imaging, and the possible solution by PA mammography, are introduced in **Chapter 1**. PA imaging relies on the strong optical absorption contrast between benign and malignant tissue, which is mainly the consequence of a local abundance of hemoglobin at malignant sites.

In **Chapter 2**, this optical absorption contrast is introduced by means of a painting from Rembrandt van Rijn: 'Bathsheba at her bath' (1654). In 1983, two Australian surgeons hypothesized that Bathsheba's model had suffered from breast cancer. Their hypothesis was based on, amongst others, the blue discoloration of Bathsheba's left breast. Bathsheba has become a symbol for breast cancer ever since. In addition to medical and historical doubts regarding this 'on-art-diagnosis', the question arose if it is optically possible that a breast cancer mass causes such bluish discoloration of the overlying skin; just like blood vessels under the skin can appear bluish. Breast cancer is associated with a locally increased microvessel density as a consequence of angiogenic processes, hence the analogy with blood vessels. Using Monte Carlo simulations and the Retinex theory of color vision, it was investigated if the local abundance of hemoglobin at malignant sites can indeed cause the observed bluish discoloration on Bathsheba's breast. The simulations showed that only lesions positioned shallower than 5 mm in depth can give a subtle bluish shade. Therefore, it is unlikely that the specific appearance of Bathsheba's breast was the consequence of a breast cancer mass. These simulations also illustrated, once more, the influence of the increased hemoglobin concentration on the local light-tissue interactions for malignancies that are positioned within the penetration depth of the chosen wavelength.

In **Chapter 3**, different techniques that image the tumor vasculature for diagnosis and detection of breast cancer are described. Currently, the gold standard in this field is Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI), which has very high sensitivity for visualizing breast cancer. However, the technique suffers from poor specificity, requires the intravascular injection of contrast agents and is associated with relatively high costs. Most other techniques are still in the research setting and are not applied in daily clinical practice. Examples are Contrast Enhanced Digital Mammography (CEDM) and Contrast Enhanced Ultrasound (CEUS). Both have advantages: CEDM is relatively cheap and can be used as add-on to existing equipment and CEUS allows for real-time assessment. However, in addition to the limited sensitivity and specificity of the techniques, the requirement of contrast agents and/or ionizing radiation is a major shortcoming for widespread clinical use. Although optical imaging techniques rely on the highest available intrinsic contrast by the absorption of hemoglobin and do not use ionizing radiation, also these techniques suffer from one major disadvantage: the high scattering of

light in biological tissue leads to an unacceptable poor resolution at a few mm depth. A technique that theoretically overcomes this poor resolution, while exploiting the high optical absorption contrast, is photoacoustic (PA) imaging. In PA imaging, the tissue of interest is illuminated by short pulses of laser light, usually in the near infrared (NIR) regime. Upon absorption, the light causes a small temperature increase, which is converted into a pressure wave by the process of thermoelastic expansion. This pressure wave can be detected by ultrasound detectors with the appropriate frequency and bandwidth. The low scattering of ultrasound in tissue overcomes the poor resolution of pure optical techniques. Although PA mammography is theoretically an ideal technique for imaging the breast and first feasibility studies have shown its possibility to visualize breast cancer or healthy vasculature, more clinical data is required to determine PA's potential for use in clinical practice.

The Twente Photoacoustic Mammoscope (PAM) has been developed in the Biomedical Photonic Imaging group (BMPI) of the University of Twente. The system images the breast in forward mode, using 1064 nm light (10 ns, 10 Hz) illumination in combination with a 1 MHz (130% bandwidth) unfocused ultrasound detector array. In **Chapter 4**, PA imaging results from ten patients with malignancies and two patients with benign cysts were described. Due to technical constraints, measurements were performed in a small region of interest (ROI) of $4 \times 4 \text{ cm}^2$ (25 minutes scan duration) and the study focus was on highly suspicious palpable lesions. All ten malignancies could be visualized with high imaging contrast and such high contrast areas were absent in case of cysts. Moreover, in this small patient group, the contrast seemed to be independent of the mammographically estimated breast density. However, the PA estimated lesion size was always smaller than the true, histopathologically defined lesion size and the smooth and homogenous appearance of the PA detected lesions was not expected from MRI and histopathology literature. Part of these shortcomings might have been the consequence of the limited field of view (FOV) measurements. Therefore, an important recommendation at the end of this chapter was to increase the FOV and meanwhile decrease the measurement time before continuation of the clinical studies.

The invisibility of the two cysts described in Chapter 4 was not expected based on the high water content and the expected absorption contrast with the surrounding tissue. In **Chapter 5**, the PA appearance of breast cysts in PAM was investigated. Monte Carlo and K-wave simulations were combined to link the light propagation behavior to the sound propagation, using models that mimic the single-wavelength planar geometry configuration from PAM. Results from those simulations were compared to phantom and patient measurements using an updated version of PAM, which was then able to measure a $9 \times 8 \text{ cm}^2$ scan area within ten minutes scan duration. The homogenous optical and material properties of cysts relative to the surrounding tissue cause them to be visible in the PA imaging volume as one or two high contrast regions representing the front or the front and the back of the cyst. However, using PAM, cysts cannot always unambiguously be

discriminated from (multiple) malignancies. The signature appearance of cysts suggests that they might be differentiable from malignancies in a system using tomographic multi-wavelength imaging.

The homogenous appearance of malignancies observed in Chapter 4, led to the question into what extent the PA appearance and contrast can be related to the tumor vascularity. The answer to this question can help to assess the future potential of PA mammography in malignancy detection and in differentiating benign from malignant abnormalities. In **Chapter 6**, a first comparison between PA images and Magnetic Resonance (MR) images and vascularity stained histopathology slides (using anti-CD31 immunostaining) was made. The three lesion types observed in PA images were reminiscent of enhancement types in MR images. On an individual basis, the PA images also showed good resemblance with the MR images for lesion location, appearance and shape. In 5 of 6 malignancies, the specific PA appearance showed resemblance with the pattern of vascularity from the CD31 stained tumor slides. These results indicate that the PA appearance and contrast is indeed largely the consequence of tumor vascularity, but that the contribution of other chromophores cannot be excluded.

In **Chapter 7**, an overview is given of PA imaging of breast malignancies. PA images from 31 patients having malignancies are compared to results from conventional imaging and to patient demographics and lesion characteristics. The increased FOV ($9 \times 8 \text{ cm}^2$) made it possible to make better comparisons with x-ray and MR images and to measure on a higher variety of lesions. In total, 32 of 33 malignancies were visible with high imaging contrast, which was confirmed by a good co-localization with the lesions in x-ray and/or MR images. In some cases, lesions that were occult in x-ray images could be visualized with high contrast in the PA images. The contrast was independent of the mammographically estimated breast density, while there was (as expected) a significant decrease in contrast for the x-ray lesions in the higher density breast. There were no further significant relations between PA appearance and lesion characteristics for this relatively small patient group, but there was a positive correlation between the PA estimated size and the size from the gold standard histopathology. The study confirms the high potential of PA mammography in breast care. However, in a non-negligible number of patients, high intensity regions were observed that could not be related to the presence of known structures in the breast such as malignancies, cysts or blood vessels. This leads to an expected limited specificity of the current imaging system using a single wavelength.

This thesis shows the potential of PA mammography for the visualization of malignancies. The results also indicate that the current system requires improvements. In **Chapter 8**, the results are critically discussed and recommendations for future research and equipment are given in order to develop a system that is not only able to visualize known malignancies, but also to differentiate them from benign lesions. For development of an imaging system that can fully be exploited in a clinical setting, it is strongly recommended to specify the potential application: screening or diagnosis.

Samenvatting

In dit proefschrift worden verschillende aspecten van fotoakoestische (PA) beeldvorming van borstafwijkingen beschreven. De nadelen van de conventionele beeldvormende technieken en de potentiële oplossing in de vorm van PA mammografie worden in **Hoofdstuk 1** geïntroduceerd. PA mammografie maakt gebruik van het sterke optische absorptiecontrast tussen goed- en kwaadaardig weefsel dat hoofdzakelijk het gevolg is van de sterke toename van hemoglobine in maligne weefsel.

Dit optische absorptiecontrast wordt in **Hoofdstuk 2** geïntroduceerd met behulp van een schilderij van Rembrandt van Rijn: “De badende Bathseba” (1654). In 1983 veronderstelden twee Australische chirurgen, mede op basis van een blauwe verkleuring van Bathseba’s linkerborst, dat Bathseba’s model wellicht aan borstkanker had geleden. Sindsdien is Bathseba een symbool voor borstkanker geworden. Naast de medische en historische vraagtekens bij deze diagnose, ontstond de vraag of het optisch gezien mogelijk is dat een borstkankermassa een dergelijke blauwe verkleuring van de overliggende huid veroorzaakt. Als analogie worden de bloedvaten in de huid omschreven, die ook blauw kunnen lijken. Met behulp van Monte Carlo simulaties en de retinex theorie voor kleurwaarneming is onderzocht of de lokaal toegenomen hoeveelheid hemoglobine in maligne weefsel inderdaad de geobserveerde blauwe verkleuring van Bathseba’s borst kan veroorzaken. De simulaties laten zien dat alleen afwijkingen die ondieper dan 5 mm liggen een subtiele blauwe verkleuring kunnen geven. Het is dus onaannemelijk dat het specifieke uiterlijk van Bathseba’s borst door borstkanker is veroorzaakt. Deze simulaties illustreren nogmaals de invloed van de toegenomen hoeveelheid hemoglobine op de lokale licht-weefsel interacties.

In **Hoofdstuk 3** worden verschillende technieken omschreven die gebruik maken van de tumorvascularisatie voor detectie en diagnose van borstkanker. De gouden standaard in deze discipline is momenteel Dynamic Contrast Enhanced MRI (DCE-MRI), die een hele hoge sensitiviteit heeft voor de afbeelding van maligniteiten. Deze hoge sensitiviteit gaat echter ten koste van de specificiteit en daarnaast zijn contrastmiddelen vereist en is de techniek relatief duur. De meeste andere methoden zijn nog in de onderzoeksfase en worden nog niet algemeen in de dagelijkse klinische praktijk toegepast. Voorbeelden zijn Contrast Enhanced Digital Mammography (CEDM) en Contrast Enhanced Ultrasound (CEUS). Beide technieken hebben voordelen: CEDM is relatief goedkoop en kan als aanvulling op bestaande mammografie-apparatuur worden gezien en CEUS kan worden gebruikt voor real-time beoordeling van het weefsel. Naast de beperkte sensitiviteit en specificiteit is de afhankelijkheid van contrastmiddelen en/of ioniserende straling echter een groot nadeel voor gebruik van deze technieken in de kliniek. Hoewel de optische beeldvormende technieken gebruik maken van het hele hoge intrinsieke absorptiecontrast

van hemoglobine en geen ioniserende straling gebruiken, zijn ook deze technieken onderhevig aan een belangrijk nadeel: de verstrooiing van licht in biologisch weefsel is dusdanig groot dat de resolutie op een paar mm diepte al onacceptabel is. Een techniek die deze slechte resolutie ondervangt is fotoakoestische (PA) mammografie. In PA beeldvorming wordt het weefsel belicht met korte pulsen van, meestal nabij infrarood, laserlicht. De absorptie van het licht leidt tot een kleine temperatuuroptoe, die op zijn beurt een drukgolf veroorzaakt via het proces van thermoelastische expansie. De drukgolf kan worden gedetecteerd met ultrageluidsdetectoren met de geschikte frequentie en bandbreedte. De veel lagere verstrooiing van ultrageluid in weefsel, voorkomt de slechte resolutie waarmee puur optische technieken te maken hebben. Hoewel PA mammografie in theorie een ideale methode is om de borst af te beelden en eerste haalbaarheidsstudies hebben laten zien dat het borstkanker en gezonde bloedvaten kan afbeelden, zijn meer klinische data nodig om de potentie te bepalen.

De Twente Photoacoustic Mammoscope (PAM) is in de Biomedical Photonic Imaging vakgroep van de Universiteit Twente ontwikkeld. Het systeem beeldt de borst af in transmissie (belichting van de craniale kant en detectie aan de caudale kant van de borst) en gebruikt daarbij 1064 nm gepulst licht (10 ns, 10 Hz). De ultrageluidssignalen worden opgevangen met een 1 MHz (130% bandbreedte) ongefocuste ultrasound detector array. In **Hoofdstuk 4** worden de PA resultaten van tien patiënten met maligniteiten en twee patiënten met cystes beschreven. Vanwege technische beperkingen was het meetgebied maar 4x4 cm² (25 minuten scantijd) en daarom lag de focus van deze studie op zeer verdachte palpabele afwijkingen van beperkte grootte. Alle tien maligniteiten konden worden afgebeeld met een hoog beeldcontrast en zulke hoog-contrast gebieden waren afwezig in het geval van cystes. In deze kleine patiëntgroep leek het contrast van de afwijking bovendien onafhankelijk van de mammografisch bepaalde dichtheid van het borstweefsel. De PA geschatte grootte van de afwijking was echter altijd kleiner dan de ware, histopathologisch bepaalde, grootte van de afwijking en de relatief gladde en homogene verschijning van de PA afwijkingen was niet verwacht op grond van MRI en histopathologie literatuur. Deze tekortkomingen konden deels zijn veroorzaakt door het beperkte meetgebied. Een belangrijke aanbeveling aan het einde van dit hoofdstuk was dan ook om het meetgebied te vergroten en tegelijkertijd de scanduur te verkorten alvorens de klinische studie te vervolgen.

De onzichtbaarheid van cystes, zoals omschreven in Hoofdstuk 4, was niet verwacht gezien de hoge waterconcentratie en het verwachte absorptiecontrast met het omliggende weefsel. In **Hoofdstuk 5** is de PA weergave van cystes onderzocht. Met de combinatie van Monte Carlo en K-wave simulaties, kon het lichtgedrag gekoppeld worden aan de ultrageluidspropagatie. Hierbij werden modellen gebruikt die de belichting en detectie van PAM nabootsten. De resultaten van deze simulaties werden vervolgens vergeleken met de resultaten van fantoom- en patiëntmetingen die werden verkregen met een verbeterde versie van PAM. Deze verbeterde versie kon een interessegebied van 9x8 cm² afbeelden in tien

minuten scantijd. De homogene optische en materiaaleigenschappen van cystes ten opzichte van het omliggende weefsel, maken dat deze goedaardige afwijkingen zichtbaar kunnen zijn met PAM als één of twee hoog-contrast gebieden die de voor- of de voor- en achterkant van de cyste representeren. Met deze representaties kunnen cystes echter niet altijd onomstotelijk worden onderscheiden van maligniteiten. De kenmerkende verschijning van cystes suggereert wel dat ze te onderscheiden zijn van maligniteiten als er aanpassingen worden gedaan aan de beeldvormende apparatuur. Tomografie en het meten met meerdere golflengten zijn daarbij van groot belang.

Het onverwachte homogene uiterlijk van maligniteiten in het PA mammogram, zoals omschreven in Hoofdstuk 4, leidde tot de vraag in hoeverre de PA verschijning kan worden gerelateerd aan de tumorvascularisatie. Het antwoord op deze vraag kan ons helpen om de toekomstige potentie van PA mammografie voor detectie en diagnose van maligniteiten verder te bepalen. In **Hoofdstuk 6** is een eerste vergelijking gemaakt tussen PA afbeeldingen enerzijds en MR afbeeldingen en histopathologische coupes waarop een speciale vaatkleuring (anti-CD31) is toegepast, anderzijds. De drie typen afwijkingen die werden waargenomen op de PA afbeeldingen werden herkend van de aankleuringspatronen op MRI. De individuele vergelijking tussen PA afbeeldingen en MR afbeeldingen toonde grote overeenkomsten met betrekking tot de positie van de afwijking en de afwijkingsvorm. Daarnaast kon de PA verschijning worden gerelateerd aan het histopathologische beeld en in 5 van de 6 patiënten ook aan de aankleuring op de CD31 gekleurde tumorcoupes. Deze resultaten laten zien dat het PA uiterlijk en contrast inderdaad met name het gevolg zijn van tumorvascularisatie, maar dat de bijdrage van andere chromoforen niet kan worden uitgesloten.

In **Hoofdstuk 7** wordt een overzicht gegeven van de PA beeldvorming van borstmaligniteiten. De PA resultaten van 31 patiënten met maligniteiten zijn vergeleken met de resultaten van conventionele beeldvorming en met patiënt- en afwijkingskarakteristieken. Het grotere scangebied ($9 \times 8 \text{ cm}^2$) heeft het mogelijk gemaakt om een betere vergelijking met röntgenmammografie en MRI te maken en om een grotere variëteit aan patiënten te includeren. In totaal waren 32 van de 33 maligniteiten zichtbaar met hoog beeldcontrast en werd deze zichtbaarheid bevestigd door een goede co-lokalisatie met de afwijking op röntgen- en/of MRI-beelden. In een aantal gevallen was de afwijking niet zichtbaar op één van conventionele beeldvormende technieken, terwijl het wel met hoog beeldcontrast op het PA mammogram kon worden gezien. Het PA afwijkingscontrast was onafhankelijk van de mammografisch bepaalde dichtheid van het borstweefsel, terwijl er (zoals verwacht) een afname was in het contrast van de afwijking op het röntgenmammogram voor de hogere dichtheid borstweefsel. Er was verder een positieve correlatie tussen de geschatte grootte op PA afbeeldingen en de histopathologisch bepaalde grootte van de tumor. Er waren voor deze relatief kleine groep patiënten geen verdere significante verbanden tussen het PA uiterlijk, het PA contrast en de PA grootte enerzijds en de patiënt- en afwijkingskarakteristieken anderzijds. De resultaten bevestigen de potentie

van PA mammografie in de klinische praktijk. De kanttekening moet worden gemaakt dat in een niet-verwaarloosbaar aantal patiënten hoog-contrast gebieden werden gevonden in het PA beeldvolume die niet konden worden gerelateerd aan de aanwezigheid van bekende structuren in de borst, zoals maligniteiten, cystes of bloedvaten. Dit leidt tot een te verwachte beperkte specificiteit voor het huidige systeem dat maar één golflengte gebruikt.

Dit proefschrift laat de potentie zien van PA mammografie voor de afbeelding van maligniteiten. De resultaten laten echter ook zien dat het huidige systeem moet worden verbeterd. In **Hoofdstuk 8** worden de resultaten kritisch bediscussieerd en worden er aanbevelingen voor toekomstig onderzoek en toekomstige apparatuur gegeven om een systeem te kunnen ontwikkelen dat niet alleen in staat is om bekende maligniteiten af te beelden, maar ook om deze van goedaardige afwijkingen te kunnen onderscheiden. Om een systeem te kunnen ontwikkelen dat volledig tot zijn recht komt in een klinische setting, wordt het sterk aangeraden om de potentiële toepassing te specificeren: screening of diagnose.

Tot slot

In september 2009 kwam ik in BMPI (destijds nog BPE) als PhD kandidaat. Er is veel gebeurd sindsdien. Nu, vier-en-een-half jaar later, mag ik mijn proefschrift in het openbaar verdedigen. Het zijn leerzame jaren geweest, maar vooral jaren waarin ik met enthousiasme en motivatie naar de UT en het MST ging. In dit hoofdstuk wil ik iedereen bedanken die heeft bijgedragen aan de uitbreiding van mijn kennis, het resultaat daarvan in de vorm van dit boekje en het plezier waarmee ik heb gewerkt: bedankt!

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Michelle Poelman-Heijblom,

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About the author

Michelle Heijblom was born on February 24th, 1985 in Hardinxveld-Giessendam, The Netherlands. She received her Bachelor and Master degree in Technical Medicine from the University of Twente, Enschede, The Netherlands in 2006 and 2009 respectively. Her graduation project was performed in the Medisch Spectrum Twente in Enschede, where she started a feasibility study regarding the ‘visualization of tinnitus using EEG and fMRI’. In this project, she strongly collaborated with Pento Audiological Center in Hengelo and the Donders Institute for Neuroimaging in Nijmegen.

From September 2009 to January 2014, she was a Ph.D student in a joined project from the Biomedical Photonic Imaging Group of the University of Twente and the Center for Breast Care of the Medisch Spectrum Twente under the supervision of Prof. Wiendelt Steenbergen, Prof. Ton G. van Leeuwen and Dr. Srirang Manohar. In the work on photoacoustic breast imaging, she was mainly responsible for the clinical part of the project.

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