Genaesthics
Breast Surgery in *BRCA1/2* Gene Mutation Carriers

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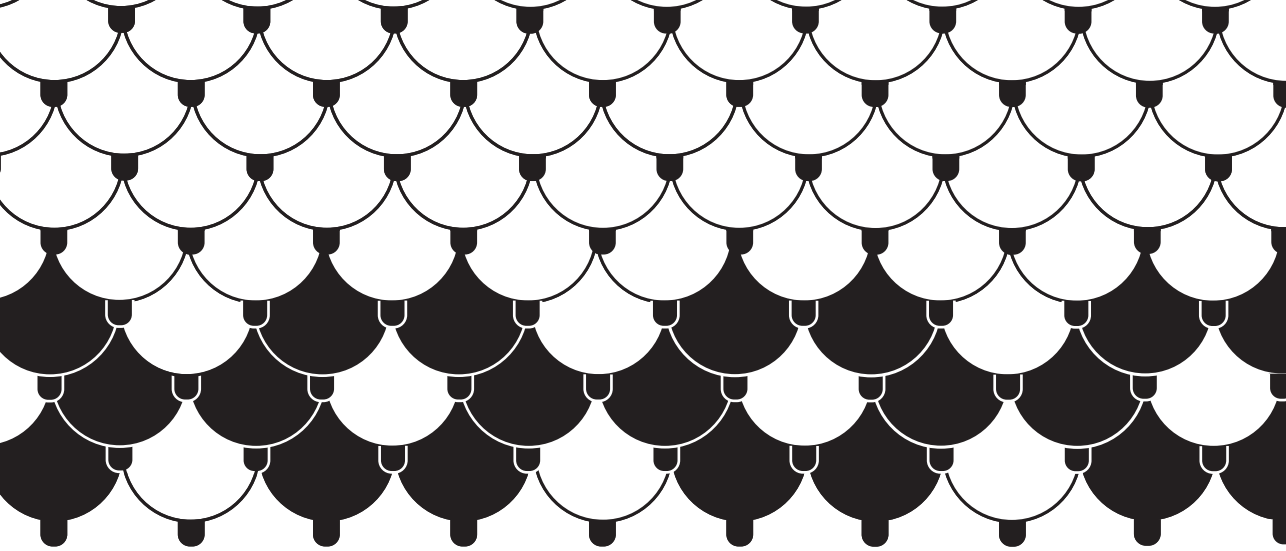
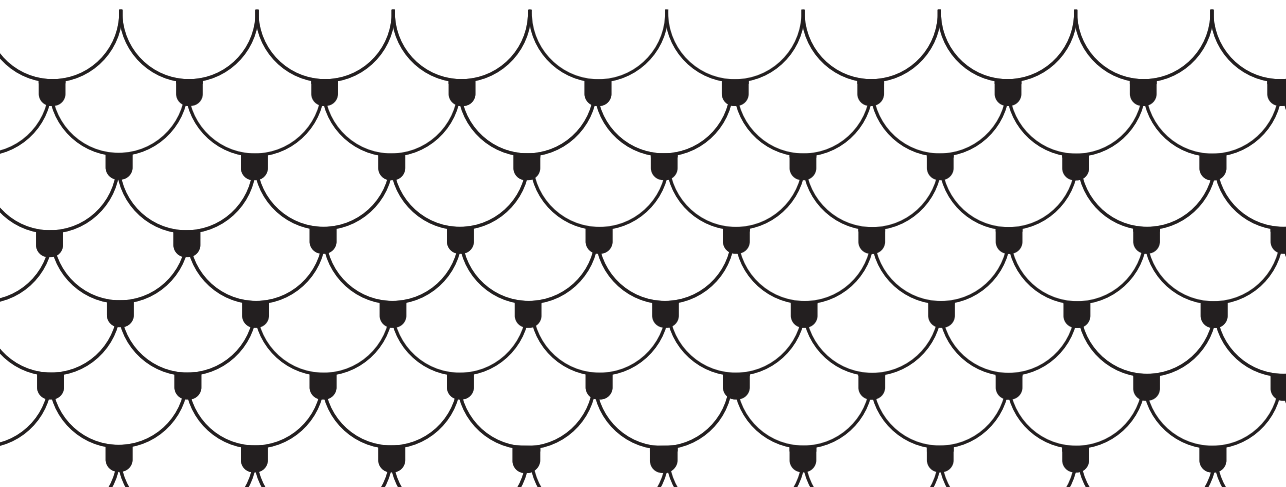
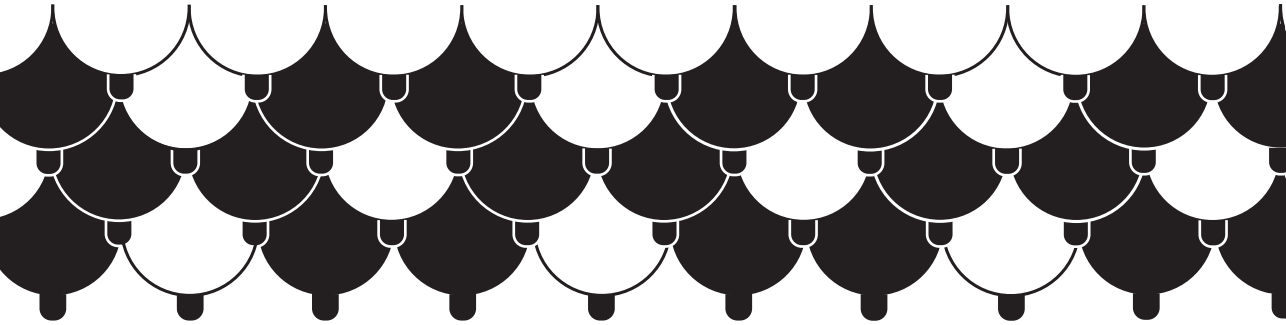
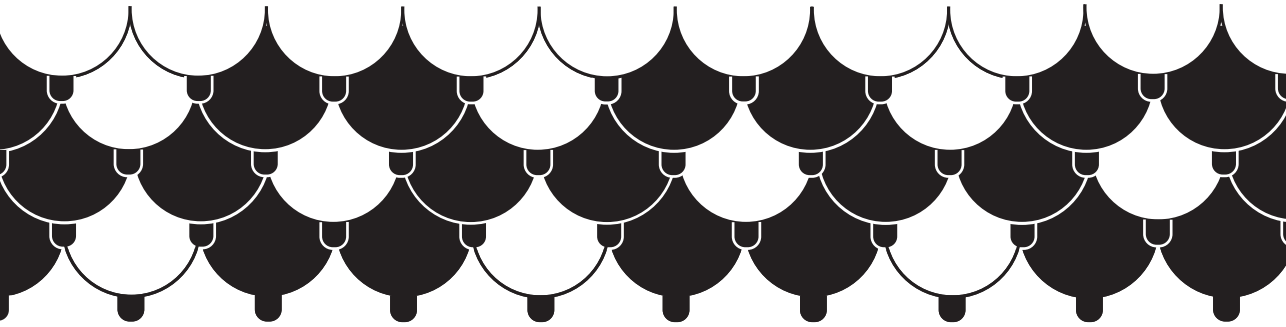
CONTENTS





INTRODUCTION





CHAPTER 1

General introduction and outline of this thesis

The present thesis focuses on breast surgery in *BRCA1/2* gene mutation carriers. The topics that are studied vary broadly, representing the multiple disciplines that are involved in the diagnostic work-up and treatment of *BRCA1/2*-associated breast cancer. The first part contains studies on molecular and prognostic tumor characteristics in breast cancer. The thesis continues with an anatomical study on safety of prophylactic mastectomy, and finishes with studies on aesthetics and patient reported outcomes of prophylactic breast surgery and breast reconstruction. The title of this thesis 'Genaesthics' is a merger of the two extremities of this spectrum: 'genetics' and 'aesthetics'.

HISTORY AND EPIDEMIOLOGY OF GERM LINE *BRCA1/2* GENE MUTATIONS

Already in 1866 the French physician and surgeon Paul Broca suspected the heredity of cancer. A family with an extraordinary high breast cancer incidence, which was supposedly his wife's family, prompted Broca to draw a four-generation pedigree. The pedigree showed an incidence of fifteen out of twenty-six family members with cancer who were thirty years or older at the time of the pedigree drawing. Fourteen of them were women; nine of them had breast cancer whereas the other four had 'abdominal' or 'liver' cancer[1-3]. Finally, in 1990 the evidence for Broca's suspicions was provided by the discovery of the BReast CAncer 1 *(BRCA1)* gene and, in 1994, the *BRCA*2 gene[4, 5]. *BRCA1* and *BRCA2* germ line mutations, as we now estimate, are accountable for about 5% of all breast cancers and for about 16% of hereditary breast cancers[4, 6-8]. Furthermore, about 12% of all ovarian cancers are likely due to a germ line mutation in the *BRCA1/2* genes[9].

As reflected by the composition of many study populations, the incidence of *BRCA1*-associated breast cancers seems to be much higher as compared to *BRCA2*-associated breast cancers in most populations. This is of interest, because the incidence of *BRCA1* and *BRCA2* gene mutations in the entire population is estimated to be equally high, as is the penetrance of both genes[8, 10-14]. An explanation for this finding may be that in some *BRCA2*-associated breast cancers, because of their more sporadic-like phenotype, an underlying *BRCA2* gene mutation remains unrecognized[10]. Further, variations in the pathogenic mutation spectrum in *BRCA1* or *BRCA2* may explain regional differences in penetrance and age of first breast cancer[15]. Generally, cumulative lifetime risks (CLTR) for women who carry a germ line *BRCA1/2* gene mutation are estimated 55-85% to develop breast cancer by the age of 70 [10-14]. The estimated CLTRs of developing ovarian cancer vary between 15-60% for *BRCA1* and 10-35% for *BRCA2* mutation carriers[10-14]. The large variation in estimated CLTRs is partly attributable to differences in study designs[16]. Population-based studies have prospective designs but relatively small sample sizes and low event rates, while family-based studies frequently are of (partly) retrospective design with concurrent recall- and ascertainment biases. A priori, women who present to genetic clinics and who are therefore included in studies have moderate to high cancer

risks that meet criteria to participate in breast cancer screening programs. Moreover, it is very likely that variations in estimated CLTRs are attributable to genetic, environmental and lifestyle factors that modify cancer risks in *BRCA1/2* gene mutation carriers[16].

In the Netherlands, women with high breast cancer risks due to a *BRCA1/2* gene mutation may choose whether they opt for frequent breast cancer screening by biannual alternating mammography and breast MRI, or whether they want to undergo prophylactic surgery.

PATHOLOGIC CHARACTERISTICS OF *BRCA1/2*-ASSOCIATED BREAST CANCER

Compared to the general population, women with a *BRCA1/2* gene mutation are younger, often younger than 40 years, when they develop breast cancer[14] and the breast cancers they develop more frequently have aggressive tumor characteristics[17]. Biannual breast cancer screening consists of alternating mammography and MRI. Due to intensive screening, typically, screen-detected *BRCA1/2*-associated breast cancers are early stage (i.e. small and node-negative) breast cancers[18], but the majority is poorly differentiated (Bloom Richardson grade 3)[17, 19, 20]. *BRCA1*-associated breast cancers are typically basal-like with a triple-negative receptor status (negative for estrogen receptor; ER, progesterone receptor; PR, and HER2 receptor) in up to 70%, whereas *BRCA2*-associated breast cancers resemble more the sporadic breast cancer phenotype and more frequently are ER positive[17, 19, 20]. The relative uniformity of *BRCA1/2*-associated breast cancers in stage and tumor characteristics limits the use of established prognostic markers such as nodal status, grade, and hormone receptor status in the work-up and risk-assessment of *BRCA1/2* gene mutation carriers with breast cancer. Moreover, effective targeted therapy is applicable only in selected cases due to the lack of hormonereceptor and HER2 positivity, especially in *BRCA1*-associated cancers.

THE RISK OF OVARIAN CANCER AND RISK-REDUCING BILATERAL SALPINGO-OOPHORECTOMY

As mentioned before, beside a very high breast cancer risk, *BRCA1/2* gene mutation carriers have up to 60% CLTR of developing ovarian cancer. Despite of numerous attempts to develop efficient screening programs to detect *BRCA1/2*-associated ovarian cancer in an early stage, the safest and most efficient measure to prevent morbidity and especially mortality from ovarian cancer remains to undergo a risk-reducing bilateral salpingo-oophorectomy (RRSO)[21-23]. It is therefore recommended to women with a *BRCA1/2*-gene mutation, especially when they have a family history for ovarian cancer, to undergo RRSO as soon as they have completed childbearing, but preferably around 35-40 years for *BRCA1* gene mutation carriers and 40-45 years for *BRCA2* gene mutation carriers[24, 25].

The biological downside of RRSO includes that the patient enters menopause at young age, with possible adverse physical and psychological effects[26]. A potential benefit of RRSO is that it may have a preventive effect on the development of breast cancer. However, controversies remain around the true extent of risk-reduction as achieved by RRSO and on the mechanism of risk-reduction by estrogen depletion on the development of ER negative breast cancer (as typically associated with *BRCA1*)[27]. Hypothesizing that post-RRSO breast cancers may lack the prognostic adverse characteristics typical for *BRCA1/2*-associated tumors, we study tumor characteristics of breast cancers in *BRCA1/2* gene mutation carriers before and after RRSO in Chapter 3.

POTENTIAL PROGNOSTIC MARKERS IN *BRCA1/2*-ASSOCIATED BREAST CANCERS

There are several tumor characteristics reported to be of possible prognostic value in other types of solid cancer and/or in sporadic breast cancer, which may be of prognostic value in *BRCA1/2*-associated breast cancers as well.

Tumor-associated stroma

In sporadic breast cancers, the amount of tumor-associated stroma has been reported to be a prognostic marker, with stroma-rich breast cancer being an independent predictor of poor prognosis as compared with stroma-poor breast cancer[28, 29]. Stroma surrounding cancer cells is thought to have various functions concerning the genesis and the behavior of tumors. The prognostic role of tumor stroma in *BRCA1/2*-associated tumors, specifically, has not been investigated so far.

Tumor-associated inflammation

A lymphocytic infiltrate surrounding a tumor consists of numerous different inflammatory agents (i.e. B-lymphocytes, T-lymphocytes, macrophages, dendritic cells, plasma cells, mast cells) that display different – possibly anti-tumor as well as pro-tumor – effects[30]. Effects of tumor infiltrate therefore may vary depending on the subset of infiltration and possibly even on the tumor subtype[30]. Infiltration of lymphocytes is especially marked in medullary breast cancers and therefore in *BRCA1*-associated breast cancers[17]. The role of various prognostic markers such as density of tumor-associated inflammation and of tumor-stroma ratio is discussed in Chapter 4.

β-Catenin expression

In colon cancer, activity of the Wnt-pathway plays an important role in tumorigenesis[31]. Mutations in the *APC* gene cause the hereditary cancer syndrome familial adenomatous polyposis (FAP). A concurrent second hit leads to a dysfunctioning APC protein, there-

fore to inappropriate stabilization of cytoplasmic β-catenin and eventually to signaling of the nuclear Wnt target genes[31]. Also in other solid tumors, such as breast cancer, activity of the Wnt pathway may play a role in tumorigenesis[31]. However, the role of Wnt in breast cancer is much less established than in colon cancer[32-36]. In Chapter 6, we assess the presence and prognostic significance of β-catenin expression in *BRCA1/2*-associated breast cancers.

PREVALENCE OF MULTIPLE TUMORS IN *BRCA1/2* GENE MUTATION CARRIERS

Beside high CLTRs of breast and ovarian cancer, *BRCA1/2* gene mutation carriers possibly have a higher susceptibility for other cancer types, as well. This frequently results in the prevalence of multiple tumors that may occur and/or metastasize either synchronously of metachronously. In all forms, it is of utmost importance to differentiate between primary tumor origins, and between primary versus recurrent or metastasized disease, since this strongly determines prognosis and guides surgical and systemic treatment options[37, 38]. On top of diagnostic modalities to determine tumor origins such as tumor morphology and immunohistochemical tests, DNA next generation sequencing (NGS) is an upcoming diagnostic modality that may be helpful in differentiating between tumors and their origins. In Chapter 5, we analyze the additional value of NGS in the diagnostic workup of *BRCA1/2* gene mutation carriers with multiple tumors.

RISK-REDUCING SURGERY: BILATERAL MASTECTOMY

As an alternative to intensive breast cancer screening, *BRCA1/2* gene mutation carriers and other women with high breast cancer risks may choose to undergo risk-reducing bilateral mastectomy mostly followed by direct breast reconstruction. Common procedures are the skin-sparing mastectomy and nipple- (and skin-) sparing mastectomy. In skin-sparing mastectomy, all breast tissue is removed including the nipple-areola complex, leaving a skin envelope that is used to cover a direct breast reconstruction. In nipple-sparing mastectomy, the nipple-areola complex is spared together with the skin envelope. However, since the nipple-areola complex is the center of the breast gland, this may leave more glandular tissue in situ. Although it is imperative to remove as much breast glandular tissue as possible to minimize any residual breast cancer risk, the impact of microscopic amounts of residual breast tissue on breast cancer risk is difficult to estimate. In Chapter 2, we review the literature on oncologic safety of risk-reducing nipple-sparing mastectomy versus skin-sparing mastectomy. In Chapter 7 a pathological study on breast specimens is described, in which we assessed and compared the amount of breast glandular tissue left behind the NAC to the amount of breast glandular

tissue behind the skin flap (left in situ in skin-sparing mastectomy and nipple-sparing mastectomy).

AESTHETIC OUTCOME AFTER PROPHYLACTIC MASTECTOMY

When choosing a prophylactic mastectomy technique, it is necessary to balance any remaining oncological risk versus the expected aesthetic outcome. Nipple-sparing mastectomy may lead to a more natural and thus more desirable aesthetic outcome and possibly increases patient satisfaction[39-41]. In skin-sparing mastectomy the nipple-areola complex needs to be reconstructed. Current techniques of nipple-areola complex reconstruction consist of a small skin flap and specialized tattoo techniques. When performed well, this frequently leads to aesthetic outcomes that can hardly be distinguished from a native nipple-areola complex. Nipple-areola complex reconstruction may even have a superior aesthetic outcome since it can be delayed until the final breast shape is achieved and shape and place on the breast therefore can be adjusted to it. Patient satisfaction with nipple-sparing mastectomy and skin-sparing mastectomy and the function of the nipple-areola complex (either spared or reconstructed) after risk-reducing bilateral mastectomy were assessed and compared in Chapter 9. Another point of interest is the reconstruction modality used after prophylactic mastectomy. Autologous reconstruction techniques yield natural aesthetic outcomes but require more extensive surgery with concurrent morbidity[42]. Implant reconstruction may be preceded by tissue expansion using an inflatable tissue expander placed beneath the pectoral muscle. Direct-to-implant breast reconstruction is performed immediately after mastectomy without previous tissue expansion. The definitive prosthesis is placed partly subpectoral and sometimes partly covered with a pedicled latissimus dorsi muscle. In Chapter 10, the long-term results of quality of life and aesthetic outcome after risk-reducing mastectomy and direct-to-implant breast reconstruction are described. An expert panel assessed photographs of postoperative breast reconstruction results and patients were asked to complete the Breast Q reconstruction questionnaire. Chapter 8 describes a validation study of the Hopwood Body Image Scale (BIS) for the Dutch Language. The BIS is a 10-item scale suitable for daily practice to better assess patient satisfaction and body image as a quality of life parameter after cancer surgery. The BIS was concurrently used in Chapter 9.

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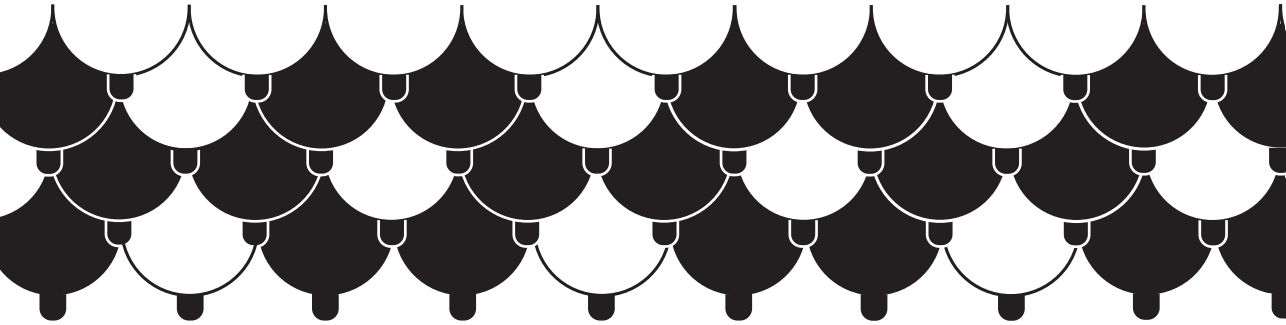

CHAPTER 2

Oncological safety of prophylactic breast surgery: skin-sparing and nipple-sparing versus total mastectomy

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ABSTRACT

Women with a *BRCA1/2* gene mutation and others with a high breast cancer risk may opt for bilateral prophylactic mastectomy. To allow for immediate breast reconstruction the skin envelope is left in situ with or without the nipple-areola complex (NAC). Although possibly leading to a more natural aesthetic outcome than the conventional total mastectomy, so-called skin-sparing mastectomies (SSM) and nipple-sparing mastectomies (NSM) may leave some breast glandular tissue in situ. The oncological risk associated with remaining breast glandular tissue is unclear. We present a case of primary breast cancer after prophylactic mastectomy followed by a review of the literature on remaining breast glandular tissue after various mastectomy techniques and oncological safety of prophylactic mastectomies.

INTRODUCTION

BRCA1/2 mutation carriers have a cumulative lifetime breast cancer risk of 55-85% by the age of 70[1-5]. As an alternative to surveillance, *BRCA1* and *BRCA2* mutation carriers and other women with a high breast cancer risk may choose to undergo bilateral prophylactic mastectomy, reducing breast cancer risks by 90-100% after 3-13 years of follow-up[6-10]. The prophylactic character of the bilateral mastectomy emphasizes the importance of a natural aesthetic outcome[11], which can be achieved by various immediate autologous and implant breast reconstruction techniques. Instead of the conventional total mastectomy, to allow for an immediate breast reconstruction and to achieve a natural aesthetic outcome so-called conservative mastectomies are increasingly performed for risk reduction. In conservative mastectomies, all breast glandular tissue is removed while leaving the skin envelope and, if spared, the nipple-areola complex (NAC) in situ (respectively, skin-sparing mastectomy; SSM and nipple-sparing mastectomy; NSM).

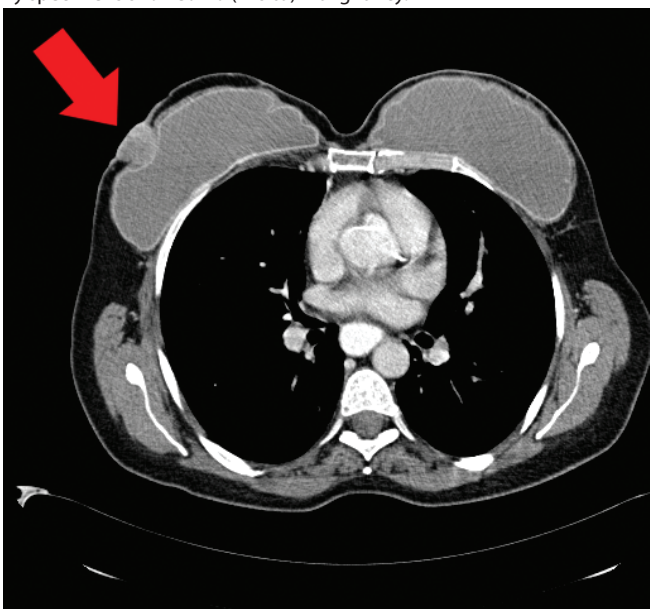
Safety of conservative mastectomies in women at high breast cancer risk is subject to an ongoing debate. The presumed oncological risk of the conservative technique lies in potential remaining breast glandular tissue with the skin flap and, if spared, with the NAC. Smaller incisions that are tailored to individual reconstruction wishes, however, may result in a technically difficult surgical approach. Therefore, the oncological safety of the conservative mastectomy remains a challenge for the surgeon. We present a case of primary breast cancer developed after prophylactic conservative mastectomy. Further, we provide a review of the literature on the oncological safety of prophylactic conservative mastectomies.

CASE: A 43-YEAR OLD WOMAN WITH PRIMARY BREAST CANCER IN THE PROPHYLACTIC MASTECTOMY SCAR

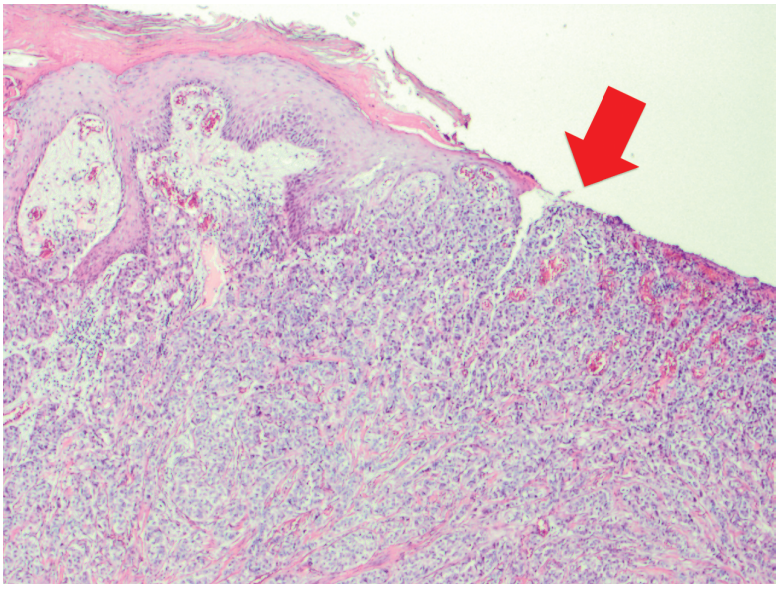
In 2011 a 43-year old woman presented a lesion clinically suspicious of breast cancer. In 1982 at the age of 15, she had been successfully treated for stage IIa Hodgkin's disease in her neck and mediastinum with 40 Gy mantle field radiation. After 10 years there were no signs of recurrence and she was discharged from follow-up.

In 1998, a mammography - performed because of a wish for breast reduction - revealed suspect microcalcifications in the left breast. The suspect lesion was excised by upper outer quadrantectomy. Pathological examination of the lumpectomy specimen showed grade 2 ductal carcinoma in situ. No adjuvant radiotherapy was administered due to the history of mantle field radiation. Initially, physicians and patient agreed to frequent radiological screening instead of a completing mastectomy. However, after several additional diagnostic procedures due to suspect lesions of the left breast, in 2001, the patient chose to undergo a SSM and immediate implant reconstruction. In

Figure 1. 43-year old woman presented with a primary, ulcerous breast cancer in the right prophylactic mastectomy scar. Eight years before presentation she had undergone prophylactic mastectomy and immediate breast implant reconstruction because of a history of Mantle field radiation at the age of 15. Histology of the mastectomy specimens showed no (in situ) malignancy.



A Computer-assisted Tomography (CT) scan of the thorax shows the tumor of 2.1 x 2.7 cm that invades the skin and causes dimpling of the subpectoral implant.



B Microscopic examination showed a grade 3 invasive ductal carcinoma with skin involvement, indicated by the arrowhead. Haematoxylin and eosin stained (H&E); 4x objective.

2003, this was followed by a prophylactic SSM of the right breast and bilateral implant reconstruction. In both cases, histologic investigation showed no (in situ) malignancy.

In 2011, she returned with an ulcerous lesion in the right mastectomy scar. On CT-scan a superficial tumor of 21 x 27 mm was seen (Figure 1A). Ultrasonography of the axilla did not show pathological lymph nodes. A wide local excision with axillary lymph node dissection was performed and the implants were removed. Histological examination of the excised specimen showed an invasive ductal carcinoma with a diameter of 2.4 cm, Bloom Richardson grade 3, estrogen receptor (ER) positive, progesterone receptor (PR) and human epithelial growth factor-2 receptor (HER2 receptor) negative (Figure 1B). Adjacent to the tumor, normal glandular breast tissue was found. One out of eight dissected axillary nodes showed a metastasis. According to our national protocol, she received adjuvant chemotherapy, hormonal therapy and re-irradiation with hyperthermia of the chest wall. At the time of writing the patient is alive without breast cancer recurrence.

SURGICAL TECHNIQUES OF CONSERVATIVE MASTECTOMIES: SKIN-SPARING MASTECTOMY (SSM) AND NIPPLE-SPARING MASTECTOMY (NSM)

Examples of conservative mastectomies include skin-sparing mastectomy (SSM) and nipple-sparing mastectomy (NSM). In SSM, a periareolar incision is used with caudal or lateral extension if necessary ('racquet' incision). The skin envelope is created by subcutaneously excising the breast glandular tissue while preserving a thin subcutaneous layer to support skin vascularization. Nipple-papilla and surrounding pigmented areola (nipple-areola complex; NAC) are removed. In NSM, the skin envelope is created through a semicircular periareolar or an inframammary incision. The NAC is dissected as thin as possible by macroscopically removing all breast glandular tissue while preserving vascularization. The nipple-papilla is 'cored' by inverting it and excising residual breast glandular tissue. The NAC is then left in situ adherent to the skin envelope. A breast reconstruction is performed during the same procedure. The oncological safety of SSM in the prophylactic setting is generally acknowledged, whereas safety of NSM is still subject to debate.

In the last two decades of the past century it was common to perform a so-called subcutaneous mastectomy. Although subcutaneous mastectomy encompassed a skin- and nipple-sparing technique as well, it is likely that this was not comparable to current NSM and SSM techniques. A description of the 'state of the art' subcutaneous mastectomy in 1983 mentions that a plaque of one centimeter of breast glandular tissue should be left in situ with the areola[12]. In contrast, current NSM and SSM techniques aim for skin flaps <5 mm and NACs of 2-3 mm thickness[13].

BREAST GLANDULAR TISSUE OR TERMINAL DUCT LOBULAR UNITS (TDLUS): RESIDUALS AFTER MASTECTOMY

The hazard of remaining breast glandular tissue after mastectomy for development or recurrence of breast cancer has been a recurring subject to debate since more than half of a century. Anatomically the NAC is a continuation of the mammary gland and therefore should be removed when pursuing a complete mastectomy. Therefore, especially sparing of nipple and areola in NSM has been a controversial topic. However, the growing ability of more specifically identifying women at high breast cancer risk and the consequently increasing interest in prophylactic mastectomies has revived the discussion. Breast cancer is thought to originate in terminal duct lobular units (TDLUs), defined as a terminal duct combined with an associated lobule[14-16]. Consequently, theoretically any remaining TDLUs may represent a lifelong potential breast cancer hazard. To estimate the remaining risk after prophylactic mastectomy, some authors have studied whether TDLUs are left in situ. Several others have simply examined the presence of remaining ductal or lobular structures or more non-specifically the presence of glandular tissue.

Residual breast glandular tissue after total mastectomies

The first study to investigate the amount of glandular tissue left in situ after a conventional total mastectomy was already in 1940 by Hicken et al.[17] The authors had been triggered by two cases of women who developed breast cancer and mastitis of residual axillary breast tissue 15 and 10 years, respectively, after an ipsilateral mastectomy for a benign indication. Mammographies of 385 breasts using intraductal contrast showed that mammary ducts frequently extend beyond regular mastectomy resection planes. In 95%, mammary ducts extended into the axillary fossa, in 15% downward into the epigastric region, in 2% beyond the lateral limits of the latissimus dorsi muscle and in two cases even past the midsternal line to the contralateral side[17]. A histological analysis of17 total mastectomies was performed in the same study by preoperatively injecting methylene blue dye into the ducts of the nipple-papilla. Any resection plane that colored blue during surgery meant that ducts had been cut and the resection site was defined as 'irradical'[17]. Results showed that breast glandular tissue had been excised irradically underneath the skin flap in 94% of cases, in 12% the axillary tail had been removed irradically, in 23% the ducts had been cut in the sternal region and in 11% in the epigastric region[17]. The authors therefore concluded that, even when it is intended to perform a total mastectomy, it is seldom accomplished[17].

In 1991, a small study was performed in 10 total mastectomies in five women[18]. Frozen sections of skin flaps, pectoral muscle and axillary tail were examined. Similar to the results of Hicken, residual breast glandular tissue was found in caudal skin flaps, the axillary tail and even in the pectoral fascia[18]. Another small study separately resected specimens specifically of the inframammary fold (IMF) and encountered small amounts

of residual breast tissue in 13/24 IMF specimens (with breast glandular tissue volume / IMF specimen volume rates of 0.04%)[19].

In 2013, Griepsma et al. studied the superficial dissection planes of 206 – mostly total – mastectomy specimens[20]. Per mastectomy 36 biopsies were obtained from standardized locations of the subcutaneously dissected part of the total mastectomy specimens. In 76% of mastectomies, one or more biopsies contained breast glandular tissue at the resection plane. Areas of predilection were the lower outer quadrant (15% positive biopsies) and halfway the subcutaneous dissection plane between the peripheral pectoral muscle margin and central skin margin (12% positive biopsies)[20].

Residual breast glandular tissue after conservative mastectomy: SSM and NSM

Three decades after the first report on total mastectomies by Hicken et al, Goldman and Goldwyn picked up on the issue of conservative prophylactic mastectomy by performing 12 subcutaneous (skin- and nipple-sparing) mastectomies in six cadavers through an inframammary incision[21]. Biopsies of post-mastectomy skin flaps, resection planes and any fibrous or adipose tissue remaining elsewhere showed residual breast glandular tissue after 83% of mastectomies[21]. In all cases even, residual breast glandular tissue was found behind the spared NAC. However, the authors do not describe which biopsy sites were positive for breast glandular tissue, nor the surgical technique used for dissection of the NAC[21].

Aiming to investigate the potential value of NSM in the treatment of lobular carcinoma in situ (LCIS), Rosen and Tench[22] vertically sectioned 101 nipples in conventional mastectomies performed for breast cancer. In 17% of the nipples lobules were found and in 13% (in situ) carcinoma was encountered. The authors propose that 'coring' of the nipple-papilla in NSM, which had been described before[23], is necessary to remove as much glandular tissue as possible. The NAC was further examined in 1993[24]. By inverting the projected center of the NAC - the nipple-papilla - and grossly removing all glandular tissue inside the papilla, the nipple was cored. Despite nipple-coring the authors did encounter mammary ducts in the areolar dermis[24].

In 1991, Barton et al. compared 27 conservative mastectomies with 28 modified radical mastectomies[25]. Post-mastectomy biopsies were taken at the inframammary fold, parasternal region, infraclavicular chest wall, latissimus dorsi muscle border, anterior lower axilla and skin flaps. The NAC was not examined. No differences were found between the number of biopsies containing residual breast glandular tissue after conservative mastectomy (22%) and after total mastectomy (21%)[25]. After conservative mastectomy, most positive biopsies (50%) originated in the skin flap. In contrary, after total mastectomy, most positive biopsies (38%) originated at the latissimus dorsi border[25].

The skin flap after conservative mastectomy was further examined in 1998[26]. The authors removed 114 small (0.5 x 2.0 cm) strips of skin from the remaining skin flap in 32

patients for complete histological examination. In none of the strips ductal breast tissue was encountered[26], however, regarding the size of the strips, this negative finding may be due to a sampling error. Somewhat larger skin flaps have been examined in a more recent study[27]. In 66 SSMs, skin specimens that had been removed additionally to the SSM specimen to facilitate reconstruction were examined for residual glandular tissue. Skin specimens had a mean volume of 93.9 cm^3 and in specimens of only four patients (6%) residual breast tissue was found[27]. However, since only a minimum of 3 sites per skin specimen was analyzed, again in this study a sampling error cannot be ruled out. A study of 168 SSMs for therapeutic indication analyzed the superficial margin to the dermis just above the tumor that would have been left in situ otherwise. In contrast with the two studies described above, in 89 (53%) of the cases benign breast ducts were present in the superficial margin specimen[28].

Residual terminal duct lobular units (TDLUs) after conservative mastectomy: SSM and NSM

Several studies have more specifically studied whether TDLUs remain after SSM or NSM [22, 29-31]. The only study on SSM was by Torresan et al in 2005[32]. In 42 total mastectomies, they resected the skin flap that would have been left in situ if it were a SSM and submitted 80 slides per skin specimen for examination. In contrary to the two studies mentioned earlier, they found TDLUs in 60% of the skin flaps[32]. The risk of finding TDLUs strongly increased for skin flaps thicker than 5 mm[32].

The other five studies focus on NSM. Stolier et al. examined the nipple-papilla for presence of TDLUs in 2008[29]. During mastectomies, 32 nipple-papilla's were transected at the junction of papilla and areola. Nipple-papilla's were sectioned, entirely embedded and examined microscopically for presence of TDLUs. Only in three out of 32 nipple-papilla TDLUs were found. Therefore, it was concluded that TDLUs are scarce in the nipple-papilla[29]. Reynolds et al. collected 62 mastectomy specimens from 33 *BRCA1/2* mutation carriers and excised the NAC for histologic evaluation[30]. In 24% of the NACs, TDLUs were found; only 8% was located in the papilla[30]. Similarly, Kryvenko et al. studied 105 NACs from mastectomy specimens[31]. Sixty-five NACs were entirely embedded for examination of presence of TDLUs; of 40 NACs only one vertical section was examined. TDLUs were found in 26% of NACs but most frequently were located in the papilla[31] - in contrast to the results of Reynolds and Stolier[29, 30]. It has been suggested that an areola-sparing mastectomy rather than a NAC-sparing mastectomy should be performed for risk reduction. Removing the nipple-papilla might reduce any remaining breast cancer risk. However, this is not supported by the abovementioned studies since two of the three show a higher incidence of TDLUs in the areola versus the nipple-papilla. Recently, our own group compared presence and numbers of TDLUs between skin flap and NAC [33]. In 105 total mastectomies, the NAC and an adjacent skin-island were dissected as if an

NSM was performed, and the papilla was cored. TDLUs were found in 61% of the NACs vs. 24% of the skin islands[33]. Also after adjustment for volume of the excised specimens, density of TDLUs was significantly higher in the NACs as compared with the skin. Further, risk factors for presence of TDLUs were younger age and parity (vs. nulliparity)[33]. We concluded that NACs, as well as skin flaps might harbor a risk for developing breast cancer, albeit very small.

ONCOLOGICAL SAFETY OF PROPHYLACTIC MASTECTOMY: CLINICAL STUDIES

In addition to the histopathological studies, we assessed whether there are any oncological consequences of the residual glandular tissue. We performed a systematic PubMed search using the term 'prophylactic mastectomy [Title/Abstract] OR skin-sparing mastectomy [Title/Abstract] OR nipple-sparing mastectomy [Title/Abstract] OR subcutaneous mastectomy [Title/Abstract] OR conservative mastectomy [Title/Abstract] OR risk-reducing mastectomy [Title/Abstract] AND breast cancer [Title/Abstract], yielding 680 titles. Titles and abstracts were checked for relevance. Reviews and case reports were excluded, as were articles that were not in English. Also excluded were: studies that focused (1) on merely therapeutic mastectomy and/or comprised <20 prophylactic mastectomies and/or did not report clinical follow-up outcome of prophylactic mastectomies; (2) on survival benefits of contralateral prophylactic mastectomy or oophorectomy and (3) on uptake, counseling and decision-making of prophylactic surgery.

Twenty-four studies from 1976-2014 met our criteria and are summarized in Table 1. All are observational studies describing prospective or retrospective cohorts or a case-control series. In 24 studies, 7,173 mastectomies are described of which 1,392 were for therapeutic indications and which were not considered in further analysis. Most prophylactic mastectomies were performed in *BRCA1/2* gene mutation carriers and other women at high breast cancer risk. Average follow-up periods range from 10.4-168 months. Most recent studies focus on NSM rather than SSM, while in older studies conservative mastectomies are defined as 'subcutaneous mastectomy', suggesting that the NAC is – partly – spared. However, as described above, it is likely that in subcutaneous mastectomy the NAC and skin are not dissected as thin as modern NSM or SSM techniques dictate.

As reported by the 24 studies in Table 1, grossly, 21 primary breast cancers occurred after 6,044 prophylactic mastectomies. Of these, three occurred after a total mastectomy (0.6% of all total mastectomies), 17 occurred after a conservative mastectomy (0.3% of all subcutaneous mastectomies, NSM or SSM) and for one breast cancer the prophylactic mastectomy technique was not specified. Besides, four patients presented with distant metastases with unknown primary site. Most prophylactic mastectomies included in these studies, as well as the ones in which a primary breast cancer developed, were

Table 1. Primary breast cancers after prophylactic total mastectomy, nipple-sparing and skin-sparing mastectomies: an overview of studies

1st author	Year	Study population	Mastectomies	Therapeutic mastectomy *Not taken into account*	Prophylactic mastectomy (PM)		
					Conservative PM		Total PM
					SSM	NSM	
			n	*n*	*n*	*n*	*n*
de Alcantara Filho	2011	125 unknown 36 *BRCA1/2+* 39 non-*BRCA1/2*	353	157	-	196	-
Arver	2011	129 *BRCA1/2+* 94 non-*BRCA1/2* or unknown	446	-	100	338[1]	8
Colwell	2014	285 patients	482	222	-	260	-
Contant	2002	63 *BRCA1+* 13 *BRCA2+* 36 non-*BRCA1/2* or unknown	207	-	193	-	14
Evans	2009	202 *BRCA1/2+* 348 non-*BRCA1/2* >25% BC risk	864	-	346[2]		200[2]
Garcia-Etienne	2009	25 patients	42	7	-	34	-
Hagen	2014	267 *BRCA1/2+* (104 history of BC)	449	-	166[2]	49[2]	5[2]
Harness	2011	6 *BRCA1/2+* 37 non-*BRCA1/2* or unknown	60	40	-	20	-
Hartmann	1999 and 2001	26 *BRCA1/2+* 150 non-*BRCA1/2* high risk 38 not tested 425 moderate risk	1278	-	1146[3]		132
Heemskerk-Gerritsen	2013	156 *BRCA1+* 56 *BRCA2+*	424	-	384	40	-
Jensen	2010	99 patients	149	99	-	50	-
Kaas	2010	179 *BRCA1+* 75 *BRCA2+*	401	-	NR (Majority)	NR	NR
Meijers-Heijboer	2001	64 *BRCA1+* 12 *BRCA2+*	152	-	148	-	4
Munhoz	2013	158 patients, genetic status unknown	233	114	-	119	-
Peled	2014	53 *BRCA1/2+* 53 non-*BRCA1/2*	212	108	-	104	-

	Follow-up after PM	Primary BC after PM	Distant metastases after PM	Location primary breast cancer after PM
	Months (range)	*n (% of all PM)*		
	10.4 (0–109)	0	-	0
	79.2 (25.2-168.0)	0	1 metastatic disease 9 yrs after PM (0.2%)	Distant metastases
	26.0 (10.8-71.0)	0	-	N/A
	30.0 (12.0-70.8)	0	-	N/A
	73.2 (range NR)	0	-	N/A
	10.5 (0.4-56.4)	0	-	N/A
	35 (3-336)	**1** primary BC 6.6 yrs after subcutaneous PM (0.2%)	-	NR
	18.5 (6-62)	0	-	N/A
	168 (range NR)	**6** primary BC: 2, 3, 5, 6, 15 and 25 yrs after subcutaneous PM (0.5%)	1 metastatic disease 12 yrs after subcutaneous PM	2 yrs: chest wall 3 yrs: lateral side chest wall 5 yrs: left breast 'above areola' 6 yrs: left nipple 15 yrs: left breast 25 yrs: chest wall
	75.6 (1.2-208.8)	0	1 metastatic disease 3.5 yrs after prophylactic SSM (0.1%)	N/A
	60.2 (12–144)	0	-	N/A
	Bilateral: 63.5 Unilateral: 65.0 (ranges NR)	**1** primary BC 2 yrs after PM (history of contralateral BC) (0.2%)	-	Axillary tail which was incompletely removed
	33.6 (1.2-68.4)	0	-	N/A
	65.6 (6-130)	0	-	N/A
	51 (8.3-132.8)	0	-	N/A

Table 1. Primary breast cancers after prophylactic total mastectomy, nipple-sparing and skin-sparing mastectomies: an overview of studies (continued)

1st author	Year	Study population	Mastectomies	Therapeutic mastectomy *Not taken into account*	Prophylactic mastectomy (PM)		
					Conservative PM		Total PM
					SSM	NSM	
			n	*n*	*n*	*n*	*n*
de la Peña-Salcedo	2011	52 patients	64	-	-	42	22
Pennisi	1976	1244 patients	NR	64[2]	1180[2,3]		-
Rebbeck	2004	105 *BRCA1*/2+	210	-	58[3,4]		100[5]
Sacchini	2006	3 *BRCA1*/2+ 1 non-*BRCA1*/2 119 unknown	219	68	-	151	-
Skytte	2011	67 *BRCA1*+ 29 *BRCA2*+	192	-	NR	NR	NR
Spear	2011	22 *BRCA1*/2+ 79 non-*BRCA1*/2 or unknown	162	49	-	113	-
Wagner	2012	7 *BRCA1*/2+ 3 *BRCA1*/2- 23 unknown 33 patients	54	17	-	37	-
Warren Peled	2012	19 *BRCA1*/2+ 411 non-*BRCA1*/2 or unknown	657	412	-	245	-
Wijayanayagam	2008	43 patients; partly *BRCA1*/2+	75	35	-	29	11
Total numbers of primary BC after conservative and total PM:					5,548 conservative PM 496 total PM		

(6-9, 13, 34-52)

BC, breast cancer; PM, prophylactic mastectomy; SSM, skin-sparing mastectomy; NSM, nipple-sparing mastectomy; NR, not reported; N/A, not applicable;

BRCA1/2+, female *BRCA1/2* gene mutation carrier; BCT, breast conserving therapy

[1] 202 of 338 NSM concerned SSM with retransplantation of the nipple but removal of the areola

[2] Women with unilateral and bilateral mastectomies; exact numbers of mastectomies not reported and are analyzed as one mastectomy per woman

[3] Conservative mastectomy = subcutaneous mastectomy

[4] Of 26 patients (52 mastectomies) mastectomy techniques were unknown

	Follow-up after PM	Primary BC after PM	Distant metastases after PM	Location primary breast cancer after PM
	Months (range)	*n (% of all PM)*		
	144 (range NR)	0	-	N/A
	84 (range NR)	**6** primary BC after subcutaneous mastectomy	-	NR
	66 (0-373)	**2** BC 2 and 9 yrs after subcutaneous mastectomy in *BRCA2+* and *BRCA1+*, respectively (1.0%)	-	1 in axillary lymph node, 1 in residual right breast tissue (exact location NR)
	24.6 (2.1-570.4)	**2** BC 2 and 5 yrs after prophylactic NSM (1.3%)	-	1 in the axillary tail 1 in the upper-outer quadrant
	47.3 (range NR)	**3** BC in *BRCA1+*; 2, 5 and 7 yrs after total mastectomy (1.6%)	-	2 at the thoracic wall, 1 in the axilla (lymph node metastasis or ectopic breast tissue)
	43 (5-246)	0	1 metastatic disease of unknown primary after 9 yrs (0.9%)	N/A
	15.0 (1–29)	0	-	N/A
	28	0	-	N/A
	NR	0	-	N/A
		17 BC after conservative PM (0.3%) **3** BC after total PM (0.6%) **1** BC after unknown PM technique		

subcutaneous mastectomies, NSM or SSM. Nonetheless, the majority of primary breast cancers did not originate near the NAC or skin flap. Of the 21 breast cancers that developed after prophylactic mastectomy, five were encountered at the chest wall, four in the axilla, (two in the axillary tail, one in an axillary lymph node, one in an unknown location), one in the outer quadrant, one in the nipple and one 'above the areola' (not further specified). In nine cases the location was unclear or not reported.

The 21 loco-regional primary breast cancers correspond with an incidence of 0.7% per woman who undergoes bilateral prophylactic mastectomy (0.35% per mastectomy). Most breast cancers that developed after conservative mastectomy were found at the chest wall or in the axilla. Although the chest wall and the axilla may be at risk in total mastectomy as well, two things should be considered: First, the origin of the breast cancer may have been the skin flap, even though it was described as 'chest wall'. Most breast implants in immediate breast reconstruction are placed underneath the pectoral muscle. Consequently, skin-flap and chest wall are in direct contact. Therefore, although we have no information on the reconstruction techniques used in these studies, it is possible that the breast cancers developing at the chest wall actually did originate in the skin flap. Second, as mentioned before, the surgical technique of SSM and NSM using small peri-areolar or inframammary incisions can be challenging. A suboptimal exposure may impede thorough removal of remaining breast glandular tissue in all quadrants and in the axillary tail.

In four cases, breast cancer presented as metastatic disease and the primary tumor site was never found. Pathological findings specific for breast cancers, the high a priori breast cancer risk of the patient and elimination of other potential first sites because of negative radiological examinations may all have led to the conclusion that the metastatic disease most probably originated from breast cancer. The possibility that the primary tumor already may have been present in the prophylactic mastectomy specimen emphasizes the importance of standardized pathological examination of the excised specimen, and – even more – thorough radiological screening by MRI before prophylactic mastectomy.

In conclusion, the incidence of primary breast cancers after prophylactic mastectomy is very low after total as well as after conservative mastectomies. However, theoretically, according to these data, approximately one out of 140 women undergoing bilateral prophylactic mastectomy for breast cancer prevention will develop a primary breast cancer over time. Oncological surgeons should be aware of this risk and may minimize it by putting extra care in dissecting all glandular tissue, especially in the axillary tail and chest wall, and by dissecting skin flaps and NAC as thin as possible. More studies are warranted that further assess long-term oncological safety. Further, it is important to more specifically study patient satisfaction after NSM and SSM and potential differences

in patient expectations. Ultimately, surgeons and patients may be able to balance any remaining oncological risk against expected benefits of NSM or SSM.

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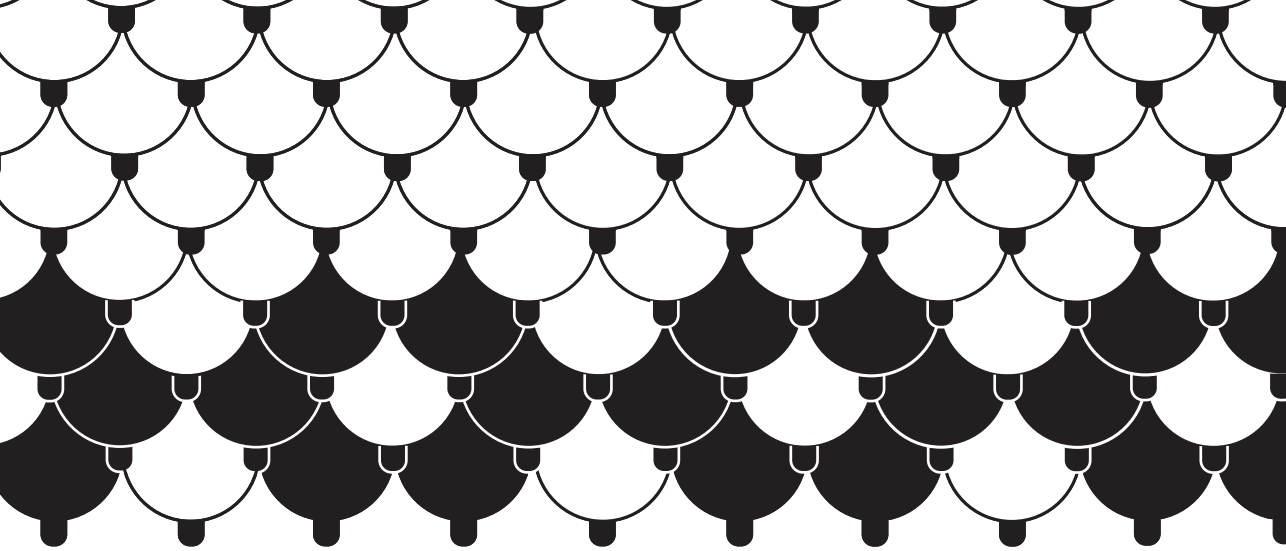
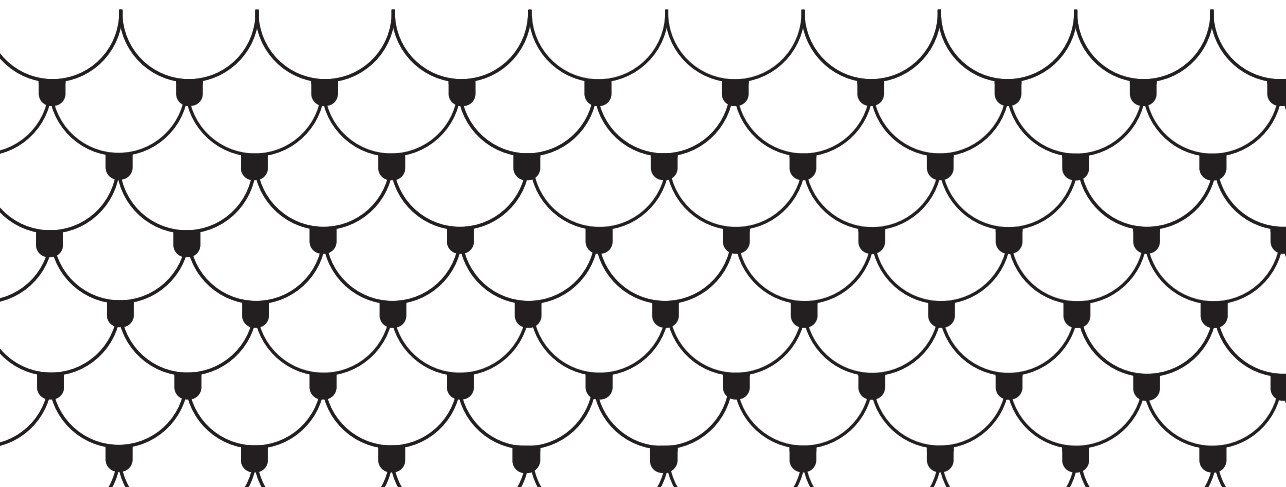
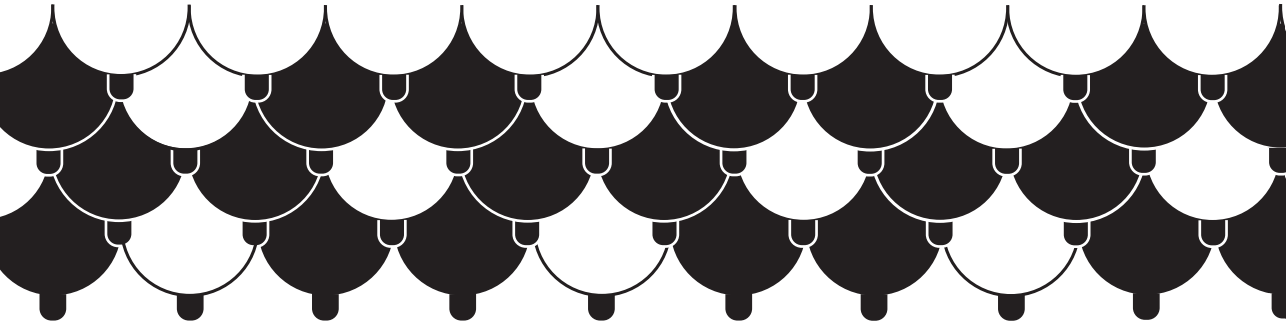




PATHOLOGY OF *BRCA1/2*-ASSOCIATED BREAST CARCINOMAS



CHAPTER 3

Lower mitotic activity in *BRCA1/2*-associated primary breast cancers occurring after risk-reducing salpingo-oophorectomy

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ABSTRACT

Risk-reducing salpingo-oophorectomy (RRSO) is associated with 50% reduction of *BRCA1/2*-associated breast cancer (BC) risk, possibly through decreased growth activity. In this pilot study, tumor characteristics and growth rates of *BRCA1/2*-associated primary BCs (PBCs) detected after RRSO were compared with those of PBCs originating without RRSO. From a cohort of 271 women with *BRCA1/2*-associated screen detected BC, we selected 20 patients with PBC detected ≥12 months after RRSO (RRSO group). Controls were 36 *BRCA1/2* mutation carriers with PBC detected without RRSO (non-RRSO group) matched for age at diagnosis (±2.5 years) and for *BRCA1* or *BRCA2* mutation. Pathology samples were revised for histological subtype, tumor differentiation grade, mitotic activity index (MAI), estrogen receptor (ER), progesterone receptor (PR) and HER2 status. Tumor growth rates, expressed as tumor volume doubling times (DT), were calculated from revised magnetic resonance and mammographic images. Median age at PBC diagnosis was 52 years (range 35-67). PBCs after RRSO had lower MAIs (12 versus 22 mitotic counts/2mm, P=0.02), were smaller (11 versus 17 mm, P=0.01), and tend to be PR-positive more often than PBCs without RRSO (38% versus 13%, P=0.07). Differentiation grade, ER and HER2 status were not different. Median DT was 124 days (range 89-193) in the RRSO group and 93 days (range 54-253) in the non-RRSO group (P=0.47). BC occurring after RRSO in *BRCA* mutation carriers features a lower MAI, suggesting a less aggressive biological phenotype. When confirmed in larger series, this may have consequences for BC screening protocols after RRSO.

BACKGROUND

BRCA1/2 mutation carriers face increased lifetime risks by the age of 70 years of developing breast cancer (BC; 55-85%), contralateral breast cancer (CBC; 20-60%) and ovarian cancer (18-54% for *BRCA1* and 3-23% for *BRCA2* mutation carriers)[1-5]. In view of the increased ovarian cancer risk, and the unavailability of an adequate screening tool, the majority of *BRCA1/2* mutation carriers opt for risk-reducing salpingo-oophorectomy (RRSO), mostly before 50 years of age[6, 7]. RRSO significantly reduces the risk of ovarian/fallopian tube cancer by more than 95%[6, 8, 9], while it is also associated with a primary BC (PBC) risk-reduction of about 50%, being most pronounced when performed at premenopausal age[7, 10].

BRCA1/2-associated BCs are often diagnosed at young age and are more often poorly differentiated than sporadic BCs (grade 3 in 50-75% versus 35%, respectively)[11, 12]. The *BRCA1* BC phenotype is mainly estrogen receptor (ER) and progesterone receptor (PR) negative, and does not express HER2, resulting in approximately 60% of the BCs being triple negative.[12] The *BRCA2* BC phenotype is quite similar to sporadic BCs regarding ER, PR and HER2 status.[12] Furthermore, shorter tumor volume doubling times (DT), expressing faster tumor growth, have been described for both *BRCA1*- and *BRCA2*-associated tumors as compared to non-*BRCA1/2*-associated tumors in patients of similar age.[13] At increasing age, *BRCA1/2*-associated tumors have longer DTs,[13] a more favorable differentiation grade and are more often ER positive possibly due to changes in ovarian hormone production[12, 14, 15]. In view of the mentioned observations and the reduced BC risk after RRSO, we hypothesized that PBCs developing after RRSO-induced menopause might show altered characteristics and decreased tumor growth. The latter is also an observation at our institute, although an earlier study on tumor growth did not find a correlation of menopausal status with tumor growth.[13] To our knowledge, no detailed data are available on this topic. The finding of a lowered growth rate of BCs occurring after RRSO might have consequences for BC screening protocols for the subgroup of *BRCA1/2* mutation carriers who underwent RRSO.

We performed a pilot study in a matched cohort of *BRCA* mutation carriers, and compared tumor characteristics and tumor growth rates of PBCs developing after RRSO with PBCs originating without RRSO.

METHODS

Patients

Since the start of the Rotterdam Family Cancer Clinic (FCC, approximately 1991), women at increased risk of hereditary breast and/or ovarian cancer are prospectively followed. From this cohort, we identified *BRCA1/2* mutation carriers with a PBC detected at least

12 months after RRSO (RRSO group, cases). Patients were matched for age at PBC (+/- 2.5 years) and type of mutation (*BRCA1* or *BRCA2*), with an intended ratio of 1:2, to obligate or proven *BRCA1/2* mutation carriers with a PBC developing without RRSO (non-RRSO group, controls) (Figure 1).

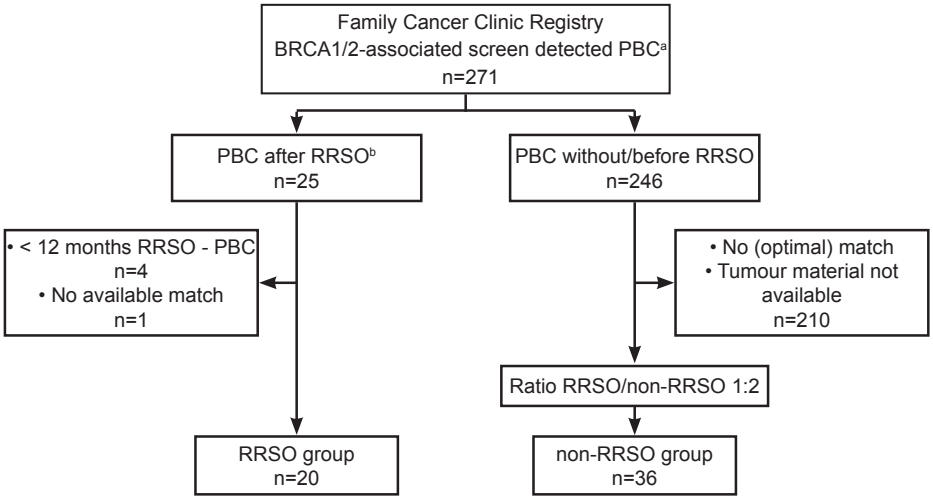

Figure 1. Patient inclusion from the Rotterdam Family Cancer Clinic
A PBC: primary breast cancer
B RRSO: risk-reducing salpingo-oophorectomy

Further eligibility criteria included (a) PBC detected at screening or presenting as interval carcinoma between two screening examinations (previous examination within 2 years before diagnosis), and (b) availability of tumor material for pathology revision. Exclusion criteria were risk-reducing mastectomy prior to PBC, neoadjuvant chemotherapy and/or a history of ovarian cancer. Detailed data on hormonal status and reproductive factors including menarche, number of pregnancies and childbearing, breast feeding, use of oral contraceptives and age at RRSO and/or menopause were collected from medical records.

Written informed consent was obtained according to research protocols approved by the Medical Ethical Committee.

Radiological tumor measurements and growth rate assessment

Radiological images of serial screening examinations (magnetic resonance imaging (MRI) or mammography; previous and at PBC diagnosis) of selected patients were collected. Eligibility criteria for this research question included (a) invasive carcinoma and (b) the availability of at least two well interpretable imaging examinations of the same

screening modality (MRI, preferably, or mammography), one made at diagnosis and one within two years prior to PBC diagnosis.

Images were revised by a breast radiologist (I.O.) being unaware of RRSO status of the patients, regarding visibility of the lesion and perpendicular tumor diameters. If the tumor was visible on ≥ 2 comparable, consecutive examinations, the first and the last examination were used for tumor volume calculations. If the tumor was clearly visible on MRI, three perpendicular tumor diameters were measured. On mammography, three perpendicular diameters were measured if possible, but if only two diameters could be measured, the smaller of the two diameters was used as third diameter. Tumor volumes were calculated by using a formula for obloid spheroids: $V = \frac{4}{3}\pi \cdot \frac{1}{2}a \cdot \frac{1}{2}b \cdot \frac{1}{2}c$ [13, 16]. Because of the assumed exponential growth pattern of small tumors,[17] an exponential formula was used to calculate tumor volume doubling time: $DT = \frac{\ln 2}{\beta}$ with β being the slope of the straight line between the logarithms of the tumor volumes versus time[13, 16]. If the tumor was only visible on MRI or mammography at diagnosis, the tumor volume of the preceding examination was set corresponding with the assumed lower detection limit of that imaging examination, being 2 mm for MRI, corresponding with a volume of 0.004 cm^3, and 4 mm for mammography, corresponding with a volume of 0.033 cm^3 [13].

Histological tumor characteristics

Pathology slides were revised by a breast pathologist (C.v.D.) unaware of the RRSO status of patients. Items scored concerned: tumor subtype according to the World Health Organization classification, grade according to the modified Bloom & Richardson score (based on tubule formation, nuclear pleomorphism and Mitotic Activity Index (MAI)),[18] and ER, PR and HER2 status. For categorization of MAI, thresholds of the modified Bloom & Richardson grade were used resulting in three categories (low 0-7 mitoses/2 mm^2, moderate 8-12 mitoses/2 mm^2 and high ≥13 mitoses/2 mm^2)[19]. For ER and PR, histoscores (H-scores) were calculated as the sum of the percentages of immunoreactive staining of tumor cells, multiplied by ordinal values corresponding to the intensity levels of the staining: *H-score (0-300) = % weakly immunoreactive cells x 1 + % moderately immunoreactive cells x 2 + % intensely immunoreactive cells x 3.* An H-score of ≥10 was considered positive, since 10% of immunoreactive staining of tumor cells, independent of intensity, is the cut-off point for ER/PR positivity according to Dutch national guidelines[19]. Patients with carcinoma in situ without an invasive component were also included. Data on tumor size and nodal status were obtained from the database and/or pathology reports.

Statistical analysis

Differences between the RRSO and non-RRSO groups were tested by using Chi-square and Fisher's exact tests for categorical variables, and by using Mann-Whitney U tests for

continuous variables. The SPSS computer package (version 20.0) was used for statistical analyses.

RESULTS

From a cohort of 271 patients with screen detected *BRCA1/2*-associated BC retrieved from the FCC database 21 female *BRCA1/2* mutation carriers were identified with a PBC detected at least 12 months after RRSO. One woman with BC after RRSO was excluded from further analysis because no match was available (Figure 1). Of 246 proven or obligate mutation carriers with BC without RRSO, 36 appropriate matches (including two obligate mutation carriers) were found for the non-RRSO group (Figure 1). For four RRSO women only one appropriate match was found.

Patient characteristics and demographics are listed in Table 1. As year of diagnosis was not a matching criterion, median year of PBC diagnosis in the RRSO group was 2009 vs. 2001 in the non-RRSO group (P=0.001). RRSO was performed at a median age of 50 years, and four women (20%) were postmenopausal at RRSO. Nine women (45%) of this group had used hormone replacement therapy (HRT) between RRSO and PBC diagnosis. In the non-RRSO group, 18 women (50%) were postmenopausal at PBC diagnosis, none of them having used HRT. More PBCs were detected by MRI in the RRSO group (14 out of 20, 70% by MRI) than in the non-RRSO-group (8 out of 36, 22% by MRI, P=0.001) as compared to mammography (Table 1). Both groups were comparable regarding age at PBC (due to matching), parity and other hormonal factors.

Radiological tumor measurements and growth analysis of invasive carcinomas

Tumor volume doubling times (DTs) of invasive BCs, as an expression of tumor growth rate, could be calculated for 12 of 17 tumors (71%) in the RRSO group and for 18 of 34 tumors (53%) in the non-RRSO group (Table 2). In total, 13 tumors (43%) were only visible on the imaging examination at diagnosis (5 on MRI, 8 on mammography), concerning 10 patients of the non-RRSO group. Twelve tumors (40%) were on revision visible on two consecutive examinations, and 5 tumors (17%) were visible on three or more consecutive examinations performed over a time period of 0.5-3.5 years (4 in the RRSO group, 1 in the non-RRSO group). Median DT of the PBCs was 124 days (IQR 89-193) in the RRSO group and 93 days (IQR 54-253) in the non-RRSO group (P=0.47) (Figure 2 and Table 2).

Table 1. Patient characteristics

	RRSO [a] group		Non-RRSO group		P value
N	20		36		
BRCA1	17	85%	31	86%	
BRCA2	3	15%	5	14%	1.0
Age PBC[b] (median, IQR [c]), years	52.0	46.0-62.5	50.0	46.0-57.0	0.44
Year PBC diagnosis (median, IQR)	2009	2005-2011	2001	1996-2006	0.001
Screening method that detected PBC					
MRI	14	70%	8	22%	
Mammography	6	30%	28	78%	0.001
Menopausal status at PBC					
Pre-	0	0%	17	49%	
Post-	20	100%	18	51%	
Unknown	0		1		
Age RRSO (median, IQR), years	50.0	41.0-55.0	-		
Menopausal status at RRSO					
Pre-	14	78%	-		
Post	4	22%	-		
Unknown	2		-		
Months RRSO-PBC (median, IQR)	19.0	14.5-118.0	-		
Hormone replacement therapy (HRT)					
Yes	8	45%	0	0	0.003
No	12	55%	18	100%	
Age menarche (median, IQR), years	14.0	12.5-15.5	14.0	12.0-14.0	0.93
Oral contraceptive use					
Yes	15	88%	29	97%	0.54
No	2	12%	1	3%	
Unknown	3		6		
Years of oral contraceptive use (median, IQR)	18.0	9.5-22.0	9.0	4.0-18.0	0.42
Parity (mean, standard deviation)	1.9	1.0	1.6	1.1	0.43
Nulliparity					
Yes	3	15%	8	26%	0.49
No	17	85%	23	74%	
Unknown	0		5		
Age at 1st child (median, IQR), years	26.0	21.5-28.0	25.5	24.0-30.0	0.61
Breastfeeding					
Yes	6	40%	17	57%	0.35
No	9	60%	13	43%	
Unknown	5		6		
Months breastfeeding (median, IQR)	4.0	0.5-9.5	6.0	3.0-8.0	0.51

[a] RRSO: risk-reducing salpingo-oophorectomy

[b] PBC: primary breast cancer

[c] IQR: interquartile range

Hormone replacement therapy, after RRSO (RRSO group) or menopause (non-RRSO group)

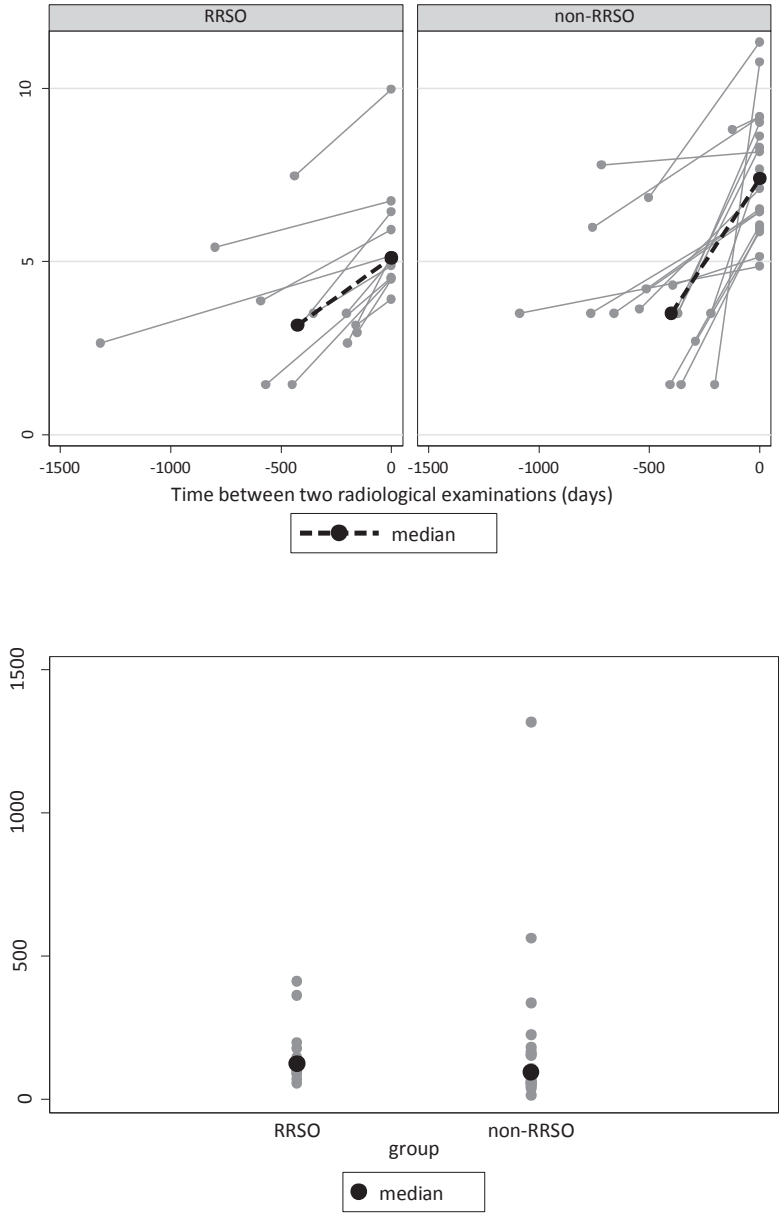

Figure 2. **A** Tumor volumes over time and **B** tumor volume doubling times (DTs) for primary breast cancers occurring after risk-reducing salpingo-oophorectomy (RRSO) and without RRSO (non-RRSO)
ln: natural logarithm
Tumor volumes: V= 4/3π*1/2a*1/2b*1/2c; a, b and c are perpendicular tumor diameters on MRI or mammography
Tumor volume doubling time (DT): DT= (ln2)/β; β= slope between natural logarithms of tumor volumes

Table 2. Radiological tumor growth analysis of invasive carcinomas

	RRSO[a] group		Non-RRSO group		P value
Invasive carcinomas (n)	17		34		
Eligible for growth rate analysis	12	71%	18	53%	
BRCA1	11		17		
MRI screening	9	82%	7	41%	0.05†
Mammography screening	2	18%	10	59%	
BRCA2	1		1		
MRI screening	1	100%	0	-	
Mammography screening	0	-	1	100%	
Tumor on revision visible on					
1 examination[b]	3	25%	10	56%	0.164†
2 examinations	5	42%	7	39%	
≥3 examinations	4	33%	1	5%	
Time between two screening examinations (median, IQR[c]), days	344	243-433	397	286-676	0.212¶
Time between examinations used for tumor growth calculation (median, IQR), days	427	201-586	400	341-676	0.719¶
Tumor volume doubling time (DT) (median, IQR), days	124	89-193	93	54-253	0.472¶

All percentages are of invasive carcinomas
[a]RRSO: risk-reducing salpingo-oophorectomy
[b]Tumor growth rate is calculated combined with one baseline examination with no visible tumor (baseline tumor volume 0.004cm (MRI) or 0.033 cm3 (mammography))
[c]IQR: interquartile range
†Fisher's Exact Test
¶Mann-Whitney U Test

Histological tumor characteristics

Tumor characteristics are presented in Table 3. The RRSO group comprised three cases of DCIS (two *BRCA1*, one *BRCA2*) and 17 invasive PBCs (15 *BRCA1*, two *BRCA2*), concerning 15 ductal carcinomas, one lobular and one metaplastic carcinoma. The non-RRSO group comprised two cases of DCIS (one *BRCA1*, one *BRCA2*) and 34 invasive ductal carcinomas (30 *BRCA1*, four *BRCA2*), including one with metaplastic characteristics. Median tumor size of the invasive PBCs was 10.0 mm (interquartile range (IQR) 6.5-16.0) in the RRSO group, versus 17.0 mm (IQR 10.0-25.0) in the non-RRSO group (P=0.01). The majority of invasive PBCs in both groups was node negative (15/17 in the RRSO group and 25/34 in the non-RRSO group, P=0.30).

Table 3. Histological tumor characteristics

	RRSO[a] group		Non-RRSO group		P-value
N	20		36		
DCIS[b]	3	15%	2	6%	0.34†
Invasive carcinoma	17	85%	34	94%	
T status*					
T1a/b	9	53%	10	30%	0.16‡
T1c	6	35%	13	38%	
T2	2	12%	11	32%	
Size, mm (median, IQR[c])*	11.0	6.0-17.0	17.0	10.0-25.0	0.01¶
N status*					
Negative	15	88%	27	74%	0.29†
Positive	2	12%	9	26%	
Tubule formation*					
> 75%	0		1	3%	0.74‡
10-75%	2	12%	3	9%	
< 10%	14	88%	28	88%	
Unknown	1		2		
Nuclear pleomorphism*					
Minimal	0		0		
Moderate	6	38%	8	25%	0.50†
Extensive	10	62%	24	75%	
Unknown	1		2		
Mitotic count /2mm^2 (median, IQR)*	12	1-20	22	14-29	0.02¶
Mitotic Activity Index* (mitoses/2mm^2)					
0-7	6	38%	6	19%	0.008‡
8-12	3	19%	0		
≥13	7	43%	26	81%	
Unknown	1		2		
Bloom & Richardson grade*					
1	1	6%	0		0.16‡
2	6	38%	7	22%	
3	9	56%	25	78%	
Unknown	1		2		
Lymphovascular invasion*					
Yes	1	7%	6	19%	0.40‡
No	14	93%	26	81%	
Unknown	2		2		
ER[d] H-score[e] (median, IQR)*	0	0-270	1	0-41	0.63¶
Positive (H-score ≥10)	7	47%	9	29%	0.33†
Negative (H-score <10)	8	53%	22	71%	
Unknown	2		3		
PR[f] H-score (median, IQR)*	3	0-150	0.0	0-1	0.05¶
Positive (H-score ≥10)	6	38%	4	13%	0.07‡
Negative (H-score <10)	10	62%	27	87%	
Unknown	1		3		

Table 3. Histological tumor characteristics (continued)

	RRSO[a] group		Non-RRSO group		P-value
HER2 status*					
Positive	1	6%	0		0.36†
Negative	15	94%	28	100%	
Unknown	1		6		
Triple-negative[g] *					
Yes	7	47%	19	68%	0.21†
No	8	53%	9	32%	
Unknown	2		6		

[a] RRSO: risk-reducing salpingo-oophorectomy
[b] DCIS: ductal carcinoma in situ
[c] IQR: interquartile range
[d] ER: estrogen receptor
[e] H-score: histoscore (0-300) = % weakly immunoreactive cells x 1 + % moderately immunoreactive cells x 2 + % intensely immunoreactive cells x 3
[f] PR: progesterone receptor
[g] Triple-negative: negative status for ER, PR and HER2
*Invasive carcinomas only
†Fisher's Exact Test
‡Chi-square Test
¶Mann-Whitney U Test

MAI of the PBCs was significantly lower in the RRSO group than in the non-RRSO group, with a median of 12 mitoses/2mm^2 (IQR 1-20) and 22 mitoses/2mm^2 (IQR 14-28.5), respectively (P=0.02). No differences were found in the amount of tubule formations, nuclear pleomorphism, overall Bloom & Richardson grade, ER status or HER2 status. The proportion of PR positive PBCs (PR H-score ≥10) was higher in the RRSO group than in the non-RRSO group (38% vs. 13%) without reaching statistical significance (P=0.07), while median PR H-score was significantly higher in the RRSO than in the non-RRSO group (3 vs. 0, P=0.05). As a consequence, the percentage of triple negative PBCs was lower in the RRSO group than in the non-RRSO group (47% vs. 68%) without reaching statistical significance (P=0.21).

DISCUSSION

In this pilot study in an age-matched cohort consisting of *BRCA1/2*-associated BC patients, PBCs occurring after RRSO were featured by significantly lower mitotic counts, a trend for more PR positivity, and (non-significantly) more often ER positivity as compared to PBCs without RRSO. Tumor volume doubling time (DT) was non-significantly longer in the RRSO group. To our knowledge, this is the first report comparing tumor characteristics and growth patterns of PBCs occurring after RRSO to those without RRSO.

The significantly lower mitotic count in PBCs occurring after RRSO as compared to PBCs without RRSO suggests that estrogen depletion induced by RRSO decreases cell proliferation. As the majority of PBCs in this study was ER negative, the mechanism behind this observation remains unclear. Various authors confirm that the development of *BRCA1*-associated triple negative BCs is susceptible to estrogen depletion or inhibition as achieved by RRSO or tamoxifen[20-22]. It has been hypothesized that the explanation lies in high ER expression of early stages of triple negative BC genesis[23, 24]. Estrogens may facilitate *BRCA1*-mutant cell proliferation and tumor development in premalignant mammary tissue until ER expression extinguishes in later stages, possibly after the loss of transcriptional ER-activation by the second *BRCA1* allele[25]. By this mechanism, estrogen depletion by RRSO may inhibit tumor development of triple negative BC in a very early stage. Furthermore, there is some evidence suggesting that in a later stage of tumor development estrogen may induce changes even in ER-negative BCs by affecting the microenvironment of the tumor[26].

Interestingly, a recent study found that also RRSO performed after natural menopause was associated with BC risk-reduction. The authors suggest that androgens, being produced by the ovaries after menopause, may affect cell proliferation either directly or indirectly through the aromatization to estrogens[27], and possibly play a role in the risk-reduction of hormone receptor negative breast cancer.

As 85% of the study patients were *BRCA1* mutation carriers, our findings are majorly driven by *BRCA1*. *BRCA1*-associated BCs are known to have higher mitotic counts than *BRCA2*-associated and sporadic BCs[28], possibly because proteins associated with normally functioning *BRCA1* genes inhibit cell proliferation[29, 30]. Separate analyses of *BRCA1* carriers alone revealed comparable results as for the overall group (data not shown). To our knowledge, reduced cell proliferation in *BRCA1*-associated BCs after RRSO or menopause has not been described so far. Although tubule formation and nuclear pleomorphism, two other components of the Bloom & Richardson grade scoring system, and overall differentiation grade were not significantly different in PBCs after versus without RRSO, there is evidence that MAI is the most important prognostic factor in early stage BCs[31, 32]. The finding of lower MAI in PBCs after RRSO therefore suggests a less aggressive biological growth pattern of this subgroup.

Still, 43% of the tumors in the RRSO group had high mitotic counts (≥13 mitoses/2 mm^2). An explanation may be that the time period of 12 months between RRSO and BC diagnosis considered in this study was relatively short, and that some tumors already had developed before RRSO. Of interest, PBCs with a high MAI were detected at a median of 24 months after RRSO, while this was 69 months for tumors with a lower MAI (0-12 mitoses/2 mm^2; data not shown). This supports previous data suggesting that the maximum level of risk reduction by RRSO is effective more than 12 months post-RRSO, although some risk reducing effect is already present one year after RRSO.[7]

We observed a trend for more PR positivity in the RRSO group, but without significant difference in ER status (Table 3). Consequently, fewer tumors in the RRSO group (47%) were triple negative, as compared to 68% in the non-RRSO group. The latter percentage is in accordance with data from the literature for *BRCA1*-associated BC[12], and mirrors the fact that the majority of our patients were *BRCA1* mutation carriers. Earlier studies found that the proportion of ER and PR positive tumors in *BRCA1*-associated BC increases with increasing age at diagnosis, but is still lower than the percentage of ER-positivity in sporadic tumors irrespective of age[12, 14, 15]. In these studies however, menopausal status and history of RRSO were not taken into account. As patients in the current study were matched for age, the increased expression of PR in PBCs in the RRSO group, in our opinion, suggests transcriptional activation by ER and therefore can be a sign of increasing ER-functionality[33-35]. Therefore, in a larger series we expect not only increase of PR positivity, but also of ER positivity in PBCs after RRSO. To our knowledge, only one study reported on BC characteristics after RRSO[36], but due to a different study design and patient cohort, the outcomes of both studies are not comparable.

Tumor size at surgery as reported in pathology reports was significantly smaller in the RRSO group than in the non-RRSO group. This is most likely a reflection of the differences in screening regimens between the two groups. In the RRSO group all women knew their *BRCA* mutation status prior to RRSO and consequently were screened by means of annual MRI and mammography, according to Dutch guidelines. The non-RRSO group was more heterogeneous with respect to radiological screening, since 19 of the 36 women had not been genetically tested until PBC diagnosis. First, time intervals between screening examinations were longer in the non-RRSO group. Second, due to our matching criteria and evolving approaches over time regarding RRSO, year of diagnosis ranged from 1999-2012 in the RRSO group as compared to 1987-2011 in the non-RRSO group with consequently varying quality of radiological screening examinations. These differences between the two groups probably resulted in earlier detection and in smaller tumor sizes at diagnosis in the RRSO group (Table 2). In smaller tumors, mitotic counts may be lower, as has been reported for screen-detected sporadic BCs[37]. Therefore, the reduced mitotic activity we found in BCs developing after RRSO may partly have been a consequence of the smaller tumor size in this group.

Median tumor volume DT was longer in the RRSO group than in the non-RRSO group, but this difference was not statistically significant. As pointed out before, in the RRSO group women were more often screened with MRI, resulting in more precise tumor volume assessment. Because of the better imaging quality of MRI over mammography and of digital mammography in recent years as compared to previous analogue mammography, tumors in the RRSO-group may have been longer visible in retrospect, resulting in lower DTs, suggesting slower growth. Further, in both groups large (interquartile) ranges for DTs were found (Figure 2), suggesting that the formula used for DT was imprecise.

Possibly the assumptions of presumed obloid tumor shape and the exponential growth are a too simplified approach of real tumor volume and growth. In combination with small groups, this might be the reason no statistical significant DT difference was found.

Unfortunately, due to the small numbers it was not possible to take menopausal status and HRT use into account regarding differences in histological characteristics and tumor volume DT. Of the non-RRSO group, 51% was naturally postmenopausal at PBC diagnosis and growth in these tumors may already have been restrained due to declined or absent production of ovarian hormones. Moreover, 45% of women in the RRSO group used HRT before PBC. Based on our hypothesis of tumor growth stimulation by estrogens, we expect that the differences between the groups in MAI, ER and PR status and DT will increase when comparing HRT-naïve patients in the RRSO group with premenopausal PBC patients in the non-RRSO group.

Strikingly, in five patients the tumor was visible on three or more screening examinations over a time period of 0.5-3.5 years before BC diagnosis. All women were *BRCA1* mutation carriers and screened by MRI, while four of them had undergone RRSO. In all five cases the lesion was noticed earlier, but classified as "probably benign", while additional ultrasonography showed no signs of malignancy. Our observations support the fact that radiologists must be aware that in *BRCA1* mutation carriers, and especially after RRSO, BC can present during screening as small benign looking lesions.

An important strength of our pilot study concerns the matched design, chosen to adjust for age at PBC and type of mutation (*BRCA1* or *BRCA2*). Furthermore, pathology samples were revised by a breast pathologist, and all imaging examinations were revised by a breast radiologist, both unaware of RRSO status and therefore not biased regarding results.

However, we are aware of some relevant limitations. First, only 20 patients were eligible for the RRSO group due to the relatively low number of PBCs detected after RRSO. Women who consult our cancer center nowadays are encouraged to undergo RRSO as of 40 years of age, and some women already have suffered from BC by that time. Strict inclusion criteria and the matched design restricted further enlargement of the non-RRSO group. While some women in the RRSO group were relatively old at the time of PBC diagnosis (> 60 years), only few *BRCA* mutation carriers were identified with a first BC occurring at older age without prior RRSO. Groups were too small to perform multivariable analysis to correct for other variables possibly of influence on tumor biology, such as HRT use, menopausal status and tumor size at detection. Second, the group consisted mostly of *BRCA1* mutation carriers, as this is most frequently seen in the Netherlands. The number of *BRCA2* mutation carriers was too small to perform a subgroup analysis.

In conclusion, the lower MAI and the increased proportion of PR positive *BRCA1/2*-associated BCs developing after RRSO suggest a less aggressive biological phenotype compared with PBCs occurring without RRSO. This was not confirmed by significantly

longer DTs, probably due to small numbers. Our findings in *BRCA1/2*-associated PBCs occurring after RRSO are the first of this kind, but confirmation is warranted in larger sample sizes, since these findings may have consequences for less intensive breast cancer screening protocols after RRSO in mutation carriers, with possibly less outpatient clinic visits, less distress for the patient, and lower costs.

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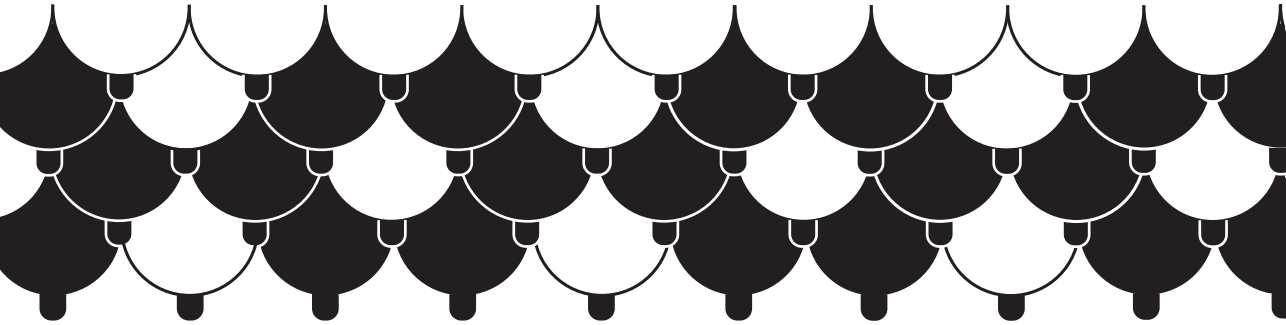

CHAPTER 4

Tumor-associated inflammation as a potential prognostic tool in *BRCA1/2*-associated breast cancer

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ABSTRACT

The prognosis of *BRCA1/2*-associated breast cancer partly depends on histologic characteristics. The majority of these breast cancers, however, is poorly differentiated. *BRCA1*-associated cancers are mainly negative for estrogen (ER), progesterone (PR) and HER2 receptor. Consequently, the use of these histologic features for risk stratification in *BRCA1/2*-breast cancer is limited. We assessed the prognostic value of additional histologic features, including tumor-associated inflammation and tumor-associated stroma in *BRCA1/2*-breast cancer patients. From the Rotterdam Family Cancer Clinic database we collected demographics, tumor characteristics and follow-up data from female *BRCA1/2*-breast cancer patients. Tumor samples were centrally reviewed including histologic subtype, differentiation grade, tumor-associated inflammation density, amount of tumor-associated stroma and intra-tumor necrosis. The impact of these factors on recurrence-free survival (RFS) was evaluated using uni- and multivariable Cox regression, adjusted for established prognostic features and year of diagnosis. We included 138 *BRCA1*- and 37 *BRCA2*-breast cancer patients. Median follow-up after diagnosis was 9.7 years. Independent prognostic factors for RFS were tumor size (HR 2.47 for >2 cm vs. ≤2 cm; 95%CI 1.10-5.57), tumor-associated inflammation (HR 0.18 for moderate/marked vs. absent/mild; 95%CI 0.05-0.61) and intra-tumor necrosis (HR 2.60 for presence vs. absence; 95%CI 1.12-6.05). Established prognostic factors as nodal status and differentiation grade were not significantly related to RFS. Subgroup analyses of 138 *BRCA1*- and 118 triple-negative breast cancer cases showed similar results. Tumor-associated inflammation density was the strongest predictor for RFS in this series of *BRCA1/2*-breast cancer patients. This provides a potential risk stratification tool that can easily be implemented in routine histological examination.

INTRODUCTION

The presence of a *BRCA1/2* gene mutation in women is associated with a primary breast cancer risk of 55-85% by the age of 70 and a contralateral breast cancer (CBC) risk of 20-60%[1-5]. Generally, the prognostic value of established histologic characteristics such as differentiation grade and hormone receptor status seems comparable for *BRCA1/2*-associated and sporadic early breast cancers[6]. However, the majority of both *BRCA1*- and *BRCA2*-associated breast cancers are poorly differentiated[7-9]. Besides, the *BRCA1* breast cancer phenotype is mostly triple-negative (negative for estrogen (ER), progesterone (PR) and HER2 receptor), limiting the role of receptor status for risk stratification[7-9], although *BRCA2*-associated breast cancers are more similar to sporadic breast cancers and frequently express ER[7-9]. Moreover, due to frequent breast cancer screening of *BRCA1/2* mutation carriers and addition of magnetic resonance imaging (MRI) to screening programs, *BRCA1/2*-associated breast cancer can be detected early resulting in a low incidence of axillary lymph node positive disease[10]. These findings limit the opportunity for using established histologic prognostic factors for risk stratification in *BRCA1/2*-associated breast cancer. Therefore, the identification of additional prognostic parameters is warranted.

In sporadic breast cancers, additional histologic features including tumor-associated inflammation density and the amount of tumor-associated stroma have recently been described as prognostic factors[11-13]. Stroma-rich breast cancer has been reported to be related to poor prognosis as compared to stroma-poor breast cancer, especially in the triple-negative subgroup[11]. So far, these characteristics have not been investigated yet in relation to prognosis of *BRCA1/2*-associated breast cancers. However, these features could especially be of prognostic relevance in the subgroup of *BRCA1/2*-associated breast cancer since these cancers have a higher frequency of dense tumor-associated inflammatory infiltrate and specifically *BRCA1* has a higher proportion of triple-negative tumors as compared to the sporadic breast cancer cohort[7, 14].

In this study, we assessed the value of several histologic parameters, including tumor-associated inflammation density, intra-tumor necrosis and the amount of tumor-associated stroma, taking into account established prognostic factors in relation to recurrence-free survival (RFS) of *BRCA1*- and *BRCA2*-associated breast cancer patients.

METHODS

Patients

Patients at increased breast cancer risk visiting the Family Cancer Clinic (FCC) of the Erasmus MC Cancer Institute for counseling and surveillance programs are registered in an institutional ongoing database after written informed consent. From this database, we

selected female breast cancer patients diagnosed between 1982 and 2008 with a proven *BRCA1/2* gene mutation and for whom breast cancer tumor slides were available. Breast cancer was mainly diagnosed by fine needle aspiration or core needle biopsy (using a 14 Gauge needle for ultrasonography-guided procedures of solid lesions and a 10 Gauge needle for MRI-guided biopsies).

Exclusion criteria were: a history of a malignancy other than BC at the time of breast cancer diagnosis except for basal cell carcinoma, neoadjuvant chemotherapy or radiotherapy given for the primary breast cancer or distant metastasis at the time of diagnosis (M1 disease). Women with CBC were included in the analysis since low stage CBCs, as typically detected during screening in *BRCA*1/2 gene mutation carriers, do not influence primary breast cancer prognosis[15]. In case of synchronous CBC, the tumor with the highest stage was included. Data on patient history, mutation carriership (*BRCA1* or *BRCA2)*, age at and year of breast cancer diagnosis and on locoregional/distant disease recurrence were extracted from the FCC database. Data on tumor size and lymph node metastases were extracted from pathology reports.

Histopathological features

Formalin-fixed paraffin-embedded primary breast tumor tissues were collected from the departments of pathology of the Erasmus MC Cancer Institute and from regional hospitals. A breast pathologist (C.v.D.) reviewed haematoxylin and eosin (H&E) stained tissue sections for assessment of histologic subtype (according to the WHO), histologic grade (Nottingham modification of Bloom and Richardson)[16] and mitotic activity index[7]. The diagnosis of medullary carcinoma required the following histological criteria: (1) syncytial growth pattern, (2) absence of glandular structures, (3) diffuse moderate to marked inflammatory response, (4) moderate to marked nuclear pleomorphisms and (5) sharply defined margins. Carcinomas were classified as 'ductal with medullary features' when most medullary features, but not all criteria for medullary carcinoma were present. Tumor-associated inflammation was defined as a diffuse lymphocytic infiltrate and semi-quantitatively scored into four categories (absent/minimal, mild, moderate or marked; Figure 1) as was described earlier by Lee and colleagues[17] and analyzed as two categories (absent/mild versus moderate/marked). Diffuse tumor-associated inflammation was scored at the tumor edge. The amount of tumor-associated stroma of the primary tumor was scored quantitatively by one observer (R.v.B.) using a 100-point grid. At 100 points with an interval of 1 mm, the presence of tumor cells or stroma was recorded. The percentage of tumor-associated stroma was calculated by dividing the number of points containing stroma by the sum of scored points containing tumor cells or stroma. Tumor-associated stroma was classified as "stroma-poor" (< 50% stroma) or "stroma-rich" (≥ 50% stroma; Figure 2). Intra-tumor necrosis was defined as groups of necrotic cells or areas of confluent necrosis. Any inflammation, necrosis or fibrosis as-

sociated with the biopsy site was not taken into account. Lymphovascular invasion was defined as presence of tumor cells within an endothelial lined space (lymphatic and/or blood vessel) outside the border of the invasive carcinoma[13].

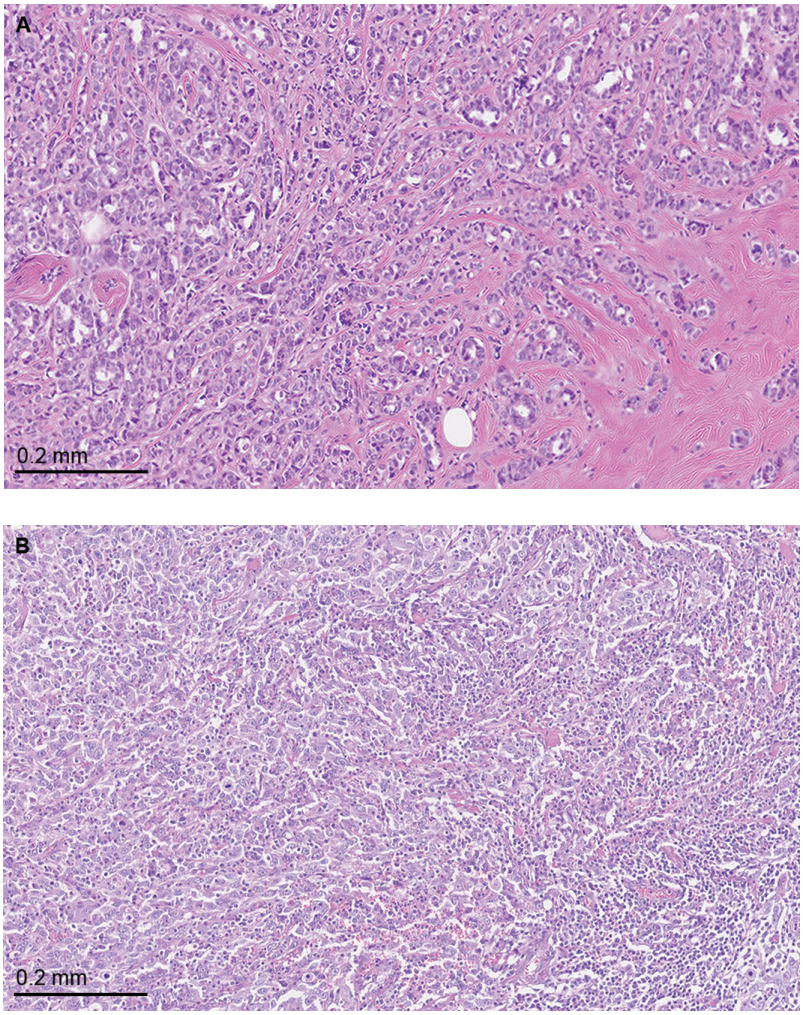

Figure 1. Breast cancer with **A** absent to mild and **B** marked tumor-associated inflammation density Haematoxylin and eosin stained (H&E); 10x objective

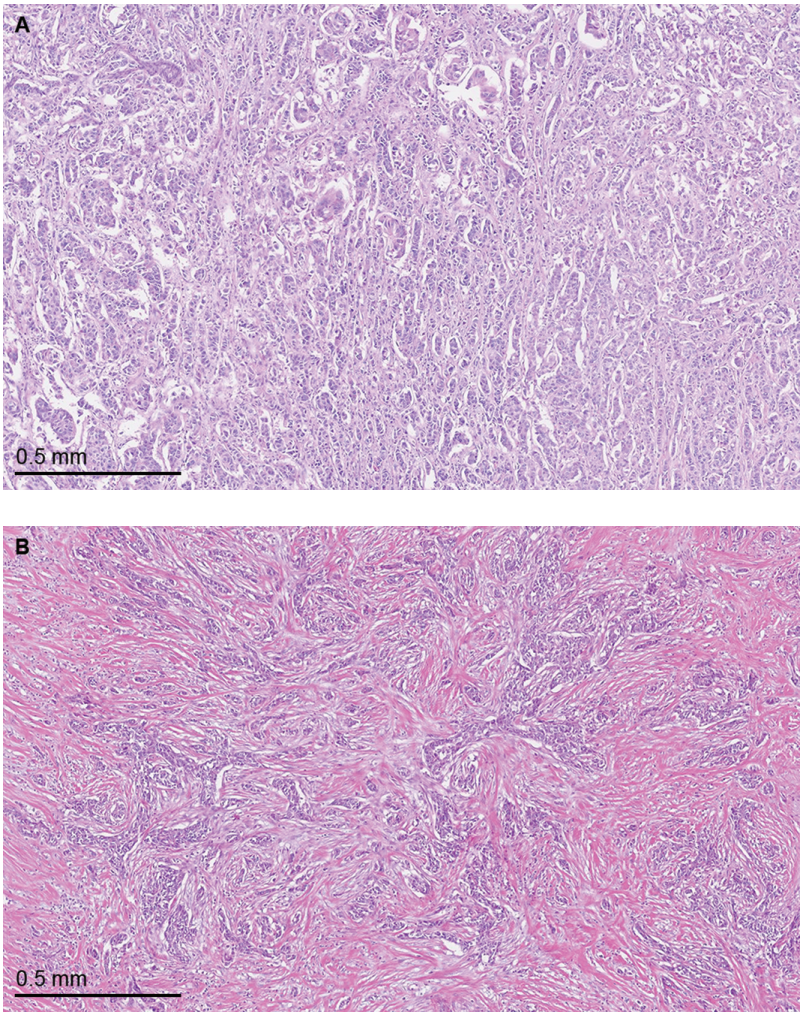

Figure 2. Breast cancer with **A** tumor-associated stroma < 50% (stroma-poor) and **B** tumor-associated stroma ≥ 50% (stroma-rich)
Haematoxylin and eosin stained (H&E); 5x objective

ER, PR and HER2 were assessed from Tissue Micro Arrays[18] using three cores per tumor to account for tumor heterogeneity. ER and PR were considered positive if staining was seen in ≥10% of the nuclei, according to the Dutch national guidelines for breast cancer treatment[19]. HER2 receptor status was scored according to international guidelines[20]. An equivocal immunohistochemical result (2+) was followed by fluorescence in situ hybridization[21].

Statistical analysis

The main outcome of interest was recurrence-free survival (RFS), defined as the time interval between date of surgery and locoregional or distant relapse of breast cancer. Left truncation was used to avoid potential survival bias due to inclusion of patients who underwent genetic testing after breast cancer diagnosis[22]. Patients were censored at last follow-up or at death from causes unrelated to their disease. We estimated hazard ratios and 95% CIs for all established prognostic factors and additional histologic parameters using Cox regression in univariate and multivariate analysis. Variables with a P-value < 0.10 in univariate analysis were included in the multivariate model. We performed subgroup analyses on *BRCA1* gene mutation carriers and on triple-negative breast cancer patients. In all analyses, age at and year of diagnosis were analyzed as continuous variables. Analyses were performed with STATA version 13.1.

RESULTS

For 175 female *BRCA1/2* mutation carriers with primary breast cancer tumor slides were available. The main characteristics of the study population are summarized in Table 1. The majority of the patients were *BRCA1* mutation carriers (138 *BRCA1* vs. 37 *BRCA2*). Median age at diagnosis was 39.0 years. Median follow-up after primary breast cancer diagnosis was 9.7 years. During follow-up, 44 patients were affected by recurrent disease (25%) and 29 patients died of breast cancer (17%). The majority of patients (58%) had a tumor size of ≤ 2.0 cm (pT1) and a negative nodal status (66%). Most *BRCA1/2*-associated breast cancers were of ductal subtype (69%) and Bloom and Richardson grade III (67%). A triple-negative phenotype was observed in 118 (67%) of the cases, of which only 6 (5%) were *BRCA2*-associated. HER2 was amplified in a minority of patients (4%).

Table 1. Patient and tumor characteristics of *BRCA1/2*-associated breast cancer patients

	N	%	*BRCA1*	%	*BRCA2*	%
Total	175		138	79%	37	21%
Age at diagnosis , years *(median, range)*	39.0	24-68	38.0	24-68	41.0	32-64
Period of diagnosis						
< 1997	53	30%	45	33%	8	22%
1997 – 2000	46	26%	38	27%	8	22%
2001 – 2003	37	21%	28	20%	9	24%
> 2003	39	23%	27	20%	12	32%
Follow-up time, years *(median, range)*	9.7	0.01-31.1	9.7	0.01-31.1	8.1	0.7-21.8
Tumor size						
≤ 2.0 cm	101	58%	80	58%	21	57%
2.0 – 5.0 cm	64	36%	54	40%	10	27%
> 5.0 cm	5	3%	2	1%	3	8%
Unknown	5	3%	2	1%	3	8%
Nodal status						
Negative	116	66%	99	72%	17	46%
Positive, 1-3 nodes	40	23%	26	19%	14	38%
Positive, 4 or more nodes	16	9%	11	8%	5	13%
Unknown	3	2%	2	1%	1	3%
Histological type						
Ductal	121	69%	93	68%	28	76%
Medullary (features)	38	22%	36	26%	2	5%
Other	14	8%	7	5%	7	19%
Unknown	2	1%	2	1%	0	0
Bloom and Richardson grade						
I	6	3%	2	1%	4	11%
II	50	29%	35	26%	15	40%
III	117	67%	99	72%	18	49%
Unknown	2	1%	2	1%	0	0
Estrogen receptor status						
Negative	121	69%	114	82%	7	19%
Positive	53	30%	23	17%	30	81%
Unknown	1	1%	1	1%	0	0
Progesterone receptor status						
Negative	134	77%	123	89%	11	30%
Positive	39	22%	14	10%	25	67%
Unknown	2	1%	1	1%	1	3%
HER2 expression						
Negative	166	95%	131	95%	35	95%
Positive	7	4%	5	4%	2	5
Unknown	2	1%	2	1%	0	0
Triple negative receptor status						
Yes	118	67%	112	81%	6	16%
No	57	33%	26	19%	31	84%

Table 1. Patient and tumor characteristics of *BRCA1/2*-associated breast cancer patients (continued)

	N	%	*BRCA1*	%	*BRCA2*	%
Vascular invasion						
No	139	79%	114	83%	25	68%
Yes	36	21%	24	17%	12	32%
Tumor-associated inflammation						
Absent/mild	109	62%	77	56%	32	86%
Moderate/marked	66	38%	61	44%	5	14%
Tumor-associated stroma						
< 50% (stroma-poor)	133	76%	109	79%	24	65%
≥ 50% (stroma-rich)	42	24%	29	21%	13	35%
Intra-tumor necrosis						
No	98	56%	67	49%	31	84%
Yes	77	44%	71	51%	6	16%

Tumor-associated inflammation was scored as moderate or marked in 38% of the cases. Stroma-rich tumors (defined as ≥50% stroma) were observed in 24% of patients. In 44% of all breast cancers, intra-tumor necrosis was observed.

As shown in Table 2, in univariate analyses prognostic factors for RFS were tumor size (HR 3.52 for >2.0 cm, vs. ≤2.0 cm; 95% CI 1.59-7.78), year of diagnosis (HR 0.86 for successive year of diagnosis; 95% CI 0.78-0.95) and tumor-associated inflammation density (Figure 3; HR 0.17 for moderate/marked vs. absent/mild 95% CI 0.05-0.56). Prognostic factors of borderline significance were nodal status (HR 1.92 for positive vs. negative; 95% CI, 0.92-4.00; P=0.08) and intra-tumor necrosis (HR 1.93; present vs. absent 95% CI 0.92-4.04; P=0.08) and therefore were included in the multivariable analysis. Bloom and Richardson differentiation grade, nor ER and PR status were of significant prognostic value in univariate analysis.

In multivariable analysis, prognostic factors for RFS were tumor size (HR 2.47 for tumor size > 2 cm vs. ≤ 2 cm; 95% CI 1.10-5.57), tumor-associated inflammation density (HR 0.18 for moderate/marked vs. absent/mild; 95% CI 0.05-0.61) and intra-tumor necrosis (HR 2.60 for presence vs. absence; 95% CI 1.12-6.05) (Table 2).

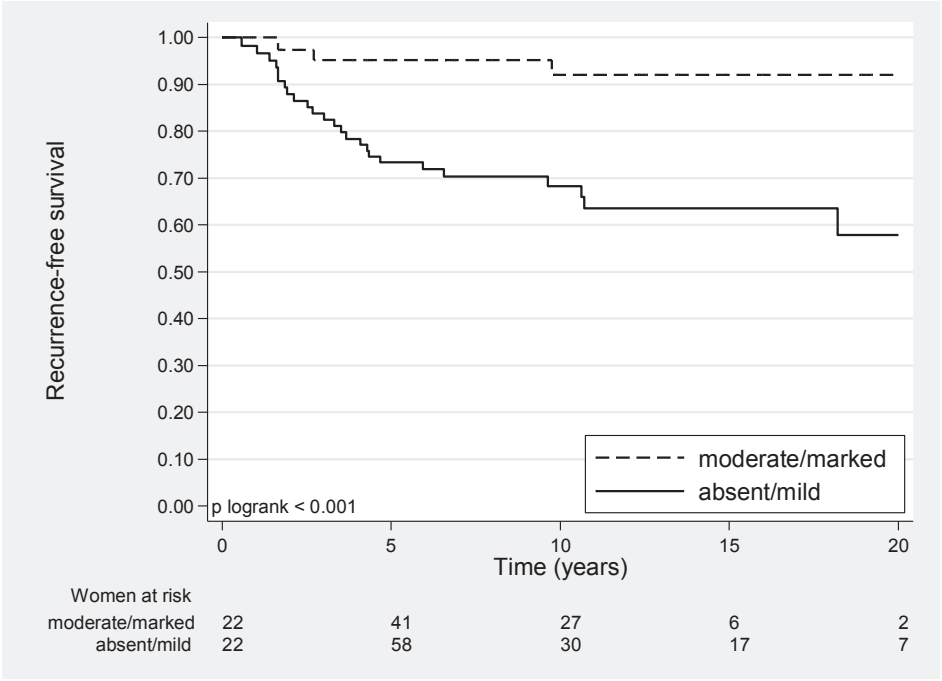

Figure 3. Recurrence-free survival (RFS) in *BRCA1/2*-associated breast cancer patients by inflammation density of the tumor

*Numbers of women at risk increase during the first years of observation because of left truncation, which was used to avoid potential survival bias due to inclusion of patients who underwent genetic testing after breast cancer diagnosis.

Subgroup of *BRCA1*-associated breast cancers

Patient characteristics of the *BRCA1* subgroup are depicted in Table 1. Median age at diagnosis was 38.0 years. The majority of these breast cancers were poorly differentiated, stroma-poor, triple-negative and ductal carcinomas. Medullary features were observed in 36 cases (26%). Almost half of the breast cancers (44%) showed moderate or marked tumor-associated inflammation.

Results on prognostic factors with respect to RFS were comparable to the results in the overall group (Table 3). In univariate analysis, prognostic factors were year of diagnosis (HR 0.87 for increasing year of diagnosis; 95% CI 0.78-0.97), tumor size (>2.0 cm vs. ≤2 cm HR 4.29; 95% CI 1.79-10.29) and tumor-associated inflammation density (HR 0.14; moderate/marked vs absent/mild 95% CI 0.04-0.47). Borderline prognostic significance was found for presence of intra-tumor necrosis (HR 2.04; 95% CI 0.91-4.59), which was included in the multivariable analysis. A medullary subtype, although frequently observed in the *BRCA1* subgroup, had no significant prognostic value with an HR of 0.44 (presence vs. no medullary(-like) features, 95% CI 0.13-1.50). Neither nodal status, grade or ER status were of prognostic significance.

Table 2. Clinicopathologic features of *BRCA1/2*-associated breast cancer patients in relation to recurrence-free survival (RFS)

	N	Univariate		Multivariable	
		HR	95% CI	HR	95% CI
Age at diagnosis (years)[1]		1.01	0.97 – 1.06		
BRCA mutation					
BRCA1	138	1.0			
BRCA2	37	0.42	0.13 – 1.41		
Year of diagnosis (years)[1]		0.86[2]	0.78 – 0.95	0.91	0.82 – 1.00
Tumor size					
≤ 2.0 cm	101	1.0		1.0	
> 2.0 cm	69	3.52[2]	1.59 – 7.78	2.47*	1.10 – 5.57
Nodal status					
Negative	116	1.0		1.0	
Positive	56	1.92[2]	0.92 – 4.00	1.82	0.82 – 4.04
Histological type					
Ductal	121	1.0			
Medullary (features)	38	0.48	0.14 – 1.61		
Other	14	1.04	0.31 – 3.51		
Bloom and Richardson grade					
I/II	56	1.0			
III	117	1.16	0.51 – 2.67		
Estrogen receptor status					
Negative	121	1.0			
Positive	53	0.79	0.34 – 1.85		
Progesterone receptor status					
Negative	134	1.0			
Positive	39	0.68	0.24 – 1.97		
Vascular invasion					
No	139	1.0			
Yes	36	0.94	0.36 – 2.48		
Tumor-associated inflammation					
Absent/mild	109	1.0		1.0	
Moderate/marked	66	0.17[2]	0.05 – 0.56	0.18*	0.05 – 0.61
Tumor-associated stroma					
< 50% (stroma poor)	133	1.0			
≥ 50% (stroma rich)	42	1.43	0.64 – 3.20		
Intra-tumor necrosis					
No	98	1.0		1.0	
Yes	77	1.93[2]	0.92 – 4.04	2.60	1.12 – 6.05

Abbreviations: HR: Hazard Ratio; 95%CI: 95% confidence interval

[1]analyzed as a continuous variable

[2]entered in multivariable analysis

*statistically significant

Table 3. Clinicopathologic features of *BRCA1*-associated breast cancer patients (N=138) in relation to recurrence-free survival (RFS)

	N	Univariate		Multivariable	
		HR	95% CI	HR	95% CI
Age at diagnosis (years)[1]		1.03	0.99 – 1.07		
Year of diagnosis (years)[1]		0.87[2]	0.78 – 0.97	0.93	0.83 – 1.03
Tumor size					
≤ 2.0 cm	80	1.0		1.0	
> 2.0 cm	56	4.29[2]	1.79 – 10.29	3.32*	1.32 – 8.38
Nodal status					
Negative	99	1.0			
Positive	37	1.89	0.86 – 4.14		
Histological type					
Ductal	93	1.0			
Medullary (features)	36	0.44	0.13 – 1.50		
Other	7	2.22	0.63 – 7.86		
Bloom and Richardson grade					
I/II	37	1.0			
III	99	1.41	0.52 – 3.83		
Estrogen receptor status					
Negative	114	1.0			
Positive	23	1.33	0.50 – 3.59		
Vascular invasion					
No	114	1.0			
Yes	24	1.04	0.36 – 3.06		
Tumor-associated inflammation					
Absent/mild	77	1.0		1.0	
Moderate/marked	61	0.14[2]	0.04 – 0.47	0.17*	0.05 – 0.57
Tumor-associated stroma					
< 50% (stroma poor)	109	1.0			
≥50% (stroma rich)	29	1.42	0.58 – 3.46		
Intra-tumor necrosis					
No	67	1.0		1.0	
Yes	71	2.04[2]	0.91 – 4.59	1.81	0.74 – 4.42

Abbreviations: HR: Hazard Ratio; 95% CI: 95% confidence interval
[1]analyzed as a continuous variable
[2]entered in multivariable analysis
*statistically significant

In multivariable analysis, tumor size and inflammation density were histologic features of significant prognostic value for RFS with an HR of 3.32 for tumor size (> 2.0 cm vs. ≤ 2.0 cm; 95% CI 1.32-8.38) and 0.17 for tumor-associated inflammation (moderate/marked vs absent/mild; 95% CI 0.05-0.57). Intra-tumor necrosis was not a significantly prognostic factor in this subgroup.

Subgroup of *BRCA1/2* mutation carriers with triple-negative breast cancers

As shown in Table 4, the triple-negative subgroup comprised 118 patients, mainly being *BRCA1*-associated cases (n=112, 95%). Median age at diagnosis was 38.0 years. Because of the high proportion of *BRCA1*-associated cases, characteristics of the *BRCA1*-subgroup (Table 1) and the triple-negative subgroup were very similar (data not shown).

Table 4. Clinicopathologic features of triple-negative breast cancer patients (N=118) in relation to recurrence-free survival (RFS)

	N	Univariate		Multivariable	
		HR	95% CI	HR	95% CI
Age at diagnosis (years)[1]		1.02	0.97 – 1.07		
BRCA mutation					
BRCA1	112	1.0			
BRCA2	6	0.85	0.11 – 6.38		
Year of diagnosis (years)[1]		0.91[2]	0.81 – 1.01	0.95	0.85 – 1.06
Tumor size					
≤ 2.0 cm	67	1.0			
> 2.0 cm	49	5.08[2]	1.86 – 13.88	4.89*	1.79 – 13.39
Nodal status					
Negative	86	1.0			
Positive	30	1.41	0.56 – 3.50		
Histological type					
Ductal	78	1.0			
Medullary (features)	36	0.46	0.13 – 1.58		
Other	3	5.86	1.27 – 27.07		
Bloom and Richardson grade					
I/II	20	1.0			
III	97	1.78	0.40 – 7.87		
Vascular invasion					
No	99	1.0			
Yes	19	0.87	0.25 – 2.97		
Tumor-associated inflammation					
Absent/mild	61	1.0			
Moderate/marked	57	0.15[2]	0.04 – 0.51	0.18*	0.05 – 0.64
Tumor-associated stroma					
< 50% (stroma poor)	102	1.0			
≥ 50% (stroma rich)	16	1.90	0.68 – 5.35		
Intra-tumor necrosis					
No	51	1.0			
Yes	67	1.75	0.71 – 4.32		

HR: Hazard Ratio; 95% CI: 95% confidence interval

[1]analyzed as a continuous variable

[2]entered in multivariable analysis

*statistically significant

Regarding prognostic factors in relation to RFS, analyses of the triple-negative subgroup showed similar results as for the overall group. In univariate analysis, prognostic factors were tumor size (HR 5.08 for > 2.0 cm vs. ≤2 cm; 95% CI 1.86-13.88) and tumor-associated inflammation density (HR 0.15 for moderate/marked vs. absent/mild; 95% CI 0.04-0.51). Year of diagnosis was of borderline prognostic significance (HR 0.91 for increasing year of diagnosis; 95% CI 0.81-1.01) and was therefore entered in the multivariable analysis. Other factors (nodal status, grade, histologic subtype) were not significantly associated with prognosis for RFS.

Independent prognostic factors were tumor size and inflammation density with HRs of 4.89 (95% CI 1.79-13.39) and of 0.18 (95% CI 0.05-0.64), respectively.

DISCUSSION

In this study, we observed that presence of moderate/marked tumor-associated inflammation, absence of intra-tumor necrosis and tumor size < 2 cm were independent favorable prognostic factors for RFS in *BRCA1/2*-associated breast cancers.

To our knowledge, this is the first study investigating the value of tumor-associated inflammation density as prognostic feature in *BRCA1/2*-associated tumors. Our finding that the presence of moderate/marked tumor-associated inflammation is a favorable prognostic marker is in line with earlier observations on sporadic breast cancer, although reported results are inconsistent. A recent systematic review on the prognostic value of tumor-associated inflammation comprised thirteen studies showing improved sporadic breast cancer prognosis associated with pronounced inflammation density, whereas seven studies reported a poorer outcome of breast cancer with pronounced inflammation and four did not find a relation with breast cancer prognosis [12].

A large proportion of *BRCA1/2*-associated breast cancers detected during adequate surveillance (including breast MRI), is smaller than 1.0 cm (pT1) and node negative [10]. Potentially, routine assessment of tumor-associated inflammation may help to improve risk stratification of these early *BRCA1/2*-associated breast cancers to further personalize (adjuvant) treatment. Further, several recent studies reported that pathological complete response and prognosis of breast cancer following neoadjuvant chemotherapy can be predicted by the amount of tumor-associated lymphocytes as assessed on tumor biopsies [23,24]. Future advancing insights on the host versus tumor inflammatory response may help in the development of new targeted therapies especially for triple-negative carcinomas.

A high tumor-associated inflammation density is associated with the medullary breast cancer subtype, which is more commonly seen in *BRCA1*-associated than in sporadic breast cancer [7]. In the current analysis, the presence of medullary features was present in 36 *BRCA1*-associated breast cancers (26%). Although medullary features seemed to

favorably affect RFS with a univariate HR of 0.48, this was not statistically significant. This may partly be explained by the prominent tumor-associated inflammation often found in the medullary subgroup [25], which as described above also favors prognosis.

Both intra-tumor necrosis and lymphovascular invasion are discriminants of poor prognosis in sporadic breast cancers [26, 27] and specifically in triple-negative carcinomas [28]. However, to the best of our knowledge, no specific data exist on the prognostic significance of these features in breast cancers of *BRCA1/2* gene mutation carriers. Intra-tumor necrosis was present in 44% of the *BRCA1/2*-associated breast cancers which is in line with the 40-60% previously described for sporadic breast cancer [21, 29]. With an HR of 2.60 the presence of necrosis was associated with a worse RFS. Hypoxia of the tumor, eventually causing necrosis, is thought to give rise to a more malignant behavior through induction of angiogenesis and migration, which among other factors favor tumor invasion and metastasis [27, 30]. A higher frequency of overexpression of HIF-1alpha, being a key transcriptional regulator of the hypoxia response, has been reported in *BRCA1*-associated breast cancer as compared to sporadic breast cancer, suggesting an important role of hypoxia in this subgroup of patients [31, 32]. The incidence of lymphovascular invasion was 21% in our series, which is in line with results of other studies on *BRCA1/2*-associated tumors (20-30%), as well as with reported incidences in sporadic carcinomas (20-25%) [33, 34]. In the current analyses, presence of lymphovascular invasion was not found to be significantly associated with RFS.

The amount of tumor-associated stroma has been reported to be an independent prognostic factor in the total group of breast cancer patients, but especially in triple-negative breast cancers with an approximately three times higher risk of relapse in stroma-rich as compared to stroma-poor tumors [11]. No significant effect of tumor-associated stroma was seen in our series, not even in the triple-negative subgroup. Potential explanations for this discrepancy include differences in assessment methods of the amount of stroma, size of the study population and a different histologic phenotype of *BRCA1/2*-associated tumors as compared to sporadic breast cancer. We chose to use a grid for the assessment of the amount of tumor-stroma in an attempt to achieve an objective, quantitative method. However, additional studies are needed to compare this method with global estimation. Our method with the 100-point grid was relatively laborious (5 minutes/patient). Therefore, in case global estimation would be as accurate as using a grid, global estimation would be more practical for everyday practice.

A larger tumor size (>2 cm) remained an independent unfavorable prognostic factor for RFS in our series of *BRCA1/2*-associated breast cancer cases. In contrast, other established prognostic factors as differentiation grade, ER/PR status and nodal status were not associated with RFS, which might partly be explained, by the finding that the majority of the tumors were poorly differentiated (grade 3), ER/PR negative and lymph node negative. Likely, the number of disease recurrences was too small to detect a

significant influence of grade, hormone receptor and nodal status in this series. ER/PR positivity may be underreported in this study compared to other series, since according to Dutch guidelines a tumor is ER or PR positive with ≥10% staining of the nuclei, while ASCO guidelines recommend ≥1% of nuclear staining as ER/PR positive.

In view of the fact that *BRCA1*- and *BRCA2*-associated breast cancers have distinct phenotypes [7] and the overrepresentation of *BRCA1*-associated cases in our series, we separately analyzed the subgroups of *BRCA1*-associated and of triple-negative breast cancers. In these subgroup analyses, presence of intra-tumor necrosis did not remain a prognostic marker for RFS, possibly because of the smaller numbers, but other results were similar to the results observed in the overall group. The proportion of *BRCA2* gene mutation carriers was too small to perform a separate analysis.

Although one of the weak points in this study concerns its retrospective nature, patient cohort data were collected in a prospective manner yielding a sufficiently long median follow-up time of 9.7 years after breast cancer diagnosis. Despite this follow-up period, there were relatively few disease recurrences, which may explain the finding that some of the examined histological features did not reach statistical significance. Another limitation is that tumor-associated inflammation, necrosis or fibrosis could be related to a pre-operative needle biopsy. However, this effect is probably limited since any inflammation, necrosis or fibrosis associated with the biopsy site was not taken into account. Besides, a substantial proportion of patients underwent fine needle aspiration.

In conclusion, in this unique series of *BRCA1/2*-associated breast cancers moderate or marked tumor-associated inflammation was the strongest independent favorable factor for RFS. Of the conventional, established prognostic factors only tumor size < 2 cm remained a favorable factor. Our findings mainly concern *BRCA1*-associated breast cancers, as the number of *BRCA2* mutation carriers was low. When confirmed in other and larger series of *BRCA1/2* mutation carriers, and more specifically in *BRCA2* mutation carriers, tumor-associated inflammation may be easily assessed in routine diagnostics and form a potential tool to improve breast cancer risk stratification in *BRCA1/2*-associated early breast cancer cases. Further research is warranted to elucidate the multifactorial process of host versus tumor immunity and to identify possible targets for adjuvant therapy with benefit for especially triple-negative *BRCA1/2*-associated carcinomas.

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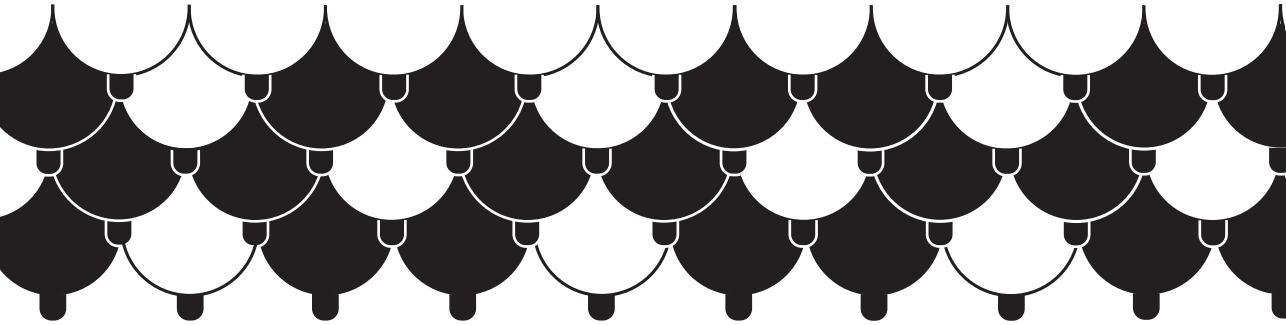

CHAPTER 5

Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast and/or ovarian cancer patients is clinically relevant

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ABSTRACT

Female *BRCA1/2* mutation carriers affected with breast and/or ovarian cancer may develop new tumor deposits over time. It is of utmost importance to know the clonal relationships between multiple tumor localizations, enabling differentiation between multiple primaries or metastatic disease with consequences for therapy and prognosis. We evaluated the value of targeted next generation sequencing[1] in the diagnostic workup of *BRCA1/2* mutation carriers with ≥2 tumor localizations and uncertain tumor origins. Forty-two female *BRCA1/2* mutation carriers with ≥2 tumor localizations were selected. Patients with inconclusive tumor origin after histopathological revision were 'cases'; patients with certain tumor origin of ≥3 tumors served as 'controls'. Tumors of cases and controls were analyzed by targeted next generation sequencing using a panel including *CDKN2A, PTEN* and *TP53*, hotspot mutation sites for 27 different genes and 143 single nucleotide polymorphisms for detection of loss of heterozygosity. Based on prevalence of identical or different mutations and/or loss of heterozygosity patterns, tumors were classified as 'multiple primaries' or 'one entity'. Conventional histopathology yielded a conclusive result in 38/42 (90%) of patients. Four cases and 10 controls were analyzed by next generation sequencing. In 44 tumor samples, 48 mutations were found; 39 (81%) concerned *TP53* mutations. In all 4 cases, the intra-patient clonal relationships between the tumor localizations could be unequivocally identified by molecular analysis. In all controls, molecular outcomes matched the conventional histopathological results. In most *BRCA1/2* mutation carriers with multiple tumors routine pathology work-up is sufficient to determine tumor origins and relatedness. In case of inconclusive conventional pathology results, molecular analyses using next generation sequencing can reliably determine clonal relationships between tumors, enabling optimal treatment of individual patients.

INTRODUCTION

Female *BRCA1/2* mutation carriers have a cumulative lifetime risk of developing breast cancer of 55-85% by the age of 70[2-5]. The cumulative lifetime risk of developing ovarian cancer varies between 15-60% for *BRCA1* and 10-35% for *BRCA2* mutation carriers[2-5]. Moreover, susceptibility for other cancers also seems to be increased in *BRCA1/2* mutation carriers[6, 7].

It has been reported that *BRCA1*-associated breast cancers more frequently develop visceral metastasis and fewer bone metastases[8, 9] and *BRCA2*-associated breast cancers tend to develop more lymph node metastases compared with sporadic breast cancer[9]. Metastatic sites of sporadic ovarian cancer mostly confine to the intraperitoneal cavity[10, 11], whereas it has been described that *BRCA1/2*-associated ovarian cancer patients frequently (74%) present with visceral metastases to liver, lung and spleen[12]. Although this can be of some help, the non-specific metastatic patterns in *BRCA1/2*-associated breast and ovarian cancer patients impede careful differentiation between breast cancer, ovarian cancer and other tumor origins when multiple cancer localizations occur in one patient. It is of clinical importance, however, to make this distinction, as it guides surgical and chemotherapeutic treatment and determines prognosis[13, 14].

A potentially helpful tool in determining clonal relationships between multiple tumors is DNA next Generation Sequencing [1, 15]. With next generation sequencing, selected genes known to be frequently mutated in specific tumor types can be analyzed. Additionally, single nucleotide polymorphisms can be analyzed to detect any DNA copy number changes present in the tumor cells. Identical molecular aberrations of different tumor localizations indicate a common tumor origin (e.g. metastatic disease), whereas different mutations and/or copy number changes in different tumor samples indicate two primary malignancies.

The aim of the current study was to evaluate the value of next generation sequencing in the diagnostic workup of *BRCA1/2*-associated breast and ovarian cancer patients with multiple tumor localizations.

MATERIALS AND METHODS

Patient selection: cases and controls

Patients at increased risk of breast and/or ovarian cancer visiting the Family Cancer Clinic of the Erasmus Medical Center Cancer Institute for counseling and surveillance programs are registered in an institutional ongoing database. All women provide written informed consent for registration of their clinical data and storage of genetic material (if relevant) for research purposes. From this database, we selected all female germline *BRCA1* or *BRCA2* mutation carriers with ≥2 synchronous or metachronous tumor localizations of which tumor material had been obtained by fine needle aspiration (FNA), biopsy or

surgical excision. Tumor localizations of which no suitable material was available for histopathological or molecular analysis were excluded. Included were *BRCA1/2* mutation carriers with multiple tumors of which at least one was located in the breast or ovary. Inclusion and exclusion criteria are depicted in Table 1.

Table 1. Inclusion and exclusion criteria

Inclusion
• Women with a proven *BRCA1* and/or *BRCA2* mutation
• With ≥ 2 synchronous or metachronous tumor localizations [1]
• Tumor material available for next generation sequencing analysis • (obtained by fine needle aspiration, histological biopsy or surgical excision)
• One of the 4 clinical scenarios: 1. Breast cancer and ovarian cancer 2. Breast cancer and second other tumor 3. Ovarian cancer and second other tumor 4. Breast cancer, ovarian cancer, and third or additional other tumor localizations
Exclusion criteria for 'other tumor localization'
• Hematological malignancies
• Dermatological malignancies (ie. melanomas, basal cell carcinomas)
• Ipsilateral lymph node metastases in the presence of breast cancer
• Premalignant lesions, such as ductal carcinoma in situ
• Contralateral breast cancer or second ipsilateral breast cancer, except in the presence of a third tumor localization
• Peritoneal tumor localization in the presence of ovarian cancer - If reported that the ovarian cancer was growing per continuum into the peritoneal cavity - Confining to the ipsilateral adnexa

[1]isolated site of invasive cancer as diagnosed by radiological examination, intra-operatively or during pathological examination

If possible, the origin of the tumor localizations was identified based on H&E staining. If tumor histology did not provide a conclusive diagnosis, immunohistochemical staining was applied. Patients for whom the origin of one or more tumor localizations remained uncertain after histological and immunohistochemical evaluation were selected for next generation sequencing molecular analysis ('cases'). Patients with ≥3 tumor localizations of conclusive origin, based on histology and immunohistochemistry, served as 'controls'. Controls were selected for next generation sequencing, as well, to validate the versatility of the next generation sequencing approach for tumor clonality determinations.

Conventional diagnostics

Tumor histology

Formalin-fixed paraffin-embedded tumor tissues were collected from the Department of Pathology of the Erasmus Medical Center Cancer Institute and from regional hospitals. Two pathologists specialized in breast and gynecological cancer (C.v.D., P.v.D.) independently reviewed haematoxylin and eosin (H&E) stained tissue sections of the tumor localizations for histology, with a subsequent consensus discussion.

Immunohistochemistry

Immunohistochemical tissue markers were chosen according to the institutional protocol and depended on clinical and histological differential diagnosis of the origin of the various tumor localizations. Estrogen receptor (ER) was used as a breast cancer marker. Immunohistochemical markers used for differentiation of ovarian cancer were cancer antigen 125 (CA125), Wilms' tumor 1 (WT1) and PAX-8, all known to be frequently expressed in ovarian cancer[16-18]. To differentiate with primary lung carcinoma, TTF-1 was used[19].

Molecular analysis

For cases and controls, p53 immunohistochemistry was performed on all tumor tissues if formalin-fixed paraffin-embedded tissue blocks were available. Nuclear expression of p53 in tumor cells was scored as either heterogeneous (no indication for *TP53* mutation), strong in all tumor cells (indication for missense *TP53* mutation) or absent in all tumor cells (indication for frameshift, nonsense, or splice site *TP53* mutation). For next generation sequencing analysis, normal and tumor tissues were manually microdissected from haematoxylin-stained tissue sections of formalin-fixed paraffin-embedded tissue blocks or if unavailable, from original routine H&E, immunohistochemical stained sections or cytological preparations. DNA was extracted using proteinase K and 5% Chelex resin, as previously described[20]; DNA concentrations were measured with the Qubit 2.0 Fluorometer. To assess the quality of DNA amplification a multiplex control PCR was performed as previously described[21]; PCR products were analyzed on an agarose gel. All DNA samples were screened with the Ion Torrent Personal Genome Machine, with supplier's materials and protocols (Life Technologies, Carlsbad, CA, USA). A custom made primer panel was used, designed using Ion AmpliSeq Designer 2.2.1, for diagnostic use in clonality determinations of various tumor types including breast and ovarian cancer. Because this panel was designed for analysis of a broad range of tumor types, it includes genes frequently mutated in breast and ovarian cancer, as well as genes rarely mutated in these tumors. The panel targets almost the entire open reading frame of *CDKN2A, PTEN* and *TP53* (coverage 95-99%), multiple hotspot mutation sites for 27 different genes and 143 single nucleotide polymorphisms at 15 different loci for the detection of loss of heterozygosity (see supple-

mentary Table 1 for primer details). In total, the panel consisted of 254 amplicons with a mean amplicon size of 160 base pairs. With this panel, libraries were created using the Ion AmpliSeq 2.0 Library Kit. Template was prepared using the Ion OneTouch 2 with the Ion OneTouch 200 Template Kit v2 DL or using the IonChef with the Ion Personal Genome Machine Hi-Q Chef Kit. Sequencing was performed on an Ion 318v2 chip with the Ion Personal Genome Machine sequencing 200 kit v2 or the Ion Personal Genome Machine Hi-Q sequencing kit. Data was analyzed with Variant Caller v4.0 or v.4.4.2.1. Annotation of the variants was previously described[22]. For mutation detection, all exonic and splice variants with a variant percentage ≥20% were reported, excluding synonymous single-nucleotide variants and variants present in patient-matched normal tissue. Variants with a total coverage of <100 reads, reference coverage <10 reads, and/or a variant coverage of <5 reads for either the forward or reverse strand were excluded. For loss of heterozygosity analysis, single nucleotide polymorphisms with a total coverage of <100 reads or a strand bias (ratio forward:reverse reads not between 1:10 and 10:1 for reference and/or variant reads) were excluded. If a mutation was detected in one or more tumor samples of a patient, the specific locus was manually checked using the integrative genomics viewer (IGV) in normal DNA as well as all tumor samples of that patient. Furthermore, *TP53* was manually checked for mutations if no mutation was detected and immunohistochemistry showed aberrant staining or was unavailable.

Samples for which the control PCR showed no signal for amplicons larger than 100 base pairs and for which next generation sequencing analysis showed <70% of reads on target and/or <70% of amplicons with at least 100 reads were defined low quality samples. For low quality samples with more than 3 variants, we focused on variants present in other tumors of the patient, or if not present, on *TP53* variants. For all low quality samples, mutations were confirmed by Sanger sequencing or by a second next generation sequencing run. For Sanger sequencing, primers from the AmpliSeq design were extended with M13 tails. PCR protocol was previously described[23], data was analyzed using Mutation Surveyor v.4.0 software (SoftGenetics).

RESULTS

Patients

Fifty-six *BRCA1/2* mutation carriers with multiple tumor localizations were selected. Fourteen were excluded due to missing or unsuitable tumor material, leaving 42 women (39 *BRCA1*, 3 *BRCA2*) for analyses. Clinical classification of tumor origins was 'breast cancer + ovarian cancer' in 31 patients, 'breast cancer + other' in nine, 'ovarian cancer + other' in one, and 'breast cancer + ovarian cancer + other' in one woman (data not

shown). Median number of tumor localizations was 2 (range 2-5), and median time from first to last cancer diagnosis was 5 years (range 0-23).

Conventional diagnostics

For 21/42 women (50%) the origin of the tumor localizations was conclusive based on histology only. In an additional 17 (40%) a conclusive diagnosis was reached after immunohistochemistry for relevant markers. Ten of 38 women with conclusive outcomes based on histology and/or immunohistochemistry had ≥3 tumor localizations (controls; 8 *BRCA1* and 2 *BRCA2* mutation carriers).

In four women (10%) one or more tumor localizations remained of uncertain origin after histological and immunohistochemical evaluation (cases; all *BRCA1* mutation carriers).

Case no. 1 presented with tumors in the right and the left breast, and a tumor in the lung seven years later. Both breast tumors were diagnosed IDC of the breast based on HE staining. The lung tumor was diagnosed non-small cell carcinoma, however, conclusive diagnosis regarding the origin of the tumor was not possible based on HE and immunohistochemistry (see Figure 2A for details).

Case no. 2 presented with a tumor in the ovary and a tumor in the breast six years later. The tumor of the ovary was diagnosed serous carcinoma of the ovary based on HE staining. The breast tumor was diagnosed adenocarcinoma based on cytological preparations; however, no tissue was available for immunohistochemistry. Therefore, tumor origin could not be determined.

Case no. 3 presented with a tumor in the breast and peritonitis carcinomatosa 10 years later. The tumor in the breast was diagnosed IDC of the breast based on HE staining. The tumor cells found in the ascites were diagnosed adenocarcinoma based on cytological preparations. CA-125 and WT-1 immunohistochemistry performed on de-stained cytological preparations was not conclusive, therefore, determining the site of the origin of this tumor was not possible.

Case no. 4 presented with a tumor in the breast and tumors in the retroperitoneal lymph nodes as well as in the ovary and uterus three years later. The tumor in the breast was diagnosed IDC of the breast and the tumor in the ovary and uterus serous carcinoma of the ovary, both based on HE staining. The tumor in the retroperitoneal lymph nodes was classified as a large cell carcinoma based on the HE staining. However, only a small biopsy was available, from which no tissue was left in the formalin-fixed paraffin-embedded tissue block for additional analyses.

Characteristics and outcomes of tumor histology and immunohistochemistry of cases and controls are outlined in Table 2. Median age at first cancer diagnosis was 41.5 years (range 33-59). Median year of first cancer diagnosis was 1997 (range 1983-2012). Clinical classification of tumor origins was breast cancer + ovarian cancer in 11, breast cancer + other in two, and breast cancer + ovarian cancer + other in one woman.

Table 2. Cases and controls: patient characteristics and outcomes of tumor histology, immunohistochemistry and molecular a

BRCA1/2+ Age	Tumor sites analyzed, timeline (*years*)			Histology (H&E) with immunohistochemistry (IHC), if applicable			
				Conclusive[2]		Diagnosis[3]	Tumor origin[4]
				H&E	IHC		
Case 1 *BRCA1*+ 41 y	0	Right breast[1]	T1	+		Invasive ductal carcinoma	Breast
	0	Left breast	T2	+		Invasive ductal carcinoma	Breast
	+7	Lung[1]	T3	-	-	Non-small cell carcinoma	Unknown
Case 2 *BRCA1*+ 55 y	0	Ovary	T1	+		Serous carcinoma	Ovary
	+6	Breast[1]	T2	-	ND[6]	Adenocarcinoma	Unknown
Case 3 *BRCA1*+ 33 y	0	Breast[1]	T1	+		Invasive ductal carcinoma	Breast
	+10	Ascites[1]	T2	-	-	Adenocarcinoma	Unknown
Case 4 *BRCA1*+ 38 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast
	+3	Retroperitoneal lymph node	T2	-	ND[6]	Large cell carcinoma	Unknown
	+3	Ovary & uterus	T3	+		Serous carcinoma	Ovary
Control 1 *BRCA1*+ 38 y	0	Right breast[1]	T1	+		Invasive ductal carcinoma	Breast
	+13	Right breast	T2	+		Invasive ductal carcinoma	Breast
	+19	Ovary	T3	-	+	Serous carcinoma	Ovary
Control 2 *BRCA1*+ 49 y	0	Right breast[1]	T1	+		Invasive ductal carcinoma	Breast
	+2	Adnexa	T2	-	+	Serous carcinoma	Ovary
	+23	Left breast	T3	+		Invasive ductal carcinoma	Breast
Control 3 *BRCA1*+ 37 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast
	+2	Ovary	T2	-	+	Serous carcinoma	Ovary
	+5	Ovary & uterus (+inguinal & cervical lymph nodes)	T3	-	+	Serous carcinoma	Ovary
Control 4 *BRCA2*+ 60 y	0	Larynx[1]	T1	+		Squamous cell carcinoma	Larynx
	+2	Lung	T2	-	+	Adenocarcinoma	Lung
	+5	Uterus & omentum	T3	+		Serous carcinoma	Ovary
	+9	Breast	T4	+		Invasive ductal carcinoma	Breast
Control 5 *BRCA1*+ 37 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast
	+6	Ovary	T2	+		Serous carcinoma	Fallopian tube
	+7	Omentum	T3	+		Serous carcinoma	Fallopian tube
Control 6 *BRCA1*+ 41 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast
	0	Cervix	T2	-	+	Adenocarcinoma	Genital tract
	+1	Uterus	T3	-	+	Serous carcinoma	Genital tract
	+1	Omentum	T4	-	+	Serous carcinoma	Genital tract

nalysis

Molecular analysis				Agreement molecular analysis with histopathology
p53 IHC[4]	Variants by next generation sequencing per tumor	Entity[5]	Agreement with loss of heterozygosity analysis	
+	TP53 c.646G>A; p.V216M	1		
-	TP53 c.637C>T; p.R213*	2	+	NA
+	TP53 c.646G>A; p.V216M	1		
-	TP53 c.158G>A; p.W53*	1		
ND[6]	TP53 c.158G>A; p.W53*	1	+/-	NA
ND[6]	TP53 c.686_687del; p.C229fs*10	1		
ND[6]	TP53 c.527G>A; p.C176Y	2	NE	NA
+	TP53 c.318_326delinsAAA; p.S106_F109delinsRN	1		
ND[6]	TP53 c.514G>T; p.V172F	2	+	NA
+	TP53 c.514G>T; p.V172F	2		
-	No mutations	1		
+	TP53 c.722C>T; p.S241F	2		
	PTEN c.176C>G; p.S59*		+	+
+	TP53 c.400T>G; p.F134V	3		
+	No mutations	1		
+	TP53 c.645T>G; p.S215R	2		
+/-	PIK3CA c.3140A>G; p.H1047R	3	NE	+
	STK11 c.484G>A; p.D162N			
+	TP53 c.817C>T; p.R273C	1		
-	TP53 c.406C>T; p.Q136*	2		
-	TP53 c.406C>T; p.Q136*	2	+/-	+
-	TP53 c.375_375+1delinsTT	1		
+	TP53 c.814G>T; p.V272L	2		
	BRAF c.1405_1406delinsTT; p.G469L		+/-	+
-	TP53 c.528C>A; p.C176*	3		
+/-	PIK3CA c.3140A>G; p.H1047R	4		
+	TP53 c.743G>A; p.R248Q	1		
-	TP53 c.395del; p.K132fs*38	2	+/-	+
-	TP53 c.395del; p.K132fs*38	2		
+	TP53 c.743G>A; p.R248Q	1		
-	TP53 c.721del; p.S241fs*6	2		
	CAPZB c.491C>A; p.T164N		+	+
-	TP53 c.721del; p.S241fs*6	2		
	CAPZB c.491C>A; p.T164N			
-	TP53 c.721del; p.S241fs*6	2		

Table 2. Cases and controls: patient characteristics and outcomes of tumor histology, immunohistochemistry and molecular analysis (continued)

BRCA1/2+ Age	Tumor sites analyzed, timeline (*years*)			Histology (H&E) with immunohistochemistry (IHC), if applicable			
				Conclusive[2]		Diagnosis[3]	Tumor origin[4]
				H&E	IHC		
Control 7	0	Ovary	T1	+		Serous carcinoma	Ovary
BRCA1+ 50 y	+4	Breast	T2	+		Invasive ductal carcinoma	Breast
	+4	Rectosigmoid	T3	+		Serous carcinoma	Ovary
Control 8	0	Ovary	T1	+		Serous carcinoma	Ovary
BRCA1+ 42 y	0	Omentum	T2	+		Serous carcinoma	Ovary
	0	Breast	T3	-	+	Invasive ductal carcinoma	Breast
	+1	Abdominal wall (scar) [1]	T4	+		Serous carcinoma	Ovary
	+1	Pleural effusion	T5	-	+	Serous carcinoma	Ovary
Control 9	0	Breast	T1	+		Invasive ductal carcinoma	Breast
BRCA2+ 59 y	+2	Ovary[1]	T2	-	+	Serous carcinoma	Ovary
	+2	Uterus	T3	-	+	Serous carcinoma	Ovary
Control 10	0	Breast	T1	+		Metaplastic carcinoma	Breast
BRCA1+ 45 y	+6	Adnexa	T2	+		Serous carcinoma	Ovary
	+6	Rectouterine pouch	T3	+		Serous carcinoma	Ovary

H&E: haematoxylin and eosin stained slides
IHC: immunohistochemical analysis
NA: not applicable
ND: not done
NE: not evaluable
[1] Low quality sample
[2] Conclusive diagnosis, based on tumor histology (H&E) and immunohistochemistry, if applicable: yes (+) or no (-)
[3] Based on tumor histology (H&E) and immunohistochemistry if applicable
[4] Nuclear expression of p53 in tumor cells was scored as either heterogeneous (+-), strong in all tumor cells (+) or absent in all tumor cells (-)
[5] Entity: tumor or tumors most probably of the same origin (clonally identical). 1 is one independent entity, 2 is a second independent entity, etc. Various tumors that form one entity may present advanced disease, cancer relapse, or distant metastases.
[6] No formalin-fixed paraffin-embedded tissue block available or no tissue left in the formalin-fixed paraffin-embedded tissue block.

	Molecular analysis				Agreement molecular analysis with histopathology
	p53 IHC[4]	Variants by next generation sequencing per tumor	Entity[5]	Agreement with loss of heterozygosity analysis	
	+	TP53 c.722C>A; p.S241Y	1		
	+/-	No mutations	2	+	+
	+	TP53 c.722C>A; p.S241Y	1		
	+	TP53 c.818G>T; p.R273L	1		
	+	TP53 c.818G>T; p.R273L	1		
	ND[6]	TP53 c.524G>A; p.R175H	2	+	+
	ND[6]	TP53 c.818G>T; p.R273L	1		
	+	TP53 c.818G>T; p.R273L	1		
	-	TP53 c.327_328dup; p.R110fs*14	1		
	-	TP53 c.112del; p.Q38fs*6	2		
		FBXW7 c.1347G>C; p.E449D		+	+
	-	TP53 c.112del; p.Q38fs*6	2		
		FBXW7 c.1347G>C; p.E449D			
	+	TP53 c.488A>G; p.Y163C	1		
	+	TP53 c.524G>A; p.R175H	2	+	+
	+	TP53 c.524G>A; p.R175H	2		

Molecular analysis

Outcomes of molecular analysis are depicted in Table 2. The formalin-fixed paraffin-embedded tissues used for DNA isolation were relatively old, ranging from 2 to 32 years old at time of isolation. Six out of 38 (16%) DNA samples isolated from formalin-fixed paraffin-embedded tumor tissue were of low quality (see Supplementary table 2 for quality parameters). For 6 tumors no formalin-fixed paraffin-embedded tissue was available and DNA was isolated from original routine HE and/or immunohistochemical sections or from cytology preparations. Four out of 6 (67%) DNA samples isolated from original sections were of low quality.

In total, 167 tumor-specific variants were detected in the 44 analyzed tumors (Supplementary table 3). Up to 27 variants were detected in the low quality tumor samples, compared to only 1 or 2 variants for good quality tumor samples. Additionally, some multinucleotide changes were incorrectly reported as 2 or 3 separate variants. Finally, 48 mutations were either detected in good quality samples or confirmed in low quality samples. In the majority of tumors (n=34, 77%), one mutation was found; 7 tumors harbored two mutations. Thirty-nine (81%) of all 48 variants concerned a mutation in

Figure 1. Loss of heterozygosity analysis →
Variant allele frequencies for single nucleotide polymorphisms at 15 different loci on 11 different chromosomes (indicated on the y-axis) for the tumors samples of all patients analyzed are shown. The variant allele frequencies for the different single nucleotide polymorphisms are indicated by different colors. The example (bottom right) shows an A/T single nucleotide polymorphism, A representing the reference allele and T the variant allele. For any informative single nucleotide polymorphism without loss of heterozygosity, a variant allele frequency of 0.5 is expected (grey). If there is loss of the reference allele, a variant allele frequency >0.5 is expected (red). Alternatively, loss of the variant allele would result in a variant allele frequency <0.5 (green). A more intense color, either red or green, represents a variant allele frequency deviating further from 0.5, indicating a higher tumor percentage. Regardless of the actual nucleotides, green represents the reference allele and red the variant allele for all single nucleotide polymorphisms. Non-informative single nucleotide polymorphisms or single nucleotide polymorphisms with a strand bias or coverage <100 reads are not shown. If multiple tumors of a patient show largely concordant loss of heterozygosity patterns (all tumors show either red or green), this indicates that these tumors are most likely clonally related. Alternatively, differences in the loss of heterozygosity patterns between multiple tumors of one patient indicate multiple primary tumors.
Twelve patients (all patients except control no. 4 and 9) are *BRCA1* mutation carriers. 10/12 patients show a concordant loss of heterozygosity pattern for the *BRCA1* locus in their multiple tumors. Control no. 2 shows an equivocal loss of heterozygosity pattern, which is probably due to the low quality of the data. For case no. 3 only 1 informative marker is available which does not show clear loss of heterozygosity for T1 (variant allele frequency of 0.41). Control no. 4 and 9 are *BRCA2* mutations carriers. Control no. 9 shows a concordant loss of heterozygosity pattern for the *BRCA2* locus for the three analyzed tumor samples. Control no. 4 shows a concordant loss of heterozygosity pattern for samples T2 and T3, a different loss of heterozygosity pattern for sample T1 and no loss of heterozygosity for T4.
Chr: Chromosome
LOH: loss of heterozygosity

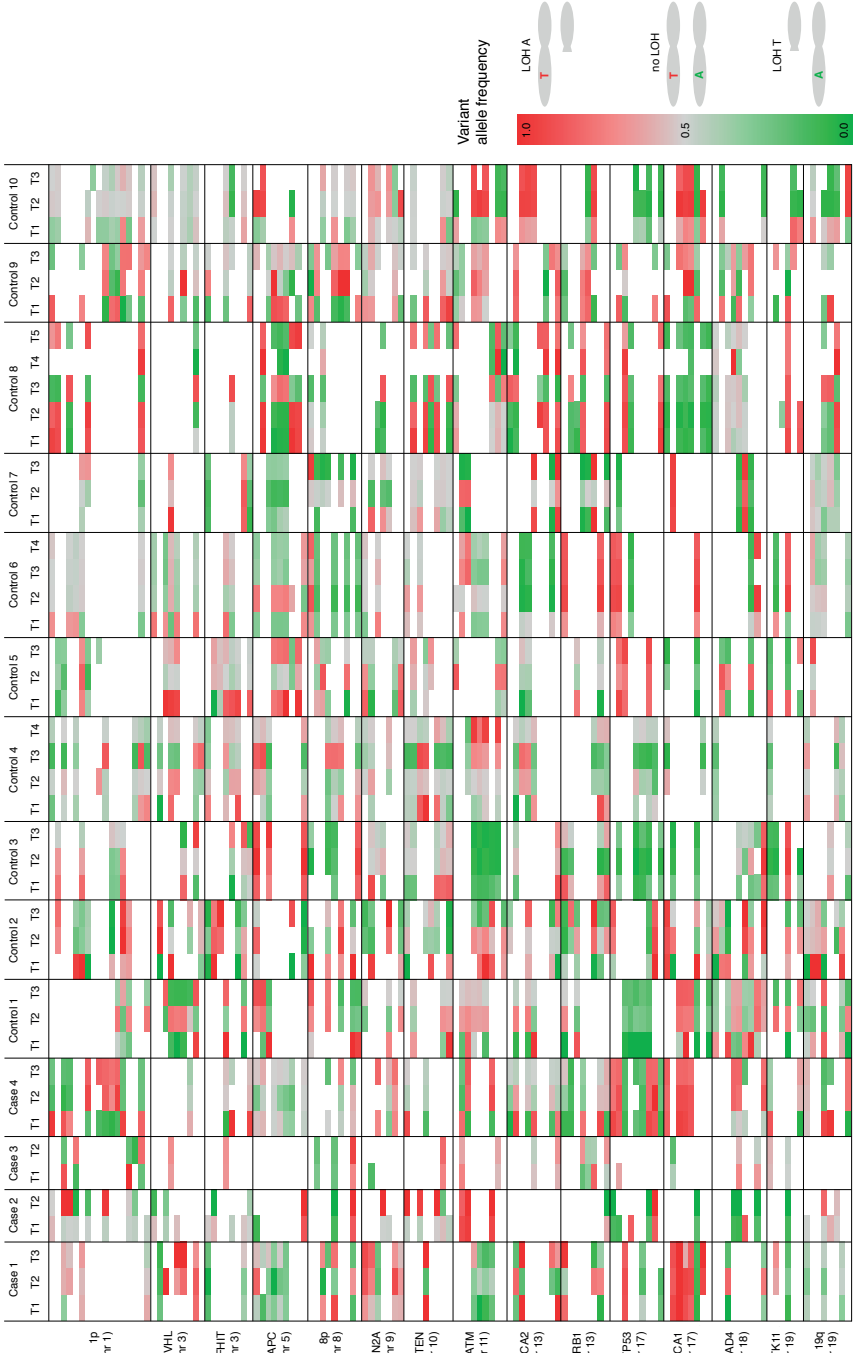

Figure 1. Loss of heterozygosity analysis

the *TP53* gene; in 39 of the 44 tumors (89%) a *TP53* mutation was found. Other variants included *PTEN*,

PIK3CA and *STK11* mutations in tumors located in the breast; a *CAPZB* mutation in tumors in the uterus and cervix; a *FBXW7* mutation in tumors in the ovary and uterus; and a *BRAF* mutation in a lung lesion (Table 2). Parallel to molecular analysis, p53 immunohistochemistry was conducted and showed results consistent with molecular outcomes (Table 2). In two low quality samples with aberrant p53 staining, no *TP53* mutation was detected, probably due to insufficient coverage of *TP53* (<100 reads for 8/19 amplicons for control no. 2, T1) or the type of *TP53* mutation (possible intronic mutation or homozygous deletion for control no. 1, T1).

As further shown in Table 2, based on the molecular analysis, all tumor localizations analyzed could be classified into one or more entities concerning their origins. Additional loss of heterozygosity analyses of the 143 single nucleotide polymorphisms at 15 different loci were confirmative of the classifications made in 8/14 patients (Figure 1; + Supplementary Table 4 showing all single nucleotide polymorphism data). In the group of cases, where conventional histology and immunohistochemistry were not conclusive, molecular outcomes were decisive for all tumors (see Figure 2 for an example). In the group of controls, all molecular outcomes matched the diagnosis given by conventional histopathological diagnostics.

Figure 2. Conventional diagnostics and molecular analysis results for case no. 1 (continued) →
A Both tumors in the breast (T1 and T2) could be conclusively diagnosed invasive ductal carcinoma of the breast based on haematoxylin and eosin (H&E) stainings only. Additionally, ER and HER2NEU stainings are shown, which were negative in both tumors. Conclusive diagnosis regarding the origin of the non-small cell carcinoma in the lung (T3) based on HE stainings and immunohistochemistry (P63 and TTF1 both negative) was not possible.
As part of the molecular analysis p53 immunohistochemistry was performed, showing strong nuclear expression in the tumor cells of T1 and T3, and absent expression in the tumor cells of T2. Scale bars represent 50 µm.
B Targeted next generation sequencing results of *TP53* exon 6 for DNA isolated from normal and tumor tissues of the patient. Each grey line represents an individual read; only aberrations from the wildtype sequence are indicated. Sequencing results are shown in reverse complement, which means that TCG is actually CGA. T1 and T3 show an identical *PT53* missense mutation (c.646G>A; p.V216M), whereas T2 shows a different *TP53* nonsense mutation (c.637C>T; p.R213*).
C Loss of heterozygosity was analyzed using single nucleotide polymorphisms, the variant allele frequencies of 17 single nucleotide polymorphisms at 5 different loci (chromosome 8p, *PTEN, BRCA2*, *BRCA1* and *SMAD4*) are shown for the three tumor samples. Loss of the reference allele is indicated in red and loss of the variant allele in green; a more intense color (either red or green) indicates a higher tumor percentage. As expected for a *BRCA1* germline mutation carrier, all tumor samples show loss of the same *BRCA1* allele. For all other loci shown, T1 and T3 show corresponding loss of heterozygosity patterns (both tumors show either red or green), whereas T2 shows a different loss of heterozygosity pattern.
Chr: Chromosome

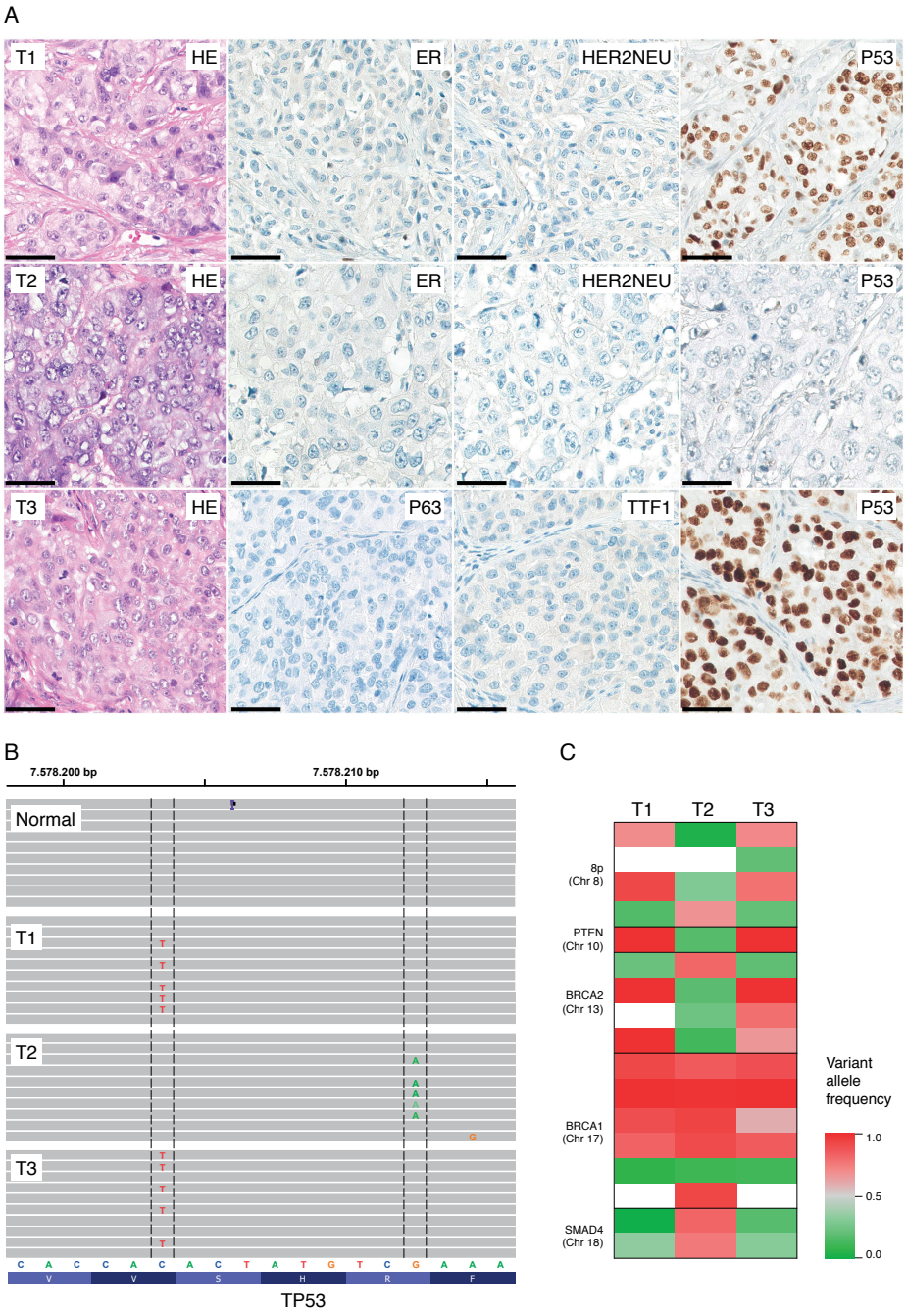

Figure 2. Conventional diagnostics and molecular analysis results for case no. 1
Case no. 1 presented with tumors of the right (T1) and the left (T2) breast, and a tumor in the lung 7 years later (T3).

DISCUSSION

For 38/42 (90%) *BRCA1/2* mutation carriers with multiple tumor localizations, conventional histopathological analyses (histology, immunohistochemistry) were sufficient to determine tumor origins. Results obtained by next generation sequencing provided decisive information in all four cases with inconclusive results from conventional diagnostics, enabling accurate differentiation between a second primary or metastatic cancer. Next generation sequencing conducted on 10 control cases with ≥3 tumor localizations, unequivocally showed the same results as obtained by conventional histopathology, and indicate that next generation sequencing analysis of multiple tumors within one patient is a versatile procedure to determine clonal relationships between the lesions. Next generation sequencing analysis can be useful in case of ambiguous histopathology results, or if no formalin-fixed paraffin-embedded tissue block is available for immunohistochemistry.

As an illustration, the results of two patients are discussed below. First, case no. 2 comprises ovarian cancer followed by thoracic wall and axillary lymph node metastases three years later. There were no signs of breast cancer, suggesting that the ovarian cancer had metastasized to the thoracic wall and the axilla. After another three years, synchronously with progressive metastatic disease, a small breast cancer was detected. After extensive diagnostic work-up it was concluded that thoracic wall and axillary lesions actually were metastases of this formerly subclinical primary breast cancer and the patient was treated accordingly. However, retrospectively, our findings of identical *TP53* variants in the ovarian cancer and breast cancer strongly suggest that the breast cancer was actually metastatic ovarian cancer. Unfortunately, no suitable material of the thoracic wall and axillary lesions was left for molecular analysis in this study. Since the primary tumor origin determines the therapy of choice for metastatic disease, it is essential to have no doubt about the origin of the metastases. The above-mentioned case is an example of how next generation sequencing can be decisive.

Second, control no. 1 comprises two ipsilateral breast cancers with a 13-year interval, both classified as invasive ductal carcinoma by histopathology, and ovarian cancer 6 years later. Histopathological analysis is not always able to differentiate between local recurrent and second primary breast cancer. The location of the breast cancer may help, but in this case, the first breast cancer was located in the medial upper quadrant while the second breast cancer was located centrally, leaving both options open. Some data suggest that *BRCA1/2* mutation carriers, especially when young (<40 years), show longer intervals to local recurrent breast cancer[24, 25]. However, since the prognosis of a second ipsilateral breast cancer occurring <5 years is worse than after >5 years, late-recurring breast cancer are probably more often second primary tumors[26, 27] and it is justifiable that they are treated accordingly. It is likely that the recurrent breast cancer after 13

years in this case was a second primary breast cancer. Molecular analysis confirmed that these tumors were two different entities.

Loss of heterozygosity -patterns were supportive of the results obtained by variant analysis in more than half of cases and controls (Figure 1). Almost all cases and controls showed corresponding loss of heterozygosity of *BRCA1* or *BRCA2* in all tumors, representing the 'second hit' of the functioning *BRCA* wild-type allele. For *BRCA1* mutation carriers, exceptions were case no. 3 with no clear loss of heterozygosity of *BRCA1* for the breast cancer and control no. 2 with no evaluable loss of heterozygosity results. For *BRCA2* mutations carriers, an exception was control no. 4 with four primary tumors showing loss of one allele of *BRCA2* in the larynx tumor, loss of the other allele in both the lung tumor and the uterus/omentum tumor, and no loss of *BRCA2* in the breast tumor. So far, *BRCA2* mutation carriers are not associated with elevated risk of lung cancer and an increased risk of laryngeal carcinoma seems improbable[28-31]. Additional Sanger sequencing showed loss of the mutated *BRCA2* allele for the tumor located in the larynx and loss of the wild-type allele for the lung lesion and the uterus/omentum tumor localizations (data not shown). The laryngeal carcinoma therefore is most likely a sporadic tumor. Loss of the wild-type *BRCA2* allele in the lung tumor may indicate either sporadic or *BRCA2*-related carcinogenesis. Furthermore, it has been described that loss of heterozygosity causes the second hit in only 80% of *BRCA1*-associated and in 60-70% of *BRCA2*-associated breast cancer[32,1,33], fitting with the fact that we did not find (clear) loss of heterozygosity in two breast tumors. Possible alternative 'second hit' mechanisms include mutations and deletions of the wild-type allele. Epigenetic silencing as a second hit, to our knowledge, is rare in germline *BRCA1/2* mutation carriers and therefore not a plausible explanation[32].

The diagnostic panel used in this study covered the exonic regions of the genes *CDKN2A*,

PTEN and *TP53* almost completely, multiple hotspot mutation sites for 27 genes, and single nucleotide polymorphisms (Supplementary Table 1). In the majority of cases and controls a conclusive diagnosis concerning tumor site clonality could be made based on different or similar *TP53* variants. A *PTEN* mutation was only found once and none of the tumors harbored *CDKN2A* mutations. Up to 97% of all high grade serous ovarian cancer, typically occurring in *BRCA1/2* germline mutation carriers, harbor somatic *TP53* mutations[13, 34]. *TP53* is affected in 16% to 84% of *BRCA1/2*-associated breast cancer, and in up to 97% of *BRCA1*-associated basal-like breast cancer[35,36]. Our finding of *TP53* mutations in 93% of all tumors (39/44 confirmed and 2/44 based on p53 immunohistochemistry) is in line with the high percentages found in the literature. It suggests that molecular diagnostic workup may simply consist of *TP53* analysis, rather than next generation sequencing of an entire panel. However, in two tumors without *TP53* mutations, we found mutations in other genes (*PIK3CA* and *STK11*), providing also a conclusive diagnosis for

these tumor localizations. Additionally, loss of heterozygosity analysis was not only confirmative of the classifications made for most of the patients, but was also helpful if 'hotspot' *TP53* mutations were found. An example is control no. 10, for which both T2 and T3 harbor a *TP53 R175H* mutation. Since according to somatic mutation databases this is a common *TP53* mutation these tumors potentially could still be different primary tumors. However, because loss of heterozygosity patterns were identical, we were able to reliably classify these tumors as one entity.

Immunohistochemical tissue markers were chosen according to institutional protocol depending on clinical and histological differential diagnosis of the tumor origin. Various different immunohistochemical markers of breast cancer have been investigated, such as GATA3, GCDFP, mammaglobin and SOX10. Although of potential value for differentiating breast cancer, as yet, their applicability seems limited or has not been validated well enough in triple negative breast cancer [37-39].

A limitation of our study was that 10/44 tumor samples analyzed with next generation sequencing were of low quality, mostly due to fixation artefacts or a low amount of starting material, resulting in less reliable variant calling. Variants in low quality samples were therefore confirmed by Sanger sequencing or by a second next generation sequencing run. Furthermore, loss of heterozygosity analysis of these samples was difficult, resulting in non-evaluable loss of heterozygosity data in two patients with one or more tumor samples of low quality. Nevertheless, using a combined approach of multiple molecular analyses resulted in reliable classification of the tumors into one or more entities for all patients. Another limitation was that, due to the specific selection criteria, the study sample size was small.

In conclusion, during diagnostic workup of *BRCA1/2*-associated breast cancer and ovarian cancer patients with multiple tumor localizations, analysis of tumor histology and immunohistochemistry by a specialized pathologist may be sufficiently conclusive in most cases. However when routine pathology is inconclusive, molecular analysis using next generation sequencing can reliably determine the relationships between the tumor localizations and as such guide the most appropriate treatment for each individual patient.

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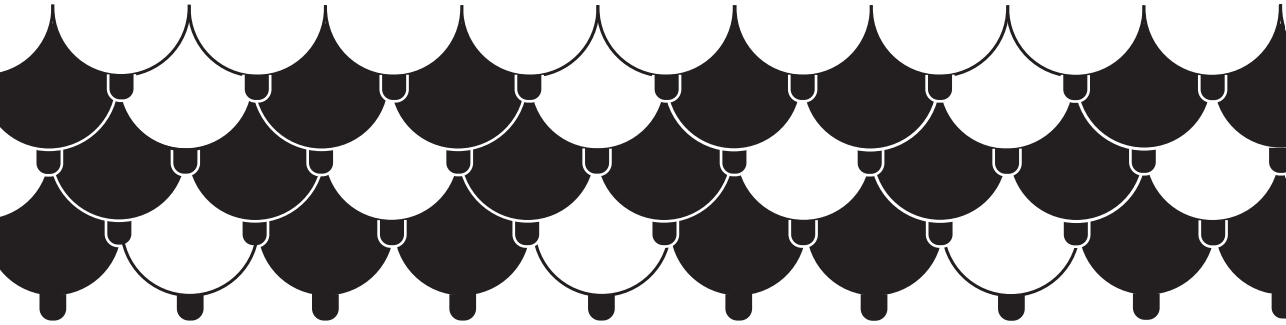

CHAPTER 6

β-catenin expression as a prognostic marker in *BRCA1/2*-associated breast cancer

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Submitted

ABSTRACT

Background The majority of *BRCA1/2*-associated breast cancers (BC) is of high histologic grade and most *BRCA1*-associated BCs are triple-negative. Therefore, the value of conventional prognostic markers such as grade and hormone receptor status is limited in these patients. The aim of the present study was to assess the presence and prognostic significance of β-catenin expression in *BRCA1/2*-associated BC patients.

Methods We included female *BRCA1/2* gene mutation carriers diagnosed with BC between 1982 and 2014. Patient demographics, tumor characteristics and recurrence-free survival (RFS) were extracted from an ongoing institutional database. BC slides were centrally reviewed for histologic subtype and grade. Immunohistochemical β-catenin staining was classified as membranous, cytoplasmic and/or nuclear staining, and scored as negative/weak, moderate or strong.

Results Ninety-two *BRCA1* (73%) and 28 *BRCA2* (27%) mutation carriers were included. Median follow-up was 7.7 years (range 0.6-32.6). Thirty-eight per cent of BCs showed nuclear β-catenin expression. In univariable analyses, tumor size and membrane-associated β-catenin staining (HR 0.11 for strong vs. negative/weak; 95%CI 0.01-0.97) were significantly associated with RFS. In multivariable analyses, the only significant prognostic factor remained strongly positive membrane-associated β-catenin staining (HR 0.19; 95%CI 0.05-0.73).

Conclusion The presence of nuclear β-catenin expression in a substantial proportion of *BRCA1/2*-associated BC suggests a potential role of Wnt signalling in hereditary breast cancer. Besides, membranous b-catenin expression was associated with a favorable RFS. Confirmation of these results in larger series may improve risk stratification in these patients.

BACKGROUND

Women with germ line *BRCA1* or *BRCA2* gene mutations have cumulative lifetime risks of 55-85% of a first breast cancer (BC) and 20-60% of a contralateral BC by the age of 70 years[1-5]. Therefore, these women may opt for either risk-reducing mastectomy or frequent BC screening. Modern screening programs for *BRCA1/2* gene mutation carriers comprise alternating mammography and magnetic resonance imaging (MRI). As a result, the majority of *BRCA1/2*-associated BC is detected in an early stage (43% is detected at stage T1a/b and 79% is lymph node negative)[6].

Both *BRCA1*- and *BRCA2*-associated BC typically are poorly differentiated[7-9] and about 70% of *BRCA1*-associated tumors are triple-negative (negative for estrogen receptor (ER), progesterone receptor (PR) and HER2[9]. These findings limit the value of established prognostic markers for risk stratification in *BRCA1/2*-associated BC. Therefore, the identification of additional prognostic parameters is warranted.

Wnt signaling, mediated by β-catenin, is known to play an important role in tumorigenesis of colorectal cancers and several other solid tumors including breast cancer[10, 11]. Beside induction of tumorigenesis, the Wnt pathway accounts for the regulation of a broad range of cell functions such as proliferation, survival and cell matrix modeling[10].

In the absence of Wnt, an intracellular complex is formed containing the proteins GSK3β, APC and Axin. This complex binds and phosphorylates membranous β-catenin, which is then ubiquitinated and destructed. When Wnt is activated, the GSK3β/Axin/APC destruction complex cannot be formed. Unphosphorylated β-catenin thus accumulates in the cytoplasm where it enters the nucleus[10, 12, 13]. Previous studies on Wnt activity as a prognostic factor in BC, based on the expression of β-catenin, yielded conflicting results[13-19]. There is some evidence that the prognostic value of Wnt signaling depends on the molecular BC subtype and may be stronger in basal-like BC[20, 21], which suggests a potential prognostic value of the Wnt/β-catenin pathway in *BRCA*-associated BC. Besides, several members of the Wnt cascade were reported to be deregulated in a substantial proportion of breast carcinomas by methylation rather than by specific gene mutations, which makes it an attractive potential target for pharmacological inhibition[22].

Another role of membranous b-catenin concerns cell-cell adhesion through the binding of E-cadherin on the extracellular cell membrane. Loss of b-catenin at the membrane may lead to epithelial-mesenchymal transition (EMT), which is required for metastasis of tumor cells[17]. The b-catenin that binds E-cadherin is likely distinct from the b-catenin that mediates Wnt signaling[23]. The aim of the present study was to assess Wnt activity, by using nuclear β-catenin expression as a read-out, in *BRCA1/2*-associated BC. Second, we tested the value of membranous, cytosolic and nuclear β-catenin expression as a potential novel prognostic marker in this subgroup.

METHODS

Patients

After written informed consent, the Family Cancer Clinic of the Erasmus MC Cancer Institute registers patients with an increased BC risk in an institutional ongoing database. From this database we selected women with a proven *BRCA1/2* gene mutation who were diagnosed with BC between 1982 and 2014 and for whom BC tumor material was available for immunohistochemical staining.

In- and exclusion criteria have been described before[24]. In short, patients with a history of a malignancy other than BC at the time of BC diagnosis except for basal cell carcinoma, neoadjuvant chemotherapy or radiotherapy administered for the primary BC or distant metastasis at the time of BC diagnosis were excluded. Women with contralateral BC were included in the analysis since low stage contralateral BCs, as typically detected during screening in *BRCA1/2* gene mutation carriers, do not influence primary breast cancer prognosis[25]. In case of synchronous contralateral BC, the tumor with the highest stage was included. Patient history, mutation carriership (*BRCA1* or *BRCA2)*, age and year of BC diagnosis and data on locoregional/distant disease recurrence were extracted from the Family Cancer Clinic database. Data on tumor size and lymph node metastases were extracted from pathology reports.

Histopathological features

Formalin-fixed paraffin-embedded primary BC tissues were collected from the departments of pathology of the Erasmus MC Cancer Institute. A breast pathologist (C.v.D.) reviewed haematoxylin and eosin (H&E) stained tissue sections for assessment of histologic subtype (according to the WHO) and histologic grade (Nottingham modification of Bloom and Richardson)[26].

ER, PR and HER2 status were extracted from pathology reports or assessed from Tissue Micro Arrays (TMA) using three cores per tumor. ER and PR were considered positive if staining was seen in ≥10% of the nuclei, according to the Dutch national BC guidelines[27]. HER2 receptor status was scored according to international guidelines[28].

β-catenin staining

A breast pathologist (C.v.D.) assessed immunohistochemical β-catenin staining (BD 610154 14/Beta-Catenin monoclonal antibody; dilution 1:200) of tumor cell membrane, cytoplasm and nucleus, using a positive and negative control (colon carcinoma and lobular BC respectively). Normal epithelial breast tissue was used as an internal reference regarding intensity of membrane staining. The scoring method was adapted from Khramtsov et al.[20], resulting in a semi quantitative final score for membrane and cytoplasmic β-catenin staining. The final score (1-7) consisted of the sum of separate scores

for intensity of staining (0: no staining – 3: maximum staining intensity) and for the percentage of tumor cells that show β-catenin staining (1:0-10%, 2: 11-30%, 3: 31-70%, 4: 71-100%). Three categories were derived from the final score: 1-3: negative/weakly positive, 4-5: moderately positive and 6-7: strongly positive β-catenin staining.

A binary score was used for nuclear β-catenin staining (0: no nuclear staining, 1: nuclear staining in any tumor cells).

Statistical analysis

The statistical methods used in this study were described before[24]. Lobular BCs were used as negative control for β-catenin staining. Therefore, to prevent confounding women with lobular BC were excluded. The main outcome of interest was recurrence-free survival (RFS), defined as the time interval between date of primary BC surgery and locoregional or distant BC relapse. To avoid potential survival bias due to inclusion of patients who underwent genetic testing after BC diagnosis left truncation was used[29]. Patients were censored at last follow-up or at death. We estimated hazard ratios and 95% CIs for established prognostic factors and additional histologic parameters using Cox regression in univariable and multivariable analysis. Variables with a P-value < 0.10 in univariable analysis were included in the multivariable model. We performed subgroup analyses on *BRCA1* gene mutation carriers and on triple-negative BC patients. Associations of categorical data were analyzed using a Chi-Square test. In all analyses, age at and year of diagnosis were analyzed as continuous variables. Analyses were performed with STATA version 13.1.

RESULTS

Patient and tumor characteristics

In total, 146 female *BRCA*1/2 mutations carriers were selected from the database. Of them, 16 women were excluded due to missing tumor material. Two had metastatic disease at the time of diagnosis. Eight had lobular BC and were excluded. Characteristics of the remaining 120 BC patients are shown in Table 1. The group consisted of 92 *BRCA1* (77%) and 28 *BRCA2* (23%) mutation carriers. Median age at BC diagnosis was 38.5 years (range 21-68). Median follow-up was 7.7 years (range 0.6-32.6) since primary BC diagnosis. During follow-up, 9 patients (8%) were affected by recurrent (local or distant) BC. The majority of the patients (83; 69%) had a pT1 tumor (≤2.0 cm diameter) and a negative nodal status (94; 78%). The majority of tumors were grade 3 (78; 65%). Seventy-seven (64%) BCs were triple-negative (71 *BRCA1*, 77%; 6 *BRCA2*, 21%).

Table 1. Patient and tumor characteristics of *BRCA1/2*-associated breast cancers

	N	%	*BRCA1*	%	*BRCA2*	%
Total	120		92	77%	28	23%
Age at diagnosis, years	38.0	21-68	37.0	23-65	42.0	21-68
(median, range)						
Tumor size						
≤ 2.0 cm	83	69%	62	67%	21	75%
2.0 – 5.0 cm	31	26%	27	29%	4	14%
> 5.0 cm	4	3%	2	2%	2	7%
Unknown	2	2%	1	1%	1	4%
Nodal status						
Negative	94	78%	76	83%	18	64%
Positive	26	22%	16	17%	10	36%
Histological type						
Ductal	111	92%	85	92%	26	93%
Other	9	8%	7	8%	2	7%
Bloom and Richardson grade						
I	5	4%	1	1%	4	14%
II	35	29%	22	24%	13	46%
III	78	65%	68	74%	10	36%
Unknown	2	2%	1	1%	1	4%
Estrogen receptor status						
Negative	79	66%	72	78%	7	25%
Positive	38	32%	18	20%	20	71%
Unknown	3	2%	2	2%	1	4%
Progesterone receptor status						
Negative	90	75%	80	87%	10	36%
Positive	6	22%	10	11%	16	57%
Unknown	4	3%	2	2%	2	7%
HER2 status						
Negative	108	90%	82	89%	26	93%
Positive	8	7%	7	8%	1	4%
Unknown	4	3%	3	3%	1	4%
Triple-negative receptor status						
No	40	33%	19	21%	21	75%
Yes	77	64%	71	77%	6	21%
Unknown	3	3%	2	2%	1	4%
Membrane-associated β-catenin						
Negative/ weakly positive	3	3%	3	3%	0	0%
Moderately positive	21	17%	11	12%	10	36%
Strongly positive	96	80%	78	85%	18	64%
Cytoplasmic β-catenin						

Table 1. Patient and tumor characteristics of *BRCA1/2*-associated breast cancers (continued)

	N	%	*BRCA1*	%	*BRCA2*	%
Negative/ weakly positive	25	21%	16	17%	9	32%
Moderately positive	73	61%	56	61%	17	61%
Strongly positive	22	18%	20	22%	2	7%
Nuclear β-catenin						
Negative	73	61%	50	54%	23	82%
Positive	46	38%	41	45%	5	18%
Unknown	1	1%	1	1%		

Nucleus-associated β-catenin staining was classified as 'negative' in 61% vs. 'positive' in 38% of cases. In one case the classification of nuclear β-catenin staining remained unclear (1%). In all cases scored as 'positive' for nuclear staining, only a small proportion of cells were positive (<5%), as illustrated in Figure 1. None of the cases showed stromal nuclear staining.

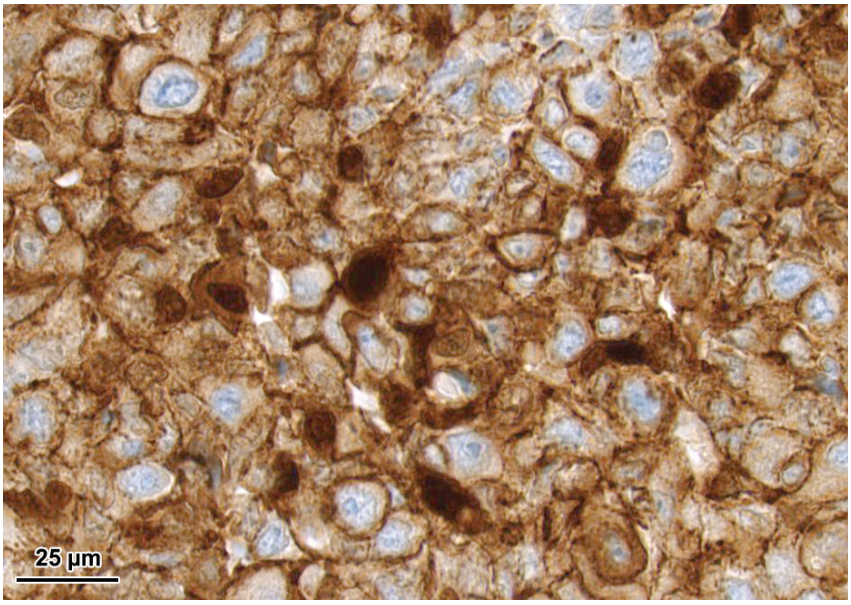

Figure 1. An example of a breast cancer case with positive nucleus-associated β-catenin staining

Cytoplasmic β-catenin staining was classified as 'moderately positive' in the majority of cases: 61%, vs. 21% 'negative/weakly positive' and 18% 'strongly positive'. Membranous β-catenin staining was classified as 'strongly positive' in the majority of cases: 80%, vs. 17% 'moderately' and 3% 'negative/weakly positive'. Figure 2 provides an example of a BC with strong membranous β-catenin staining (A) and negative/weak staining (B).

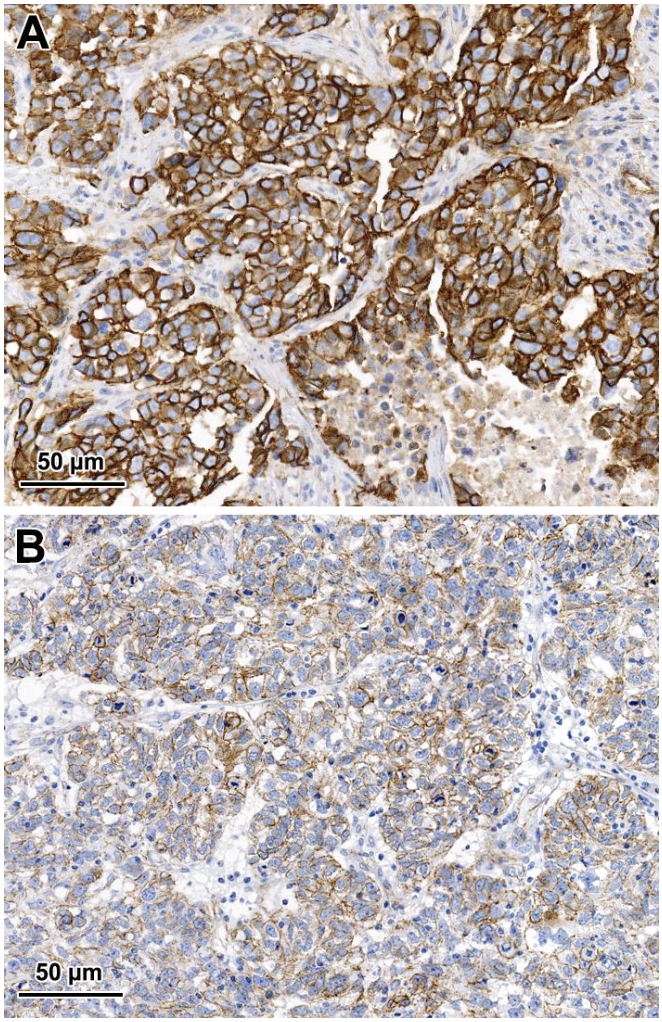

Figure 2. Examples of breast cancer cases with A. strong membranous β-catenin staining and B. negative/weak membranous β-catenin staining

Membranous b-catenin was not significantly associated with histologic grade (P= 0.17; Chi-square test). There was a significant association of nuclear b-catenin expression with triple-negative receptor status (P<0.001; Chi-square test). Loss of membranous b-catenin expression was not correlated with nuclear b-catenin expression (P 0.67).

β-catenin staining in relation to recurrence-free survival (RFS)

In univariable analyses (Table 2), a larger tumor size was related to a poor RFS (HR 1.06 per millimeter; 95%CI 1.02-1.10). Strong membranous β-catenin staining was a favorable prognostic factor (HR 0.11; 95%CI 0.01-0.97). Five-year RFS was 90% in the group

with strong membranous b-catenin staining; vs. 51% in the groups with negative/weak and moderate membranous b-catenin staining (Figure 3). Nuclear β-catenin staining was of no prognostic value.

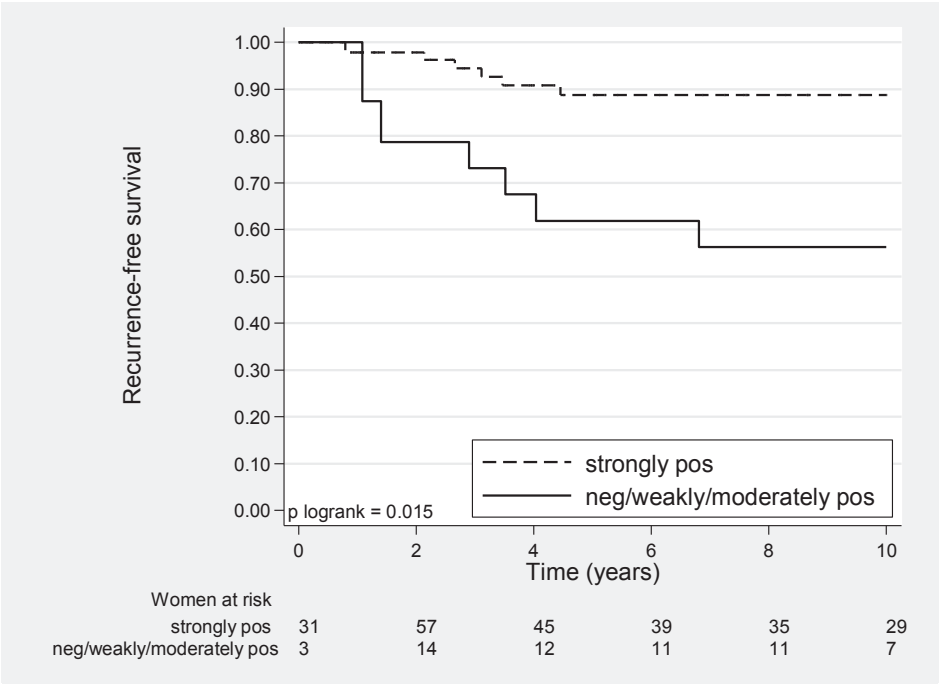

Figure 3. Recurrence free survival (RFS) in *BRCA1/2*-associated breast cancer patients by membranous β-catenin staining
Numbers of women at risk increase during the first years of observation because of left truncation, which was used to avoid potential survival bias due to inclusion of patients who underwent genetic testing after breast cancer diagnosis.

In multivariable analyses, strong membranous b-catenin staining remained the only significant prognostic factor with favorable impact on RFS (HR 0.19; 95%CI 0.05-0.73).

β-catenin staining in the subgroup of *BRCA1*-associated breast cancers

Patient characteristics of the *BRCA1*-associated subgroup are shown in Table 1. In univariable analyses (Table 3), a larger tumor size (HR 1.15 per millimeter; 95%CI 1.07-1.25) was associated with a poor RFS. Strongly positive membrane β-catenin staining was a favorable prognostic factor (HR 0.13; 95%CI 0.03-0.60). In multivariable analyses, the only significant factor remained a larger tumor size (HR 1.14 per millimeter; 95%CI 1.04-1.24).

Table 2. Clinicopathologic features of *BRCA1/2*-associated breast cancers in relation to recurrence-free survival (RFS)

	N	Univariate		Multivariable	
		HR	95% CI	HR	95% CI
Age at diagnosis (years)*		0.92	0.84 – 1.00**	0.97	0.89 – 1.05
BRCA mutation					
BRCA1	92	1.0			
BRCA2	28	0.72	0.15 – 3.48		
Year of diagnosis (years)*		0.96	0.83 – 1.13		
Tumor size (mm)*		1.06	1.02 – 1.10**	1.05	1.00 – 1.11
Nodal status					
Negative	94	1.0			
Positive	26	2.33	0.58 – 9.31		
Histological type					
Ductal	111	1.0			
Other	9	2.68	0.33 – 21.5		
Bloom and Richardson grade					
I/II	40	1.0			
III	78	1.55	0.32 – 7.49		
Estrogen receptor status					
Negative	79	1.0			
Positive	38	1.07	0.27 – 4.30		
Progesterone receptor status					
Negative	90	1.0			
Positive	26	1.09	0.23 – 5.26		
HER2 status					
Negative	108	1.0			
Positive	8	1.59	0.20 – 12.7		
Membrane-associated β-catenin					
Negative/ Weakly positive	3	1.0			
Moderately positive	21	0.58	0.06 – 5.70		
Strongly positive	96	0.11	0.01 – 0.97**	0.19***	0.05 – 0.73
Cytosolic β-catenin					
Negative/ Weakly positive	25	1.0			
Moderately positive	73	0.33	0.08 – 1.33		
Strongly positive	22	0.38	0.04 – 3.39		
Nuclear β-catenin					
Negative	73	1.0			
Positive	46	1.57	0.42 – 5.90		

HR: Hazard Ratio; 95%CI: 95% confidence interval
*analyzed as a continuous variable
**entered in multivariable analysis
***strong vs negative/weak/moderate

Table 3. Clinicopathologic features of *BRCA1*-associated breast cancers (N=92) in relation to recurrence-free survival (RFS)

	N	Univariate		Multivariable	
		HR	95% CI	HR	95% CI
Age at diagnosis (years)*		0.89	0.78 – 1.02		
Year of diagnosis (years)*		1.00	0.84 – 1.19		
Tumor size (mm)*		1.15**	1.07 – 1.24	1.14	1.04 – 1.24
Nodal status					
Negative	76	1.0			
Positive	16	4.89	1.09 – 21.9		
Estrogen receptor status					
Negative	72	1.0			
Positive	18	0.87	0.10 – 7.22		
HER2 status					
Negative	82	1.0			
Positive	7	1.51	0.18 – 12.6		
Membrane-associated β-catenin					
Neg/Weakly/Moderately positive	14	1.0			
Strongly positive	78	0.13**	0.03 – 0.60	0.49	0.07 – 3.21
Cytosolic β-catenin					
Negative/ Weakly positive	16	1.0			
Moderately/ Strongly positive	76	0.57	0.11 – 2.96		
Nuclear β-catenin					
Negative	50	1.0			
Positive	41	1.93	0.43 – 8.66		

HR: Hazard Ratio; 95% CI: 95% confidence interval
*analyzed as a continuous variable
**entered in multivariable analysis

DISCUSSION

To our knowledge, this is the first study investigating β-catenin expression in *BRCA1* and *BRCA2*-associated BC. A substantial proportion of BC cases showed nuclear staining and strong membranous staining was a potential favorable marker for RFS.

In line with our results, previous studies on sporadic BC reported patterns of reduced membranous b-catenin expression being associated with a worse outcome[16, 19, 20, 30, 31]. These studies are in conflict with reports that did not find a clear association between β-catenin expression and prognosis[14, 32]. The finding of reduced membranous b-catenin expression as an unfavorable prognostic marker is unlikely to be correlated with Wnt activation. If Wnt activation would play an important role, β-catenin expression should

not only be reduced at the membrane, but should become positive in the nucleus, since nuclear expression is seen as a read-out of Wnt activation[10]. In the present study we did not find an association of loss of membranous β-catenin expression with marked nuclear β-catenin expression. As membranous b-catenin also plays a role in cell-cell adhesion through binding of E-cadherin, reduced membranous b-catenin expression may be a marker of loss of epithelial differentiation, and in this way be a marker of poor prognosis[17, 31]. A substantial proportion of BCs (38%, mainly triple-negative cases) showed nuclear β-catenin expression, which seemed of no prognostic value. However, we never found nuclear expression in more than a few tumor cells per case. In BC, data are limited regarding nuclear b-catenin expression as a prognostic marker[13, 33], but an association between nuclear b-catenin expression and poor outcome has been reported in basal-like BC[20]. Nonetheless, in sporadic BC, there is ample evidence for a role of aberrant Wnt activation in tumorigenesis[12, 34], although the exact mechanism remains to be elucidated.

No studies have been published examining the presence and significance of b-catenin expression in *BRCA1/2*-associated BC. As described before, this subgroup of patients lacks valuable prognostic markers and therapeutic targets, due to the high rate of early-detected (node-negative) BC in combination with a high rate of high grade and triple-negative cancers. Therefore, the identification of additional prognostic parameters is warranted.

This study has several limitations. Although the group of *BRCA1/2* gene mutation carriers was relatively large and median follow-up was 7.7 years, there were only 9 events of locoregional or distant disease recurrence. This limited the amount of covariates entered in the multivariable analyses and probably has limited the statistical power of our outcomes. Further, the scoring and classification system used in this study, which was adapted from Khramtsov et al., has never been validated. Some authors advocate using a computerized quantitative scoring system[16]. A more universal and validated classification system is warranted to be able to compare and combine different study results.

In conclusion, we reported nuclear β-catenin expression in a substantial proportion of (mainly triple-negative) BC, which suggests a potential role of Wnt activity in *BRCA1/2*-associated BC. Confirmation of these results, preferably by the identification of target genes may help to identify potentially therapeutic markers. Besides, membranous β-catenin expression was associated with a favorable RFS, which could improve risk stratification in this patient population.

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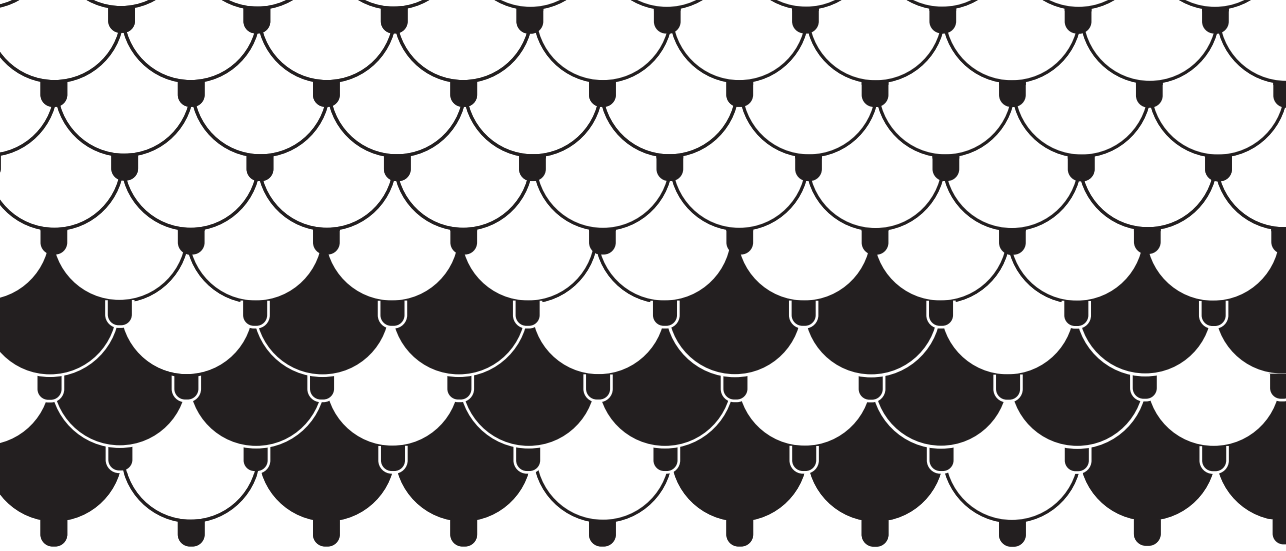
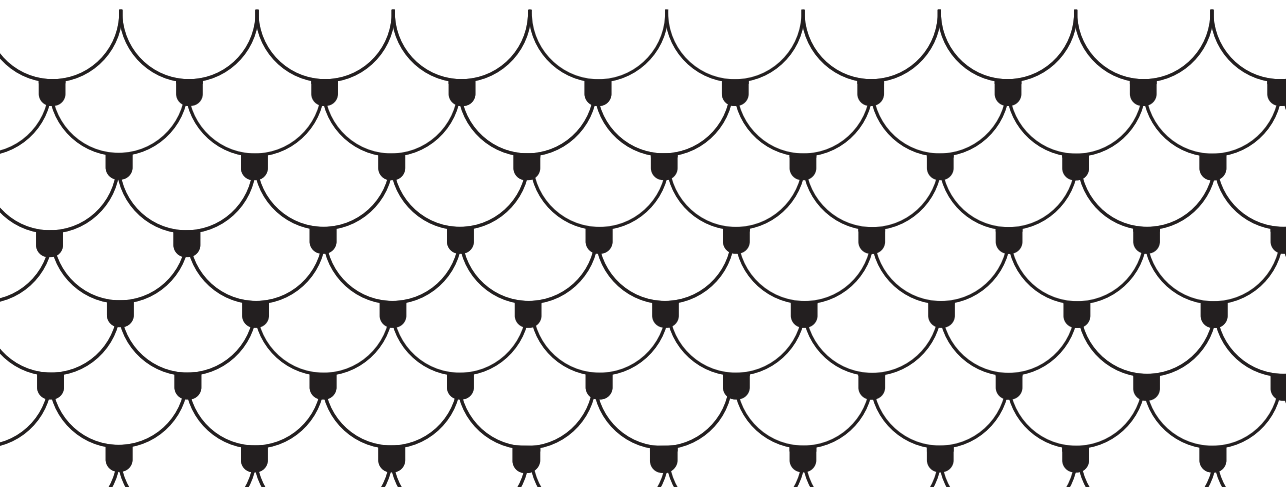
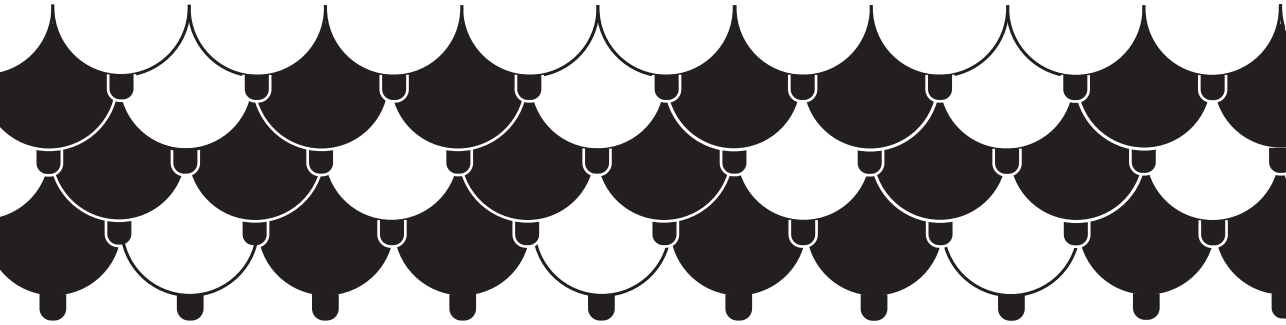




ANATOMY OF THE BREAST AND NIPPLE



CHAPTER 7

Prophylactic nipple-sparing mastectomy leaves more terminal duct lobular units in situ as compared to skin-sparing mastectomy

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ABSTRACT

Prophylactic skin-sparing mastectomy (SSM) is associated with major breast cancer risk reduction in high-risk patients. In prophylactic nipple- and skin-sparing mastectomy (NSM) it is unknown how many terminal duct lobular units (TDLUs) remain behind the nipple-areola complex (NAC) additionally to those behind the skin flap. Therefore safety of NSM can be doubted. We compared amounts of TDLUs behind the NAC as compared to the skin. In prophylactic SSM and conventional therapeutic mastectomy patients, the NAC and an adjacent skin island (SI) were resected as if it were an NSM. NAC and SI were serially sectioned perpendicularly to the skin and analyzed for the amount of TDLUs present. Slides of NAC and SI were scanned and slide surface areas (cm^2) were measured. TDLUs/cm^2 in NAC- versus SI-specimen, representing TDLU density, were analyzed pair-wise. In total 105 NACs and SIs of 90 women were analyzed. Sixty-four NACs (61%) vs. 25 SIs (24%) contained ≥1 TDLUs. Median TDLU density was higher in NAC-specimens (0.2 TDLUs/cm^2) as compared to SI-specimens (0.0 TDLUs/cm^2; $P<0.01$). Independent risk factors for presence of TDLUs in the NAC-specimen were younger age and parity (versus nulliparity). The finding of higher TDLU density behind the NAC as compared to the skin flap suggests that sparing the NAC in prophylactic NSM in high-risk patients possibly may increase postoperative breast cancer risk as compared to prophylactic SSM. Studies with long-term follow-up after NSM are warranted to estimate the level of residual risk.

BACKGROUND

BRCA1/2 mutation carriers have a cumulative lifetime breast cancer risk of 55-85% by the age of 70[1-5]. As an alternative to surveillance, *BRCA1/2* mutation carriers and other high risk patients may choose to undergo prophylactic bilateral mastectomy and breast reconstruction, achieving risk reductions of 90-100% after 3-13 years of follow-up[6-10].

To allow for direct breast reconstruction after mastectomy, the technique of skin-envelope preserving mastectomy (skin-sparing mastectomy; SSM) aims to spare the skin of the breast. In SSM, a periareolar incision is used (see Figure 1A and B, which show a circular (A) and a drop-shaped (B) incision as used in SSM). Breast glandular tissue is excised subcutaneously creating a skin envelope while preserving a thin subcutaneous layer to support skin vascularization. Nipple-papilla and surrounding pigmented areola (nipple-areola complex; NAC) are removed. Additionally, a breast reconstruction is performed. SSM is generally considered oncologically safe for prophylactic and therapeutic indications, although no prospective randomized controlled trials have been conducted[6-11].

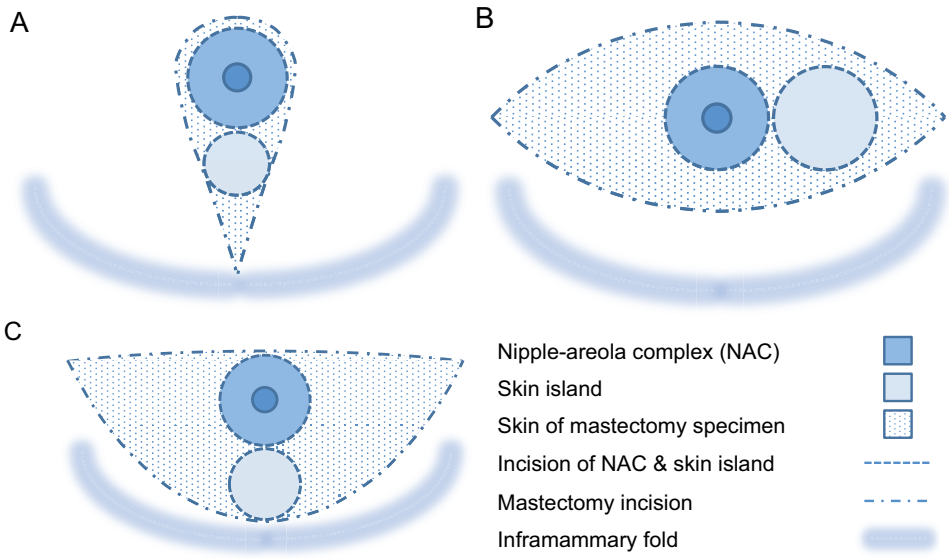

Figure 1 Examples of incisions around the nipple-areola complex (NAC) and skin island (SI) in **A** skin-sparing mastectomy, **B** and **C** in conventional, non-skin-sparing mastectomies

Preservation of the NAC in SSM (nipple-sparing mastectomy; NSM) may further improve cosmetic outcome and patient satisfaction[12]. In NSM, a semicircular incision is used to create a skin envelope as in SSM (see Figure 2C, which shows a semicircular incision as used in NSM). The NAC is dissected as thin as possible by macroscopically removing all breast glandular tissue while preserving vascularization (see Figure 3A, which shows intra-operative dissection of the NAC). The nipple-papilla is 'cored' by inverting it and

excising residual breast glandular tissue (see Figure 3B, which illustrates the 'coring' of the nipple-papilla)[13]. The NAC is then left in situ adherent to the skin envelope. NSM has been reported safe for selected patients with small, peripherally located breast cancers[14]. However, long-term oncological safety of NSM for breast cancer prophylaxis in *BRCA1/2* mutation carriers and other women with high breast cancer risk is still subject to debate. Various authors have found terminal duct lobular units (TDLUs) in and/or closely behind the NAC[15-17]. TDLUs in the mammary gland are defined as a terminal duct combined with an associated lobule and are known as the origin of invasive breast cancer[18, 19]. Theoretically, any residual TDLUs behind skin or behind NAC may remain a lifelong potential hazard for developing DCIS or invasive breast cancer. Although SSM is considered a safe risk-reducing option, residual TDLUs have also been found behind skin flaps after SSM[20]. Several studies have reported safety of NSM in high-risk patients, but they lack follow-up[21, 22]. Lifelong follow-up of *BRCA1/2* mutation carriers would be necessary to estimate the remaining oncological risk of any residual breast tissue after prophylactic surgery and particularly NSM. We compared presence and amounts of TDLUs remaining behind the NAC with presence and amounts of TDLUs behind the skin flap, to assess whether NSM would lead to a significant increase in remaining TDLUs as compared to SSM.

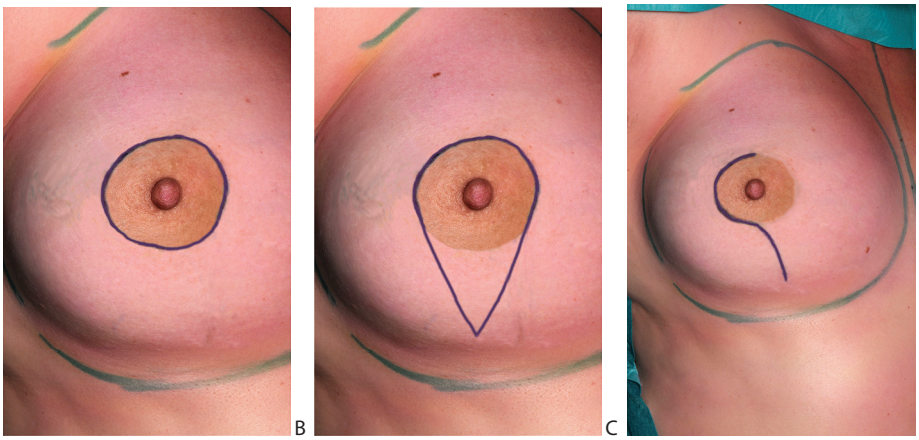

Figure 2 Incisions used for **A** and **B** skin-sparing mastectomy (SSM) and **C** nipple-sparing mastectomy (NSM)

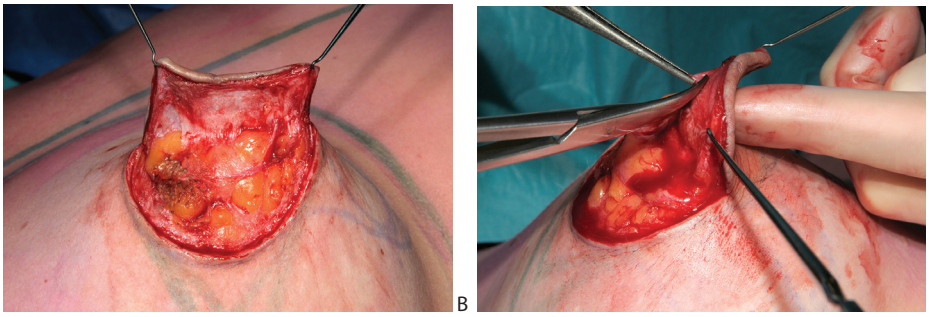

Figure 3 Sparing the nipple-areola complex (NAC) in nipple-sparing mastectomy (NSM). **A** Dissecting the nipple-areola and **B** Coring the nipple-papilla

METHODS

Patients

Women ≥18 years who underwent conventional mastectomy or non-nipple-sparing SSM for prophylactic or therapeutic indications were eligible. Exclusion criteria were gross abnormalities of the nipple (flat, inverted, retracted or Paget's disease) accountable to nipple-involvement of the tumor, a malignancy with radiological nipple-involvement, a malignancy at ≤1 cm distance to nipple or skin, a history of radiotherapy of the breast or an earlier operation in the NAC or skin area. Parameters such as age, body mass index, breast size, menopausal status, parity, history of breast feeding and history of chemotherapy were collected from medical files.

The institutional Medical Ethical Board approved the study. According to the Dutch 'Code of Conduct' for secondary use of human tissue, the use of excised tissue for research purposes after standard diagnostic procedures is part of the standard treatment agreement in the Netherlands.

Surgical technique

Oncological surgeons from four centers participated in this study. All of them were experienced in performing NSMs. The participating surgeons were assisted by one of the researchers at their first inclusions to ensure that identical techniques were used (Figure 1, Figure 3). In a conventional (non-skin-sparing) or skin-sparing mastectomy, circles were drawn around the NAC and around an as large as possible periareolar circular skin-island (SI) representing the skin flap which would be spared in SSM or NSM (Figure 1). The periareolar location of the SI was chosen because of the assumed pyramid shape of the breast gland and therefore expecting most TDLUs behind the skin near the NAC. Diameters of NAC and SI were measured before excision. Then the NAC was excised as thin as possible (i.e. without compromising blood flow and consequently induct necrosis;

Figure 3a) as if it were a real NSM. The nipple-papilla was cored (Figure 3b)[13]. The SI was excised subcutaneously as if it were a skin flap in SSM or NSM. NAC and SI excisions were completely executed intra-operatively ('in vivo'; see Figure 4, which shows intra-operative incisions around NAC and SI).

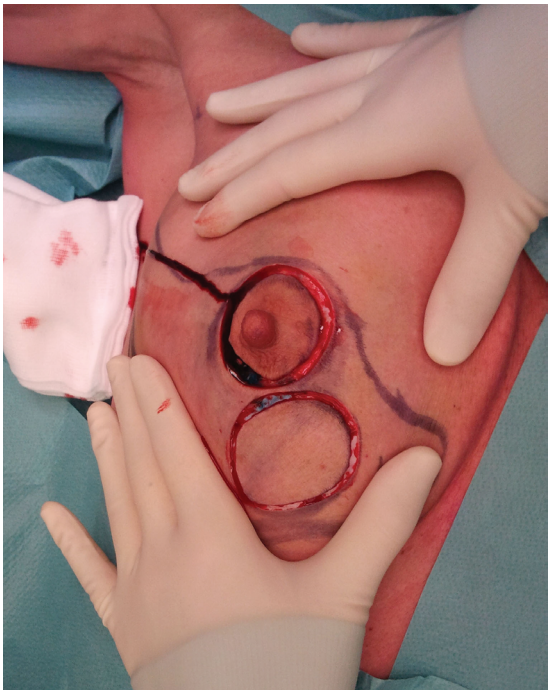

Figure 4 Intra-operative dissection of the nipple-areola complex (NAC) and skin-island (SI)

Analysis of TDLUs

NAC and SI specimens were formalin fixed, serially sectioned perpendicularly to the skin into 2 mm thick slices, paraffin embedded and entirely submitted for routine haematoxylin and eosin (H&E) staining (Figure 5). Microscopic examination was performed by two breast pathologists (C.v.D. and P.W.) after obtaining consensus on TDLU definition and specification of anatomical locations. A TDLU was defined as a terminal duct and its associated lobule (Figure 6), characterized by (1) the presence of a loose intralobular stroma that was different from the surrounding, denser, more collagenized interlobular stroma and (2) presence of a typical double cell layer confining the lobular acini[23].TDLUs were counted and their anatomical locations ('dermis', 'breast stroma' or 'fat') were noted.

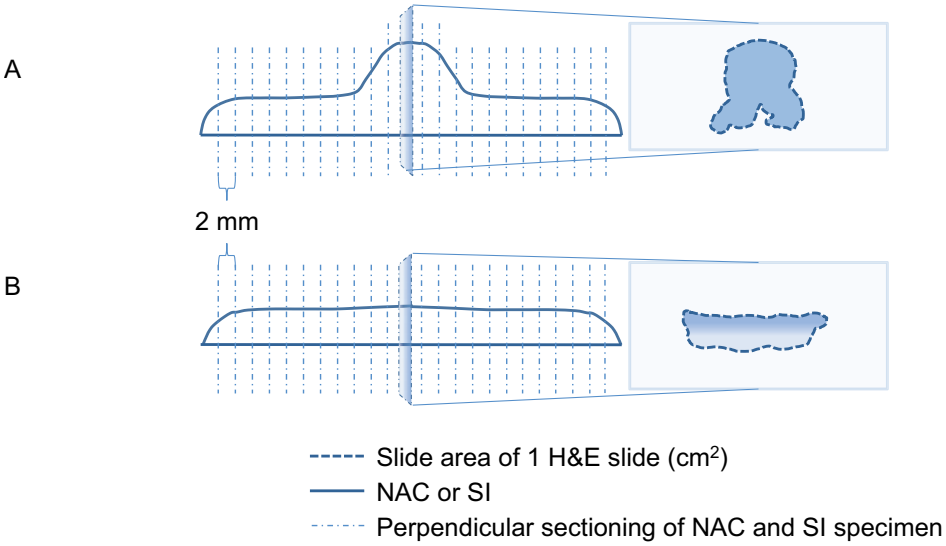

Figure 5 Perpendicular serial sectioning in 2 mm thickness for quantification of TDLUs in haematoxylin and eosin (H&E) slides of **A** nipple-areola complex (NAC) and **B** skin islands (SI). Slides were scanned and slide areas were measured in Adobe Photoshop®. TDLU density= TDLUs/Σ slide areas (cm^2)

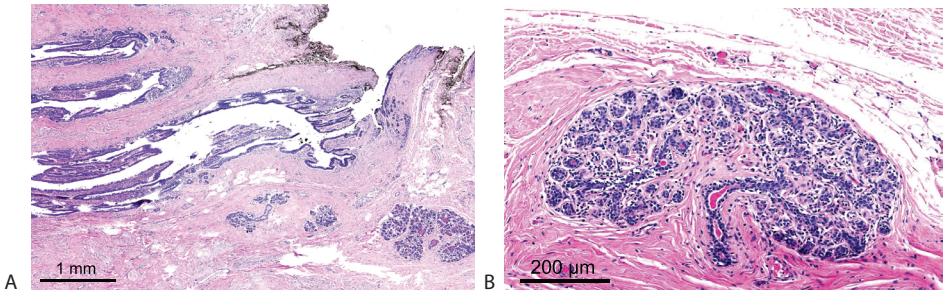

Figure 6 Terminal duct lobular unit (TDLU) consisting of a terminal duct with and associated lobule: **A** low-power view of a TDLU with associated lactiferous ducts and **B** medium-power view of one TDLU; haematoxylin and eosin (H&E) staining.

H&E slides of NAC and SI specimens were scanned. Surface areas of slides (slide area, cm^2) were calculated using Adobe Photoshop®. Total TDLU quantities were corrected for the sum of slide areas, representing density of TDLUs in NAC or in SI: TDLU density=TDLUs/Σ slide areas (cm^2) (Figure 5).

Statistical analysis

Differences in amounts of TDLUs in NAC as compared to SI were tested pairwise by using Wilcoxon's Signed Rank test. Binary logistic regression was used for multivariable analysis of factors that may influence the presence of TDLUs in the NAC, including the

interaction term age x menopausal status. Variables with a P-value of <0.1 in univariable analysis were included in the multivariable analysis. All P-values were two-sided and a significance level of α=0.05 was used. The SPSS computer package (version 20.0) was used for statistical analyses.

RESULTS

After 75 unilateral and 30 bilateral mastectomies in 90 women, 105 NACs and skin islands were available for analysis. The indication for mastectomy was prophylactic in 31, therapeutic (in situ or invasive carcinoma) in 71 and symptomatic (chronic mastodynia) in 3 cases. Median age at unilateral or bilateral mastectomy was 49 years (range 26-86) and 59 women (56%) were post-menopausal (Table 1). In 14 of them, menopause had been induced by bilateral oophorectomy at premenopausal age. Four women had undergone neoadjuvant chemotherapy, nine women had a history of adjuvant chemotherapy for breast cancer and four women had a history of chemotherapy for another indication.

TDLUs in nipple-areola complex (NAC) specimen and in skin-island (SI) specimen

Median diameter of the NAC was 4.0 cm (range 1.2-7.0 cm) and of the SI 4.0 cm (range 1.0-7.0 cm). In 64 NAC specimen (61%) ≥1 TDLUs were found, as compared to 25 SI specimen (24%, P<0.01). In 21 mastectomies TDLUs were found in both NAC and SI, in 37 mastectomies TDLUs were found neither in NAC nor in SI. In 43 mastectomies TDLUs were merely found in the NAC, whereas in four mastectomies TDLUs were found only in the SI.

Median amount of total TDLUs in the NAC specimen was 2 (range 0-186) vs. 0 in the SI specimen (range 0-48) with a median difference of 1 TDLU (P<0.01) in paired analysis. Median slide areas were 15.9 cm^2 (range 3.7-40.0) for NAC and 9.9 cm^2 (range 0.3-57.4) for SI. With adjustment for slide areas, TDLU density was 0.2/cm^2 in the NAC (range 0.0-8.5) vs. 0.0/cm^2 in the SI (range 0.0-0.5; P<0.01).

Anatomically, in the NACs most TDLUs (95%) were located in breast stroma and only 3% of TDLUs in the fat tissue. In contrast, in the SIs only 33% of TDLUs was located in breast stroma and 66% of TDLUs in the fat tissue. Location of TDLUs in the dermis was rare (2% in NACs and 1% in SIs).

Presence of TDLUs in the nipple-areola complex (NAC)

The influence on the presence of TDLUs in the NAC of the variables age, menopausal status, *BRCA1/2* mutation status, history of chemotherapy, parity, breastfeeding, body mass index (BMI), breast size and participating hospital was assessed by univariable logistic regression analysis (Table 2). Age and BMI were entered as continuous variables.

Table 1. Patient and mastectomy characteristics

Mastectomies (n)	105	
unilateral	75	(71%)
bilateral	30	(29%)
Age, years *(median, range)*	49	(26-86)
Genetic status		
BRCA1/*BRCA2* gene mutation	32	(30%)
No gene mutation or not tested	73	(70%)
Menopausal status		
Pre-	35	(33%)
Peri-*	6	(6%)
Post-	45	(43%)
Bilateral salpingo-oophorectomy (premenopausal)	14	(13%)
Not known	5	(5%)
Chemotherapy		
neoadjuvant; for ipsilateral breast cancer	4	(4%)
history of; for contralateral breast cancer	9	(8%)
history of; for other malignancy	4	(4%)
none	88	(84%)
Nullipara**	16	
Para**	57	
number of children (median, range)	2	(1-7)
Unknown	32	
History of breastfeeding		
Yes	32	(30%)
months of breastfeeding (median, range)	4	(0.3-18)
No	28	(27%)
Not known	45	(43%)
Body Mass Index, kg/m^2 (median, range)	26.1	(18.2-50.1)
Breast size		
Small (AA-B)	21	(20%)
Large (≥ C)	42	(40%)
Unknown	42	(40%)
Mastectomies included per hospital		
1	64	(61%)
2	18	(17%)
3	4	(4%)
4	19	(18%)
Diameters measured before excision, cm (median, range)		
nipple-areola complex (NAC)	4.0	(1.2-7.0)
skin-island (SI)	4.0	(1.0-7.0)

*Perimenopausal status: defined as a reported changed or irregular menstrual cycle around the age of 50
**Nullipara=no childbirth; para=≥1 childbirth

After univariable analysis the variables age, menopausal status, parity and breastfeeding and the interaction variable 'age x menopausal status' were entered in the multivariable model. Risk factors for presence of TDLUs in the NAC were younger age (OR 0.93; 95%CI 0.89-0.98) and parity (≥1 childbirths as compared to nullipara, OR 7.6; 95%CI 1.8-32.3).

Table 2. Univariable and multivariable logistic regression analyses of factors influencing the presence of TDLUs in nipple-areola complex (NAC)

	Univariable			Multivariable#		
	OR	95% CI	P	OR	95% CI	P
Age*	0.95	0.93-0.98	<0.01	0.93**	0.89-0.98	<0.01
Menopausal status						
Postmenopausal	1.0					
Pre- or perimenopausal	3.4	1.4-8.4	0.01	1.5**	0.3-8.9	0.65
History of chemotherapy (vs. no)	1.2	0.4-3.6	0.77			
BRCA1/2 gene mutation (vs. no)	2.0	0.8-4.9	0.14			
Parity						
Nulliparous	1.0					
1 or more children	4.3	1.3-13.7	0.01	7.6**	1.8-32.3	<0.01
Breastfeeding (vs. no)	3.0	1.0-8.9	0.05	1.7**	0.3-8.7	0.53
Body Mass Index*	1.0	0.9-1.1	0.93			
Breast size						
Large (≥ C)	1.0					
Small (AA-B)	1.5	0.5-4.8	0.46			
Hospital of mastectomy						
1	1.0					
2	0.8	0.3-2.3	0.63			
3	0.2	0.0-1.7	0.13			
4	0.4	0.2-1.2	0.12			

# Interaction term Age x menopause was included.
OR= Odds Ratio; 95%CI= 95% Confidence Interval
*Entered in the model as continuous variable; i.e. presented OR is per year of increasing age
**Variables included in multivariable analysis: age, menopausal status, parity, breastfeeding

DISCUSSION

BRCA1/2 mutation carriers have the option to undergo prophylactic bilateral mastectomy and breast reconstruction in order to reduce breast cancer risk. Sparing the nipple-areola complex (NAC) is an alternative to skin-sparing mastectomy, which is thought to be oncologically safe. To estimate safety of the NAC (and skin) sparing technique we assessed presence and quantities of TDLUs behind the NAC as compared to the skin in women undergoing a prophylactic skin-sparing mastectomy or a therapeutic mastec-

tomy. We compared the amount of TDLUs behind NAC and skin which would remain if NAC and skin had been spared. Since NAC and SI were surgically dissected as in NSM, results are clinically applicable.

Quantities of TDLUs in NAC and SI were analyzed pairwise to correct for possible influences on breast gland development and TDLUs such as patient related factors, hormonal factors and chemotherapy. Amounts of TDLUs were presented per cm^2 of slide surface areas of NAC or SI to correct for differences in size at excision (Figure 5). Median TDLU density was higher in NACs ($0.2/cm^2$) as compared to SIs ($0.0/cm^2$; $P<0.01$).

Several studies have reported on TDLUs in the NAC or behind the skin[15-17, 20, 24]. Reynolds et al. found TDLUs in only 24% of examined NACs, compared to 60% of NACs in this series. The NACs were retrieved *ex vivo* from mastectomy specimens, possibly using more shallow incisions creating thinner NACs as compared to our study with *in vivo* dissection of the NAC (and skin island)[15]. Stolier et al. found TDLUs in 3 of 32 (9%) nipples and recently Kryvenko et al. found TDLUs in 17 of 65 (26%) examined nipples[16, 17]. In these two studies, however, only the nipple-papilla was examined after transection at its basis. Nipple-basis and areola, possibly containing more TDLUs than the papilla, were not analyzed. One study evaluated 42 skin flaps that would have remained after SSM and that presumably were considerably larger than the SIs in the current study. Consequently, the authors found TDLUs in almost 60% of the skin flaps, in contrast with our study with TDLUs in 22% of SIs. They found that presence of TDLUs behind the skin flap was associated with a skin flap thicker than 5 mm[20]. In general, differences of our results with existing literature may partly be explained by differences in dissection methods. In our dissection method we took the viability of the spared NAC or SI into account, while trying to macroscopically remove all breast tissue. The observation that, microscopically, only 2% of TDLUs in the NAC and 1% in the SI were located in the dermis supports the idea that a thinner dissection minimizes remaining TDLUs.

Risk factors for presence of TDLUs were a younger age and a history of lactation (Table 2), but not BMI or breast size. We hypothesized that a smaller breast size and/or a lower BMI might be risk factors because the ratio breast glandular tissue/adipose tissue may be larger, resulting in a higher density of TDLUs. However, breast size and BMI did not significantly influence the presence of TDLUs behind the NAC. Hypothetically, adipose tissue is located more peripherally in the breast, while the concentration of breast glandular tissue centrally behind the nipple is independent of the amount of adipose breast tissue[25]. The highest number of TDLUs behind the NAC (186 TDLUs) was found in a 26-year old woman who had one child and who had recently lactated for 18 months (results not shown). Lactation and young age were risk factors for TDLU presence in the NAC. Moreover, this specific NAC was very large with a diameter of 7.0 cm (median diameter of the NAC was 4.0, range 1.2-7.0). All these factors may have contributed to this high amount of TDLUs. The next highest number of TDLUs we found was 121 in a NAC of 7.0

cm belonging to a 40-year old patient with two children and a history of lactation of three months (results not shown).

Since breast cancer risks in high-risk patients such as *BRCA1/2* mutation carriers do not necessarily diminish by increasing follow-up time, the strongest evidence for oncological safety of NSM can be retrieved by a study with long or lifetime follow-up after risk-reducing mastectomies. The Rotterdam series recently published by Heemskerk-Gerritsen et al. reported no primary breast cancers after 6.3 years median follow-up of 424 prophylactic mastectomies[7]. Of these, 40 were NSMs with a median follow-up of 3.3 years (unpublished data). As an alternative approach, we assessed the amount of glandular tissue remaining at risk for breast cancer if it would be a prophylactic NSM. Comparable amounts of TDLUs behind NAC and skin would suggest comparable breast cancer risks after NSM and SSM. As we found significantly more TDLUs behind the NAC as compared to the SI, that conclusion seems not applicable. On the contrary, our results show that TDLU density is higher behind the NAC than behind the SI and that sparing the NAC hypothetically may be less safe than sparing only the skin-envelope. However, the clinical relevance of these differences in TDLUs is unknown and must be placed into perspective for various reasons. First, it is unclear how many TDLUs the female breast gland comprises in total. Breast gland development and amounts of TDLUs may vary broadly inter-individually. The amounts of TDLUs we found behind NAC and SI are absolute, but their proportion to the total of TDLUs removed during NSM is unknown. Concerning pathological measurements, the quantity of TDLUs may be observer dependent. The two involved breast pathologists participating in this study (C.v.D. and P.W.) obtained consensus on TDLU quantity measurement and specification of TDLU location.

Second, in this study skin islands were examined, representing only part of the skin fold. This was an important weakness of our study. Because of the pyramid shaped breast gland TDLU density may decrease more peripherally in the skin fold. However, the total area of the spared skin fold, including the periareolar skin, is up to a few times larger than the skin island we excised (with a median diameter of 4.0 cm) and therefore is likely to contain more TDLUs than we found in the skin island. This may assume that the difference in remaining TDLUs between NAC and skin is actually smaller than we found. Third, primary breast cancers occasionally do occur after prophylactic NSM (and SSM), but are mostly located in the axillary tail or in the upper-outer quadrant of the breast and very rarely in the NAC. In the rare event of a developing malignancy after NSM, a superficially located tumor of the skin or NAC combined with closely underlying breast prosthesis might be easily detectable at physical examination, contrasting tumors of the axilla. During mastectomies it is not always easy to grossly identify the lateral edge of the breast gland and therefore glandular tissue may remain near and in the axilla[26, 27]. Sacchini et al. reported two new breast cancers located in the axillary tail and the outer upper quadrant after 24.4 and 61.8 months of follow-up, respectively, in

a series of 124 prophylactic NSMs with a mean follow-up of 24.6 months[28]. Hartmann et al. reported 6 primary breast cancers and 1 metastasis in a large series comprising 1146, partially nipple-sparing, prophylactic mastectomies, in moderate and high risk patients with a median follow-up of 14 years: 1 breast cancer in the NAC after 6 years follow-up and 1 above the areola after 5 years, while the other 4 were located near the chest wall or elsewhere in the neo-breast[29]. Another more recent article on 330 prophylactic NSMs described no new breast cancers after a mean follow-up of 22 months. Unfortunately, *BRCA1/2* mutation status in these series was unknown or not reported. Studies reporting prophylactic NSM in *BRCA1/2* mutation carriers are scarce and an overview is given in Table 3. In summary, in eight studies containing a total of 232 prophylactic NSMs in *BRCA1/2* mutation carriers with mean follow-up ranging between 10 and 63 months, no primary breast cancers were found.

Table 3. Overview of literature describing prophylactic nipple-sparing mastectomies (NSMs) in *BRCA1/2* gene mutation carriers

Author & year	Total no. NSMs	No. of prophylactic NSMs in established *BRCA1/2+*	Follow-up (months)	Primary breast cancers after prophylactic NSM	Primary breast cancers located in NAC or skin
de Alcantara et al. 2011 [19]	353	44	10.4	0	0
Harness et al. 2011 [28]	60	7	18.5	0	0
Heemskerk-Gerritsen et al. 2013* [7]	40	40	62.8	0	0
Peled et al. 2013 [29]	212	52	51.4	0	0
Spear et al. 2011 [20]	162	36	43	1 metastasis of unknown primary after 9 years, *BRCA1*/2 status unknown	0
Voltura et al. 2008 [30]	51	4	18	0	0
Warren Peled et al. 2012 [31]	657	38	28	0	0
Wijayanayagam et al. 2008 [32]	64	11	NR	0	0

NSM= nipple-sparing mastectomy; NAC= nipple-areola complex; NR= not reported
* Unpublished data

Since breast cancer incidences after NSM and SSM are very low, studies not only with long follow-up but also with a large study population are needed to possibly find a significant difference in risk reduction between NSM and SSM. Furthermore, very few studies assessed and report superiority of NSM in cosmetic outcome and quality of life as compared to SSM combined with nipple reconstruction [12]. Therefore, more studies are warranted to compare patient reported outcomes after both procedures.

Important strengths of our study concern the clinical applicability of the results because of in vivo dissection of NAC and SI by experienced breast surgeons. Additionally, breast pathologists had consensus about quantitation of TDLUs. And finally our paired analysis corrected for patient-related influences of breast gland development.

In summary, it is important that physicians and patients opting for NSM are aware that NACs may harbor risk for developing breast cancer, albeit very small. However, this may also apply to the spared skin and possibly even more for the lateral quadrants and the axillary tail. To obtain quantification of additional breast cancer risk after NSM for prophylactic indications as compared to SSM large studies with long follow-up are warranted.

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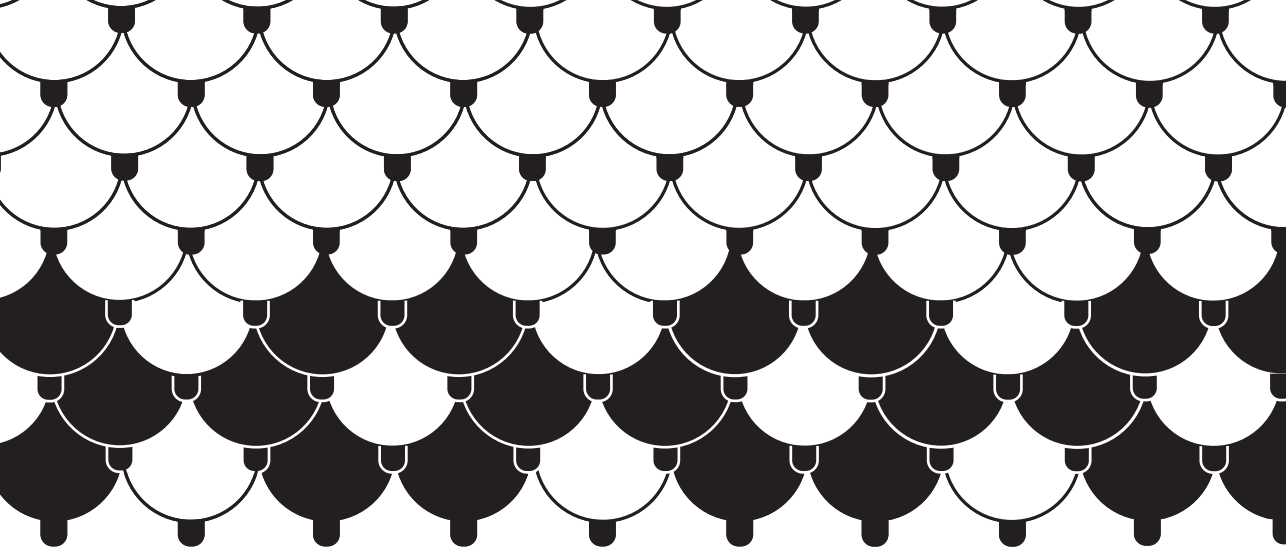
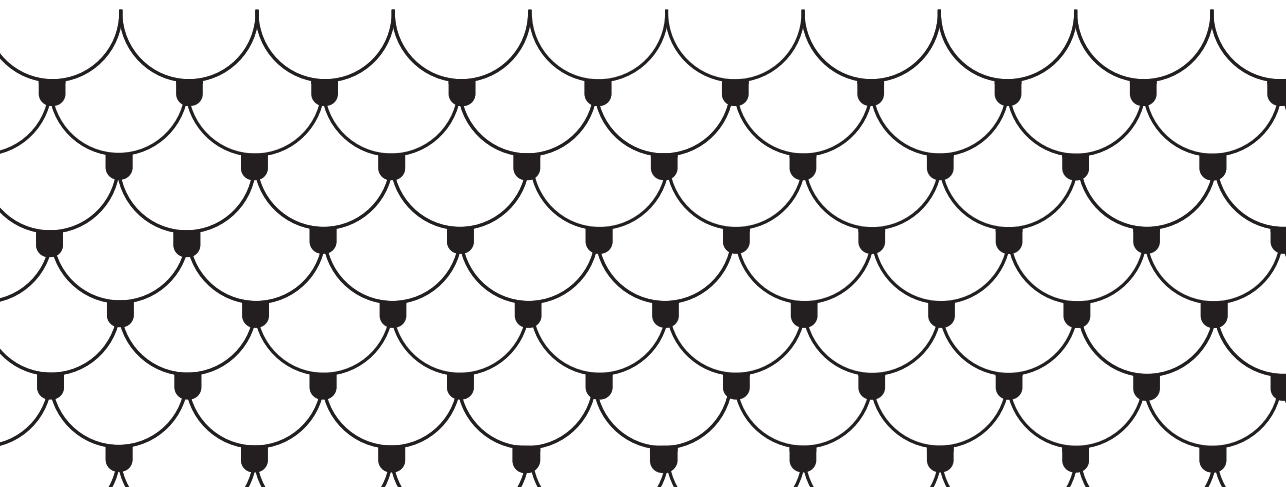
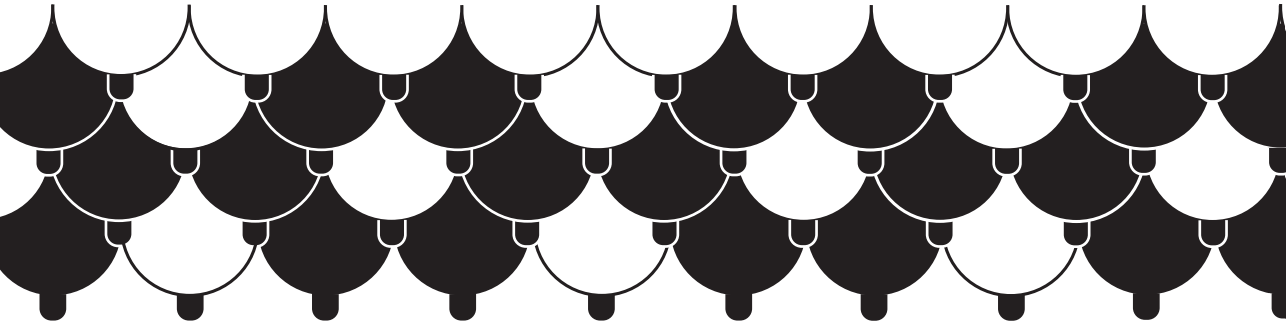




AESTHETIC OUTCOME AFTER PROPHYLACTIC MASTECTOMY AND BREAST RECONSTRUCTION



CHAPTER 8

Reliability and validity of the Dutch translated Body Image Scale

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ABSTRACT

Purpose Lacking a comprehensible and widely applicable Dutch test to assess body image changes in cancer patients, we validated Hopwood's Body Image Scale (BIS) for the Dutch language.

Methods The BIS consists of 10 items scored 0-3. Total scores range from 0 (minimum body image-related distress) to 30 (maximum distress). After forward and backward translation of the BIS we evaluated its psychometric characteristics in breast cancer patients. We assessed feasibility by missing answer rates and positive response prevalence (score ≥1) per item (criterion ≥30%), test-retest reliability with a two-week interval, internal consistence using Cronbach's α and discriminant ability by comparing body image after breast-conserving therapy (BCT) versus mastectomy.

Results Psychometric evaluation of 108 BCT and 101 mastectomy patients showed high feasibility (0.2% missing answers), high positive response prevalence of ≥30% in 9/10 items and high internal consistency (α>0.90). Test-retest reliability and correlation were high with 5.78 (test) vs. 5.75 (retest; P=0.86) and Spearman's ρ=0.92 (P<0.01). Discriminant ability was good with BIS scores of 4.56 after BCT vs. 7.19 after mastectomy (P<0.01). All results were comparable to the results of the original BIS.

Conclusion The Dutch translated BIS showed excellent psychometric results very similar to the original BIS. Its concise and simple design further supports wide application in clinical practice.

INTRODUCTION

Health care quality indicators are increasingly determined by patient-reported outcome measures (PROMs). Commonly used PROMs are quality of life (QoL) assessments of which body image is one of the presumed determinants[1, 2]. Several types of cancer and cancer treatments may cause body image changes. The majority of studies assessing body image changes in cancer patients have focused on breast cancer patients[3-6] but other types of cancer are likely to induce body image distress as well[1, 7-9]. Lacking a comprehensible and widely applicable Dutch test to assess body image changes in cancer patients, we validated Hopwood's Body Image Scale (BIS)[10] for the Dutch language.

PATIENTS AND METHODS

Hopwood's Body Image Scale (BIS)

The BIS assesses body image and body image changes after cancer treatment (Figure 1). Respondents are asked to answer questions with reference to the past week. The scale consists of ten items including affective items (e.g. feeling "self-conscious", "less feminine/masculine", "less physically attractive"), cognitive items (e.g. dissatisfied "with appearance", "with scar") and behavioral items (e.g. "avoid people", "difficult to look at yourself naked"). Response options range from "not at all" (score 0), "a little" (score 1), "quite a bit" (score 2) to "very much" (score 3). Question 10 ("dissatisfied with scar") has an additional response option "not applicable". Summing up the scores, a total score ranging from 0-30 per patient is obtained with 0 representing no distress or symptoms, whereas increasing scores represent increasing distress and symptoms.

Translation and adaptation of the BIS

The adaptation process was previously described by Bullinger et al[11]. Three Dutch native speakers with extensive knowledge of the English language provided a forward translation into Dutch. Emphasis was lying on conceptual equivalence using simple language, rather than achieving literal translation. Translators discussed difficulties with the principal investigator until consensus was reached on one optimal Dutch formulation. Two native English speakers who were fluent in Dutch provided a backward translation to English. Both backward translations were compared with the original BIS and any differences were analyzed. Finally, necessary adaptations of the Dutch version were made. The resulting version (Figure 2) was given to three patients who had been treated for breast cancer. They were asked to comment on readability and comprehension of the scale. After this last test no adaptations had to be made and the scale was administered to the study population for psychometric data collection.

Appendix 1. The Dutch translation of the Body Image Scale (BIS)

Body Image Scale items	Helemaal niet	Een beetje	Nogal	Heel erg	
1. Heeft u zich onzeker gevoeld over uw uiterlijk?	☐	☐	☐	☐	
2. Heeft u zich lichamelijk minder aantrekkelijk gevoeld door uw ziekte of behandeling?	☐	☐	☐	☐	
3. Bent u ontevreden geweest over uw uiterlijk als u aangekleed was?	☐	☐	☐	☐	
4. Heeft u zich minder vrouwelijk gevoeld door uw ziekte of behandeling?	☐	☐	☐	☐	
5. Heeft u moeite gehad om uzelf naakt te zien?	☐	☐	☐	☐	
6. Heeft u zichzelf seksueel minder aantrekkelijk gevoeld door uw ziekte of behandeling?	☐	☐	☐	☐	
7. Heeft u andere mensen vermeden vanwege hoe u zich voelde over uw uiterlijk?	☐	☐	☐	☐	
8. Heeft u het gevoel dat de behandeling uw lichaam minder compleet heeft gemaakt?	☐	☐	☐	☐	
9. Bent u ontevreden geweest over uw lichaam?	☐	☐	☐	☐	
10. Bent u ontevreden geweest over hoe uw litteken eruit ziet?	☐	☐	☐	☐	n.v.t.☐

n.v.t.: niet van toepassing

Study population and data collection

We chose to conduct psychometric evaluation in breast cancer patients, since this subgroup of patients is relatively large, homogeneous and likely to have body image related distress due the type of surgery. The fact that the original version was (partly) validated in breast cancer patients easies the comparison[10]. Female breast cancer patients ≥18 years who visited our outpatient clinic for follow-up were eligible. We aimed to include 100 breast conserving therapy (BCT) and 100 mastectomy patients. Exclusion criteria were breast reconstruction after mastectomy and insufficient knowledge of the Dutch language. In order to determine test-retest reliability, participants were asked to complete the Dutch BIS twice with a two-week interval. Patients who had to undergo breast cancer treatment between the test and the retest or who did not return the second copy by mail were excluded. Medical ethical board approval was obtained. All participants gave written informed consent.

Validation

The SPSS computer package (version 20.0) was used for statistical analyses. Psychometric results are compared to the results of the psychometric evaluation of the final version of the original BIS.

Feasibility

Missing or non-unique responses (0 or >1 box ticked, respectively) were considered invalid. Questionnaires with <9/10 valid responses to all items were excluded from analyses. When one item was not answered, the maximum score possible at that item was subtracted from the maximum achievable score of the scale (30 points). A valid score was calculated by dividing the achieved total score by the new maximum score and multiplying this by 30. Feasibility of the scale was evaluated by response rates and missing answers. Response prevalence was defined as the frequency of positive ratings (score of ≥1) for each item, indicating a change in some aspect of body image. Per item a criterion of ≥ 30% of positive ratings of the total sample was used.

Reliability

To assess whether items evaluate the same concept (e.g. body image) internal consistency of scale items was measured using Cronbach's α, which should exceed 0.70[12]. Test-retest reliability was tested using Spearman's correlation coefficient rho (ρ), paired Student's T-tests and effect size (Cohen's d=0.2: small effect, d=0.5: medium effect and d >0.8: large effect size)[13, 14].

Clinical validity

Discriminant ability between lumpectomy and the mastectomy subgroups was assessed using Student's T-tests and effect size[13, 14].

RESULTS

Response rates and feasibility

Both questionnaires were returned by 108/150 BCT patients (72%) and by 101/150 mastectomy patients (67%; Table 1). In both questionnaires, a total of 0.2% of all answers was missing. There were no non-unique answers. None of the questionnaires had >1 invalid responses. All items reached the 30% response prevalence criterion, except for item 7 ('avoid people because of the way you felt about your appearance?') which had a response prevalence of 12%. In the first version of the original BIS, three items including item 7 had a response prevalence of ≤30%, which decreased to zero items in the final version[10].

Table 1. Hopwood's Body Image Scale (BIS)

Body image scale items	Not at all	A little	Quite a bit	Very much	
1. Have you been feeling self-conscious about your appearance?	☐	☐	☐	☐	
2. Have you felt less physically attractive as a result of your disease or treatment?	☐	☐	☐	☐	
3. Have you been dissatisfied with your appearance when dressed?	☐	☐	☐	☐	
4. Have you been feeling less feminine/ masculine as a result of your disease or treatment?	☐	☐	☐	☐	
5. Did you find it difficult to look at yourself naked?	☐	☐	☐	☐	
6. Have you been feeling less sexually attractive as a result of your disease or treatment?	☐	☐	☐	☐	
7. Did you avoid people because of the way you felt about your appearance?	☐	☐	☐	☐	
8. Have you been feeling the treatment has left your body less whole?	☐	☐	☐	☐	
9. Have you felt dissatisfied with your body?	☐	☐	☐	☐	
10. Have you been dissatisfied with the appearance of your scar?	☐	☐	☐	☐	N/A ☐

N/A: not applicable

Test-retest reliability and internal consistency

Test-retest reliability was high. Mean BIS scores were 5.78 (95%CI 4.97-6.59) in Questionnaire 1 vs. 5.75 (95%CI 4.93-6.57) in Questionnaire 2 (P=0.86). This is in line with the outcomes of the original BIS with test-retest scores of 8.1 and 9.0, respectively, in the breast subgroup[10]. Correlation between the questionnaires was high for total BIS score (ρ=0.92; P<0.01) as well as for all items (Table 2), comparable to the correlation coefficient of the original BIS (ρ=0.70; P<0.01)[10]. Test-retest effect size was low (Cohen's d=0.005). Both questionnaires showed high internal consistency with Cronbach's α of 0.91 and 0.92, respectively, similar to the 0.93 of the original BIS[10].

Table 2. Patient characteristics

	BCT group		Mastectomy group	
Total patients	108		101	
Age, years (median, range)	59.0	(32.0-82.0)	60.0	(33.0-92.0)
Follow-up since surgery, years (median, range)	4.0	(1.0-13.0)	4.0	(1.0-20.0)
History of				
Chemotherapy	52	(49%)	65	(65%)
Radiotherapy	97	(91%)	39	(39%)
Hormonal therapy	49	(46%)	60	(61%)

BCT: Breast conserving therapy

Clinical validity: discriminant ability between breast conserving therapy and mastectomy

Mean total BIS scores of both questionnaires were 4.56 (95%CI 3.49-5.41) in the BCT group and 7.19 (95%CI 5.91-8.48) in the mastectomy group (P<0.01; Table 3) representing good discriminant ability. Cohen's d was 0.47, representing medium effect size. In the original BIS, this difference was even more pronounced with median total scores of 2.5 after BCT and 12.0 after mastectomy (P<0.01)[10].

Table 3. Scores of Body Image Scale (BIS) items, total BIS scores and test-retest reliability

BIS item	Questionnaire 1 – Test		Questionnaire 2 – Retest		Correlation	
	Mean score	95% CI	Mean score	95% CI	ρ	P-value[1]
1.	0.80	(0.69-0.92)	0.72	(0.61-0.83)	0.80	< 0.01
2.	0.80	(0.68-0.92)	0.79	(0.68-0.91)	0.80	< 0.01
3.	0.40	(0.30-0.49)	0.39	(0.30-0.47)	0.68	< 0.01
4.	0.59	(0.47-0.70)	0.51	(0.40-0.61)	0.74	< 0.01
5.	0.59	(0.47-0.71)	0.65	(0.52-0.77)	0.80	< 0.01
6.	0.70	(0.58-0.82)	0.75	(0.62-0.87)	0.80	< 0.01
7.	0.17	(0.10-0.24)	0.17	(0.11-0.24)	0.63	< 0.01
8.	0.65	(0.54-0.76)	0.63	(0.52-0.75)	0.73	< 0.01
9.	0.63	(0.53-0.74)	0.67	(0.56-0.78)	0.72	< 0.01
10.	0.45	(0.34-0.56)	0.47	(0.36-0.58)	0.79	< 0.01
Total BIS	5.78	(4.97-6.59)	5.75	(4.93-6.57)	0.92	< 0.01

95% CI: 95% Confidence Interval; ρ: Spearman's correlation coefficient ρ

[1]P-value of Spearman's correlation coefficient

Table 4. Discriminative ability of the Body Image Scale (BIS)

		BCT group		Mastectomy		
Total BIS score of		N= 108		N=101		
	N	Mean score	95% CI	Mean score	95% CI	P-value
All questionnaires	418	4.56	(3.85-5.26)	7.05	(6.16-7.94)	< 0.01
Questionnaire 1 – Test	209	4.45	(3.49-5.41)	7.19	(5.91-8.48)	< 0.01
Questionnaire 2 – Retest	209	4.66	(3.61-5.71)	6.90	(5.65-8.16)	< 0.01

BCT: Breast conserving therapy

DISCUSSION

The original BIS was developed in 2000 in the United Kingdom for use in clinical trials to assess body image changes in cancer patients, resulting from changes in a patient's appearance due to cancer treatment[10]. The scale assesses affective, behavioral and cognitive changes corresponding with a multidimensional approach of body image[15-17]. We validated the Dutch translated version of the BIS.

Psychometric evaluation of the Dutch BIS showed results comparable to the original version. Internal consistency was high with Cronbach's α of 0.92 and 0.91, similar to the original BIS[18]. Clinical validity based on response prevalence indicating a change in an aspect of body image also was comparable to the original tool[10]. Only one item, 'avoidance of other people because of the way a patient feels about his or her appearance', failed to reach the response prevalence criterion of ≥30% compared with none of the items in the original version[10]. This item indeed addresses an issue of body image likely not frequently impacted in breast cancer patients because the breast surgical area is hidden in most social situations. Discriminant ability was moderate to good between breast cancer patients treated by mastectomy and BCT, representing two groups with expected differences in body image changes. BIS scores differed significantly between the groups but the effect size was only moderate. The discriminant ability between BCT and mastectomy was comparable to the original BIS, although the difference between the two groups was larger in the original tool[10]. A possible explanation for the moderate effect size is that body image distress may not simply be related to the amount of breast tissue visibly removed during surgery. For example, body image distress is also found to be highly impacted after mastectomy followed by direct breast reconstruction[3]. Both the original and the Dutch BIS showed high test-retest reliability[10].

A limitation of this study was the fact that many women did not return the second questionnaire and were excluded, most likely due to unawareness that it was important to complete the same questionnaire twice. Comprehensibility of the scale did not seem to be a problem since rates of missing or non-unique answers were very low. After start-

ing to emphasize on the importance of the second questionnaire at inclusion, response rates improved.

Potential future applications of the BIS include its use as a health care quality indicator, for example in combination with assessment of QoL or satisfaction with care. Health care quality indicators, however, are not yet well-defined[19, 20]. To our knowledge, the BIS is not yet being implemented for assessment of quality of care. Potential applications of interest include screening for body image-related issues after cancer surgery and evaluation of treatment effects after psychological therapy or reconstructive surgery. Before the BIS can be used for evaluation of treatment effects, however, further specific validation is warranted.

To conclude, psychometric evaluation of the Dutch BIS showed excellent results that were comparable those of the original version. The concise and simple design makes the Dutch BIS suitable for assessment of body image issues in routine clinical practice.

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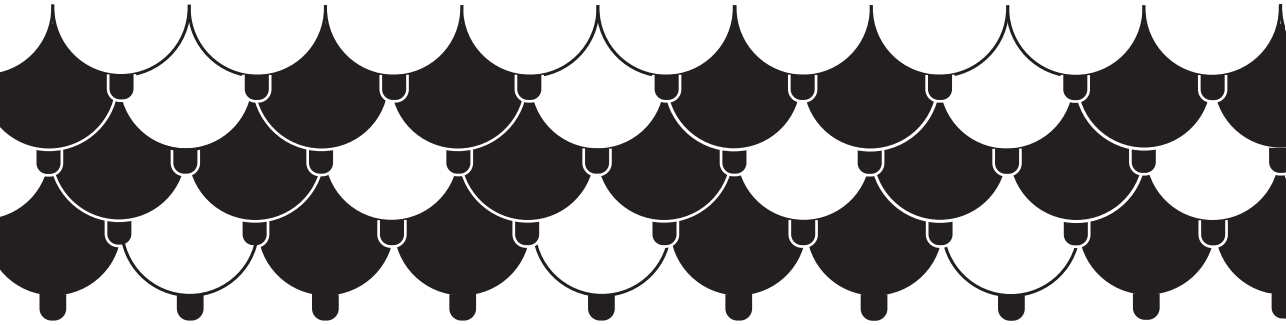

CHAPTER 9

Patient satisfaction and nipple-areola sensitivity after bilateral prophylactic mastectomy and immediate implant breast reconstruction in a high breast cancer risk population: nipple-sparing mastectomy versus skin-sparing mastectomy

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ABSTRACT

Background Prophylactic skin-sparing mastectomy (SSM) and nipple-sparing mastectomy (NSM) both are associated with major risk reduction in women with high breast cancer risk. SSM followed by nipple areola complex (NAC) reconstruction is standard of care, but NSM is increasingly being performed. Preservation of the NAC in NSM may increase patient satisfaction. Therefore, we measured NAC sensitivity after NSM and compared patient satisfaction as well as body image after SSM with NSM.

Methods Women who underwent prophylactic bilateral SSM or NSM and immediate implant breast reconstruction between 2002 and 2012 were eligible. Patient satisfaction was assessed using the Breast-Q Reconstruction questionnaire, body image using Hopwood's Body Image Scale (BIS) and satisfaction with the (reconstructed) NAC using a study-specific questionnaire. In the NSM group, NAC sensitivity was assessed using Semmes Weinstein monofilaments with a five-point scale and compared with NAC sensitivity in a non-operated control group.

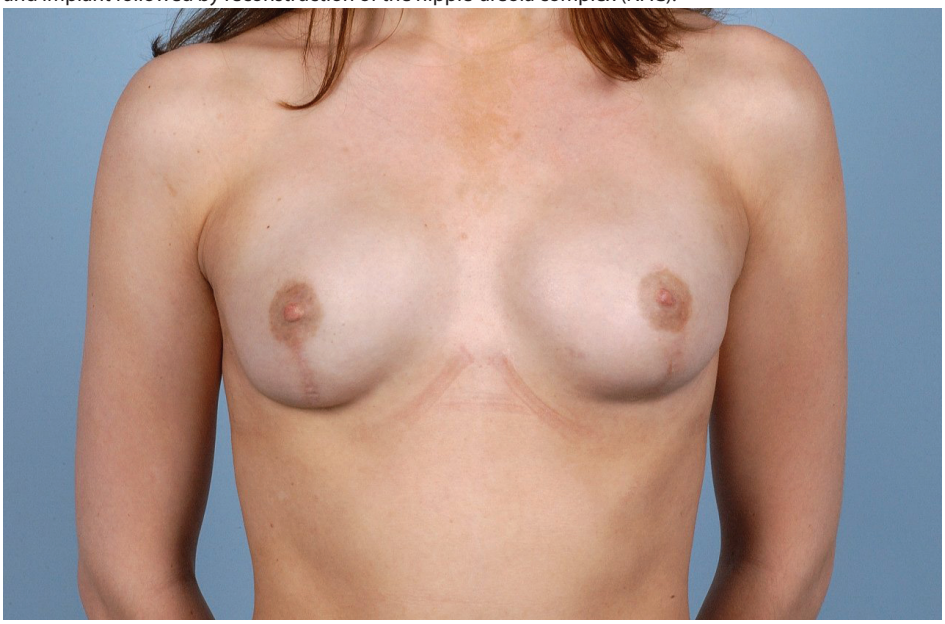
Results The SSM group comprised 25 women (50 SSMs) and the NSM group 20 women (39 NSMs). Median follow-up was 65 months in the SSM group compared with 27 months in the NSM group ($p<0.01$). In univariable analyses, Breast-Q scores were favorable in the SSM group compared with the NSM group with trends for higher 'satisfaction with breasts' (66.2 vs. 56.6; $p=0.06$) and 'satisfaction with outcome' (76.1 vs. 61.5; $p=0.09$). Mean BIS score of 7.1/30 in the SSM group and 9.3/30 in the NSM group ($p=0.35$). Adjusted for follow-up, there were no significant differences in Breast-Q scores, nor in BIS scores. Interestingly, satisfaction with the (reconstructed) NAC was similar after SSM and NSM. NAC sensitivity was lower in the NSM group (mean score 1.9, 95%CI 1.5-2.3) compared with the control group (mean score 4.7, 95%CI 4.6-4.9; $p<0.01$).

Conclusion Breast-Q scores regarding satisfaction with breasts and overall outcome were in favor of the SSM group. Residual NAC sensitivity after NSM was low. This suggests that SSM followed by NAC reconstruction is a balanced alternative to NSM. We observed no significant differences in body image and NAC-specific satisfaction between the NSM and SSM groups.

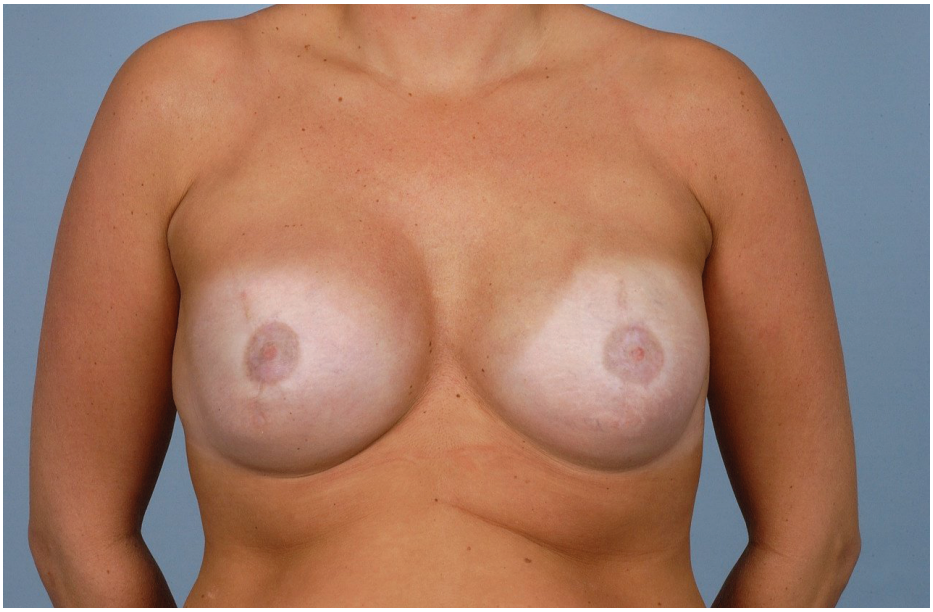
INTRODUCTION

BRCA1 and *BRCA2* gene mutation carriers and other women at high breast cancer risk may choose to undergo prophylactic bilateral mastectomy, achieving risk reductions of 90-100% after 3-13 years of follow-up[1-5]. Skin-sparing mastectomy (SSM) and nipple-sparing mastectomy (NSM) are frequently used (Figures 1 and 2). Both procedures aim to remove all breast glandular tissue while sparing the breast skin envelope to allow for immediate breast reconstruction. In SSM, breast glandular tissue is subcutaneously excised together with the nipple-areola complex (NAC). In a later stage, SSM can be followed by NAC reconstruction using local skin flaps and intradermal tattooing[6]. In NSM, the same subcutaneous procedure is carried out as in SSM while the NAC is left in situ together with the skin envelope.

Figure 1. Outcomes after skin-sparing mastectomy (SSM) and breast reconstruction by tissue expander and implant followed by reconstruction of the nipple-areola complex (NAC).



A A 24-year old patient 28 months after SSM and immediate breast reconstruction and 17 months after NAC reconstruction. An Allergan style 133 textured, anatomically shaped tissue expander filled with 420 cc was used, followed by Allergan style 410 medium height, full projection implants of 295 cc.



B A 35-year old patient 24 months after SSM and immediate breast reconstruction and 12 months after NAC reconstruction. An Allergan style 133 textured, anatomically shaped tissue expander filled with 800 cc was used, followed by Allergan style 410 full height, extra full projection implants of 690 cc.

Preservation of the NAC potentially may improve aesthetic outcome and patient satisfaction[7-9]. However, oncological safety of NSM is still subject to debate especially in women with high breast cancer risk. SSM on the other hand is generally considered oncologically safe for breast cancer prophylaxis, although no prospective randomized controlled trials have been conducted[1-5, 10]. Various authors, including our own group, have described remaining breast glandular tissue behind the NAC potentially forming a life-long breast cancer risk[11-13]. Studies with long-term follow-up after NSM in *BRCA1/2* mutation carriers are scarce. So far, data suggest that the incidence of primary breast cancer behind the NAC following prophylactic NSM is negligible[14, 15]. During the past years the proportion of prophylactic NSMs performed at our institute has steadily increased.

Although blood supply of the NAC can be preserved by the dermal plexus as well as residual subcutaneous tissue, partial or full thickness necrosis of the NAC is reported to occur in 11-30%. This can lead to scarring, pigmentation changes, loss of projection or the need for total removal of the NAC[15-18]. Other complications of NSM include malposition or asymmetry of the NACs (14-70%) and decreased NAC sensitivity[16, 19]. As an alternative to NAC-sparing, SSM followed by nipple reconstruction avoids NSM-related

complications and aims to achieve at least comparable results in patient satisfaction and aesthetic outcome. Disadvantages of nipple reconstruction are the need for an additional surgical intervention, loss of nipple projection and fading of the tattooed areola over time[6].

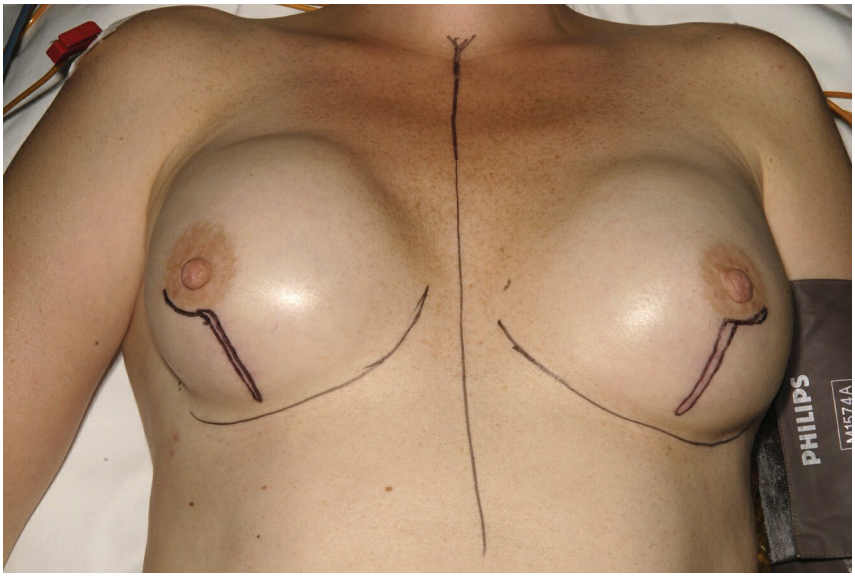
The primary aim of this study was to assess satisfaction, body image and satisfaction with the spared or reconstructed NAC in women at high breast cancer risk after prophylactic SSM as compared with prophylactic NSM. The secondary aim was to measure NAC sensitivity after NSM, compared with healthy controls.

PATIENTS AND METHODS

Patients

Women who underwent prophylactic SSM or NSM between 2002 and 2012 followed by immediate tissue expander-implant breast reconstruction with a follow-up period exceeding 12 months since mastectomy and who had completed the entire breast reconstruction course were eligible for inclusion. Participants were retrospectively collected from a prospective cohort study on prophylactic mastectomy or during outpatient follow-up visits for the cross-sectional questionnaire study and, in case of NSM, for NAC sensitivity examination. Demographics and data on smoking, body mass index (BMI), oncological history, history of radiation or chemotherapy, surgical techniques and postoperative complications were retrospectively collected from medical charts. Incisions and operative techniques were at the discretion of the oncological and plastic surgeon and mostly consisted of a circumareolar incision with caudal extension if necessary in SSM and a periareolar infero-lateral incision with caudal extension or inframammary incision in NSM (Figure 2A and B). In accordance with local protocol, Allergan style 133 textured, anatomically shaped tissue expanders with integrated Magna-Site injection site, followed by style 410 textured, highly cohesive anatomically shaped silicone-filled breast implants were used for breast reconstruction. Implant size, projection, height and width depended on preoperative patient dimensions and were at the discretion of the plastic surgeon.

A



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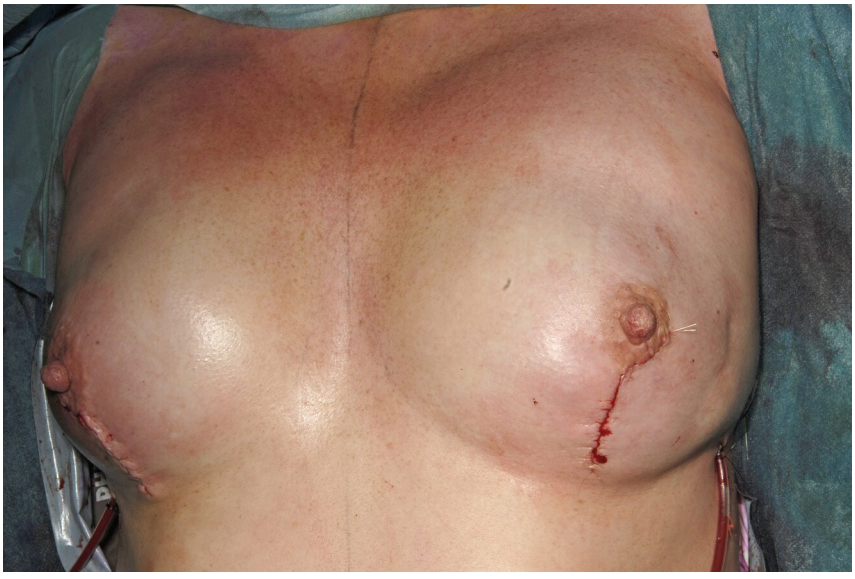

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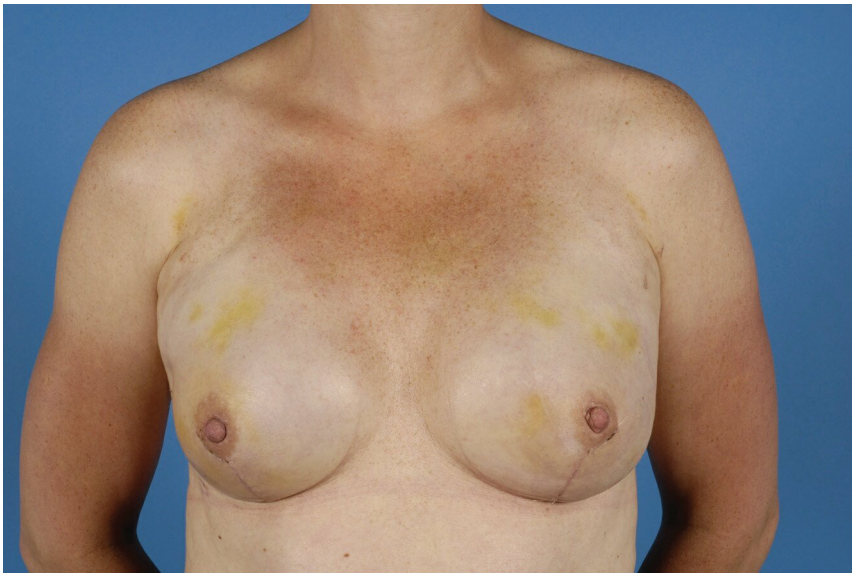

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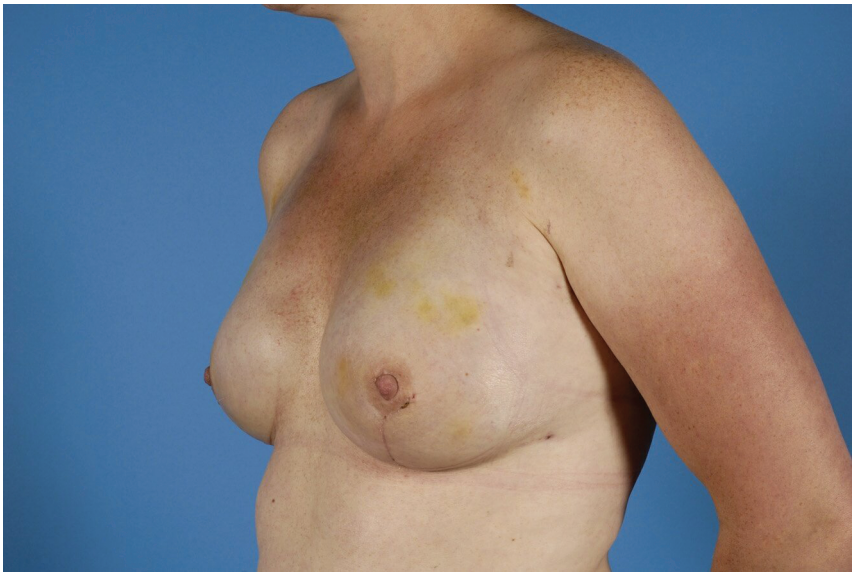

Figure 2. Nipple-sparing mastectomy (NSM) and immediate breast reconstruction in a 37-year old patient. An Allergan style 133 textured, anatomically shaped tissue expander filled with 520 cc was used, followed by Allergan style 410 full height, full projection implants of 465 cc.
A Latero-inferior incisions preoperatively, **B** shortly postoperatively, **C** and **D** outcome 6 months after NSM and breast reconstruction and 2 months after definitive implant placement.

Women were asked to participate during an outpatient clinic visit or by telephone. The institutional Medical Ethical Board approved the study. All participants signed informed consent forms for completion of questionnaires and in the NSM group for additional

examination of NAC and breast sensitivity. A control group for sensitivity of NAC and periareolar skin consisted of healthy female volunteers without a history of breast surgery.

Primary outcomes: patient reported outcomes

Women in the SSM and NSM groups were asked to complete three questionnaires at home and to return them anonymized by mail. Questionnaires were administered at the outpatient clinic or sent by mail. The Breast-Q reconstruction module assesses breast reconstruction-related quality of life (QoL) and satisfaction[20]. In this study, 5 of the 19 independent Breast-Q scales were used: (1) 'satisfaction with breasts', 16 items measuring satisfaction with appearance clothed and unclothed, with breast shape and symmetry; (2) 'satisfaction with outcome', 7 items assessing whether a patient's expectations of the breast surgery are met; (3) 'psychosocial well-being', 10 items rating a patient's social confidence, how normal and how equal to other women she feels; (4) 'sexual well-being', 6 items addressing the impact of the breast surgery and reconstruction on her sex life; and (5) 'physical well-being', 16 items on how often a patient experiences pain or discomfort in the breast area and the upper body. Additionally, Hopwood's Body Image Scale (BIS; Figure 3) consists of 10 questions concerning body image with four response options per question: "not at all" (score 0), "a little" (score 1), "quite a bit" (score 2) and "very much" (score 3)[21]. A total score ranging from 0-30 was obtained, where 0 represents no distress or symptoms while higher scores represent increasing symptoms and distress. To assess patient satisfaction with the spared or reconstructed NAC, subjective NAC sensitivity and the role of NAC and breast in sexuality, we administered a NAC-specific questionnaire comprising 8 items for the SSM group and 9 items for the NSM group that were scored on a 5-point Likert scale (Figure 4). One open-ended question was added to explore other factors that could have influenced patient feelings about their breast reconstruction, about the spared or reconstructed NAC and about changes in sexuality.

Did you/ Have you been feeling:	Not at all	A little	Quite a bit	Very much
1. Self-conscious about appearance?	-	-	-	-
2. Less physically attractive as a result of disease or treatment?	-	-	-	-
3. Dissatisfied with appearance when dressed?	-	-	-	-
4. Less feminine as a result of disease or treatment?	-	-	-	-
5. Find it difficult to look at yourself naked?	-	-	-	-
6. Sexually less attractive as a result of disease or treatment?	-	-	-	-
7. Avoid people because of how you felt about your appearance?	-	-	-	-
8. That the treatment has left your body less whole?	-	-	-	-
9. Dissatisfied with your body?	-	-	-	-
10. Dissatisfied with the appearance of your scar?	-	-	-	-

Figure 3. Hopwood's Body Image Scale (BIS)

		1	2	3	4	5
1	NAC sensitivity compared with before the operation	insensitive	less sensitive	the same	very sensitive	hypersensitive
2	Significance of breast for sexuality a) Before operation	unimportant	not very important	neither important or unimportant	important	very important
	b) Now					
3	Significance of NAC for sexuality a) Before operation	unimportant	not very important	neither important or unimportant	important	very important
	b) Now					
4	Did sexual pleasure change since the operation because of loss of NAC sensitivity?	absent	decreased a lot	substantial decrease	a little decrease	unchanged
5	Touching of the NAC is	very unpleasant	unpleasant	neither pleasant nor unpleasant	pleasant	very pleasant
6	Change of nipple reaction to cold or touch*	no reaction	a lot weaker	weaker	hardly changed	unchanged
7	a) Satisfaction with position of NAC on the breast	very unsatisfied	unsatisfied	neither satisfied nor unsatisfied	satisfied	very satisfied
	b) Not satisfied (score 1-3 in a) because NAC is too…	lateral	caudal	cranial	medial	N/A
8	Would choose the same operation again	certainly not	probably not	maybe	probably	certainly
9	Would advise this operation to other women	certainly not	probably not	maybe	probably	certainly

Figure 4. Nipple-areola complex (NAC)-specific questionnaire on satisfaction of the reconstructed or spared NAC, sensitivity and the role of the breast reconstruction and the NAC for sexuality
*Nipple-sparing mastectomy (NSM) group only

Secondary outcomes: sensitivity of the NAC and periareolar skin

In the NSM group and in the control group, tactile sensitivity was tested on 9 predetermined sites of NAC and surrounding skin using Semmes Weinstein monofilaments, starting with the thinnest and progressing to thicker monofilaments. Participants were asked to report touching of a monofilament with their eyes closed. Scores were ascribed per spot to the thinnest monofilament noticed: score 5 for the thinnest monofilament which was equivalent to 0.07 grams, score 4 for 0.4 grams, score 3 for 2.0 grams, score 2 for 4.0 grams, score 1 for 300 grams and score 0 if none of the monofilaments was noticed. Consequently, mean scores ranging from 0 to 5 per site and per breast were obtained, where score "0" represents complete absence of sensitivity and score "5" represents maximum vital sensitivity.

Statistical analysis

Outcomes of the Breast-Q Reconstruction questionnaire, the BIS and the sensitivity tests were scored and analyzed as continuous variables and of the NAC-specific questionnaire as categorical variables. Missing or non-unique answers were considered invalid.

Breast-Q scores of the independent scales were calculated using the QScore Scoring Software, which provides a total score per scale that ranges from 0-100 with a higher score representing greater satisfaction or better quality of life[20]. The Shapiro-Wilk test was used to test for normal distribution of continuous variables. Consequently, Breast-Q scores were analyzed using Student's T-tests and BIS scores and sensitivity scores were analyzed using Mann-Whitney U-tests. Categorical variables were analyzed using Chi-Square tests or Fisher's Exact tests. All p values were 2-sided and a significance level of α=0.05 was used. Outcomes were adjusted for differences in baseline characteristics with $p \leq 0.10$ using multivariable linear regression. The SPSS computer package (version 20.0) was used for statistical analyses.

RESULTS

Patients

The SSM group consisted of 25 women who underwent 50 SSMs and the NSM group comprised 20 women who underwent 39 NSMs (Table 1). All women had completed breast reconstruction with a definitive silicone implant in place at the time of inclusion. One patient who underwent unilateral SSM for breast cancer without NAC reconstruction and contralateral prophylactic NSM was analyzed in the NSM group. Median follow-up was significantly longer in the SSM group (65 months, range 43-136) than in the NSM group (27 months, range 10-58; $p<0.01$). In the SSM group, 20/25 women (80%) underwent bilateral mastectomy for prophylactic indications; 5/25 women (20%) had a history or current diagnosis of breast cancer. The majority (20/25, 80%) opted for NAC reconstruction with local transposition flaps and intradermal tattooing. Four women (16%) chose reconstruction by intradermal tattooing only and one woman decided not to have a NAC reconstruction. Mastectomy skin flap necrosis occurred in 2/25 (8%) of the cases, in one case (4%) necessitating partial resection of the affected skin flap. In the NSM group, 15/20 women (75%) underwent bilateral mastectomy for prophylactic indications; 5/20 women (25%) had a history or current diagnosis of breast cancer. In one patient who underwent bilateral NSM one NAC was removed in a later stage after finding an occult malignancy in the mastectomy specimen. In another patient both NACs had to be removed for an occult malignancy; she was excluded from sensitivity examination. Partial necrosis of the NAC occurred in 7 patients (35%) after NSM; none of them needed secondary NAC removal. Infectious complications occurred in 6 patients (25%) after SSM compared with 3 (15%) after NSM. Overall rates of reoperations were higher in the NSM group (50%); half of them were due to an unsatisfactory aesthetic outcome. The control group consisted of 21 healthy, non-operated female volunteers for assessment of NAC sensitivity at a median age of 30 years (range 20-58).

Table 1. Demographics, clinical characteristics and complication rates in nipple-sparing mastectomy (NSM) group and skin-sparing mastectomy (SSM) group

	SSM group		NSM group		
	N	(%)	N	(%)	P
Total patients	25		20		
Median age at examination (years, range)	40	(26-71)	40	(27-60)	1.00*
BRCA1 mutation	16	(64%)	10	(50%)	
BRCA2 mutation	7	(28%)	5	(25%)	
BRCA1 and *2* mutation	2	(8%)	1	(5%)	
Non-*BRCA* associated familial breast cancer risk	0	-	4	(20%)	
Mastectomy					
Bilateral prophylactic	20	(80%)	15	(75%)	
Unilateral therapeutic, unilateral prophylactic	5	(20%)	3	(15%)	
Bilateral therapeutic	0	-	2	(10%)	
Median age at mastectomy and reconstruction (years, range)	34	(21-59)	37	(26-57)	0.43*
Follow-up: months from reconstruction (median, range)	65	(43-136)	27	(10-58)	<0.01*
History of or current breast cancer	6	(24%)	5	(25%)	1.00**
Exposures, total	10	(42%)	6	(30%)	0.53**
Current smoker	8	(33%)	5	(25%)	
Chemotherapy					
prior to reconstruction	1	(4%)	1	(5%)	
after reconstruction	2	(8%)	1	(5%)	
Radiation prior to reconstruction	0	-	2	(10%)	
Comorbity, total	6	(25%)	3	(15%)	0.48**
Body mass index > 25	3	(13%)	0	-	
Malignancy other than breast cancer	2	(8%)	2	(10%)	
Other	1	(4%)	1	(5%)	
History of (prophylactic) oophorectomy	10	(40%)	6	(30%)	0.54**
Cup size at mastectomy					
A	2	(10%)	2	(14%)	0.28**
B	7	(37%)	8	(57%)	
C	6	(32%)	4	(29%)	
D	4	(21%)	0	-	
unknown	6		6		
Incisions					
Circumareolar with or without extension	25	(100%)	0	-	N/A
Inferolateral periareolar with caudal extension	0	-	15	(75%)	
Inframammary	0	-	5	(25%)	
Definitive implant size, cc (median, range)	475	(295-690)	445	(315-620)	0.89*
unknown (n)	4		1		
Nipple reconstruction					
Skin flaps and intradermal tattooing	20	(80%)	0	-	N/A
Intradermal tattooing only	4	(16%)	1	(5%)	
No reconstruction	1	(4%)	19	(95%)	

Table 1. Demographics, clinical characteristics and complication rates in nipple-sparing mastectomy (NSM) group and skin-sparing mastectomy (SSM) group (continued)

	SSM group		NSM group		
	N	(%)	N	(%)	P
Complications, total	9	(38%)	12	(60%)	0.14**
Infection	6	(25%)	3	(15%)	
Necrosis - partial or full-thickness - of NAC***	0	-	7	(35%)	
Mastectomy skin flap NAC*** loss	2	(8%)	0	-	
Due to malignancy	0	-	1	(5%)	
Due to necrosis	0	-	0	-	
Other complications	1		1		
Reoperations, total	7	(29%)	10	(50%)	0.13**
For					
infection	5	(21%)	3	(15%)	
necrosis	1	(4%)	0	-	
unsatisfactory aesthetic result	0	-	5	(25%)	
other reason	1	(4%)	2	(10%)	

*Mann-Whitney U-test

**Chi-square test

***NAC: nipple-areola complex; spared or reconstructed

Sensitivity of NAC and periareolar skin in NSM group and control group

NAC sensitivity was tested in 19 women in the NSM group with 36 spared NACs *in situ* and in 42 NACs in the control group. Results are summarized in Figure 5. In the NSM group, periareolar skin flap sensitivity was best preserved on sites medial and cranial of the NAC with a mean score of 2.7/5.0 for both sites, compared with mean scores of 4.9/5.0 in the control group ($p<0.001$). Skin sites lateral and caudal to the NAC scored 2.5 and 2.3/5.0, respectively, compared with 4.9/5.0 for both lateral and caudal sites in the control group ($p<0.01$). In line with skin sensitivity, sensitivity of the spared areola was best preserved medially and cranially (1.6/5.0 for both sites) compared with the control group (4.5 and 4.9/5.0, respectively; <0.01). Sensitivity of the spared NAC was 1.2/5.0 in the lateral areola and 1.3/5.0 in the caudal areola compared with 4.5 and 4.6/5.0, respectively, in the control group ($p<0.01$). Sensitivity of the nipple-papilla was 1.4/5.0 in the NSM group compared with 4.9/5.0 in the control group ($p<0.01$).

Body image and patient satisfaction after SSM and NSM: BIS and Breast-Q

In total, 22/25 (88%) of women in the SSM group and 18/20 (90%) of women in the NSM group returned their questionnaires after completion. Results are shown in Table 2. In univariable analyses, BIS scores did not differ between groups with a mean score of 7.1 in the SSM group (95% CI 4.6-9.6) as compared with 9.3 in the NSM group (95% CI 6.2-12.3; $p=0.24$). Breast-Q scores were favorable in the SSM group compared with

the NSM group with trends for higher 'satisfaction with breasts' in the SSM group (mean score 66.2; 95% CI 59.0-73.4) compared with the NSM group (mean score 56.6, 95% CI 51.6-61.6; p=0.06) and for higher 'satisfaction with outcome' in the SSM group (mean score 76.1; 95% CI 65.7-86.5) compared with the NSM group (mean score 61.5; 95% CI 50.2-72.9; p=0.09). After adjustment for follow-up, outcomes of Breast-Q scales and BIS did not significantly differ between SSM and NSM groups (Table 2).

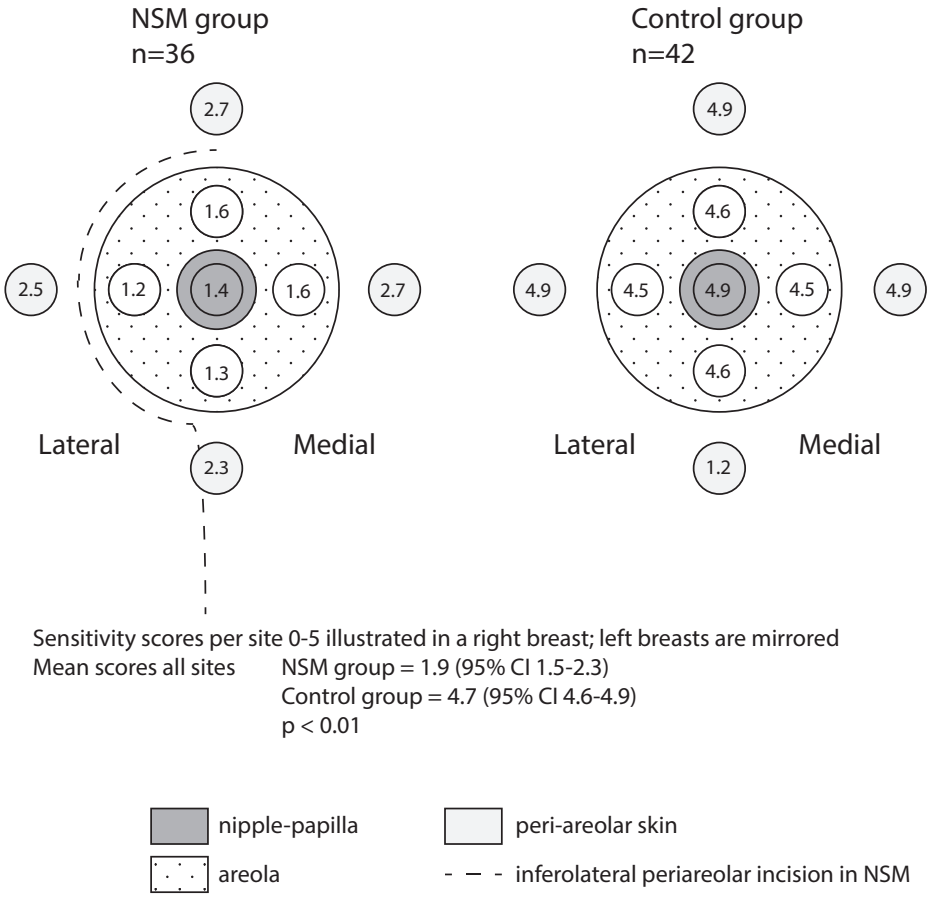

Figure 5. Sensitivity of the nipple-areola complex (NAC) and the surrounding skin after nipple-sparing mastectomy (NSM) tested using Semmes Weinstein monofilaments, as compared with a non-operated control group

Table 2. Mean scores of Body Image Scale (BIS) and Breast-Q Reconstruction Questionnaire scales after skin-sparing mastectomy (SSM group) and nipple-sparing mastectomy (NSM group)

	SSM group		NSM group				
	Mean	(95% CI)	Mean	(95% CI)	Mean Δ	P	Adjusted P-value***
Body Image Scale (BIS)	7.1	(4.6-9.6)	9.3	(6.2-12.3)	2.2	0.35*	0.21
Breast-Q Reconstruction							
Satisfaction with breasts	66.2	(59.0-73.4)	56.6	(51.6-61.6)	8.4	0.06**	0.69
Satisfaction with outcome	76.1	(65.7-86.5)	61.5	(50.2-72.9)	12.3	0.09**	0.18
Psychosocial well-being	79.2	(70.7-87.6)	69.7	(59.3-80.1)	9.2	0.14**	0.28
Sexual well-being	58.1	(47.4-68.9)	53.2	(43.7-62.6)	5.0	0.48**	0.89
Physical well-being: chest	72.0	(64.1-80.0)	63.7	(55.8-71.5)	7.7	0.16**	0.73

*Mann Whitney U test; 2-sided P-values
**Student's T-test; 2-sided P-values
***Multivariable linear regression, adjusted for follow-up time
BIS: total scores range from 0 (no distress or symptoms) to 30 (maximum distress or symptoms)
Breast-Q: scores per scale range from 0 (minimum satisfaction or quality of life) to 100 (maximum satisfaction or quality of life)

NAC-specific questionnaire in the SSM and NSM group

Subjective NAC sensitivity was reported decreased or absent since mastectomy by 17/17 women who scored sensitivity of the reconstructed NAC after SSM as compared with 16/18 women (89%) in the NSM group (p=0.49). Two women complained about NAC hypersensitivity to touch and/or cold after NSM. Before mastectomy, breasts were important or very important for sexuality in 15/22 women (68%) in the SSM group compared to 10/18 women (56%; p=0.52) in the NSM group, which decreased to 4/22 (18%) and 4/18 (22%) after mastectomy, respectively (p=1.0). Before mastectomy, the NAC had been important to very important for sexuality to 13/22 women (59%) in the SSM group and for 10/18 women (56%) in the NSM group (p=1.0), decreasing in both groups to 3/18 (17%) and 2/22 (9%), respectively, after mastectomy (p=0.64). Due to the loss of NAC sensitivity after mastectomy sexual pleasure had substantially decreased or was absent in 6/22 women (27%) in the SSM group and in 6/18 women (33%) in the NSM group (p=0.74). Touching of the NAC by themselves or by their partners was unpleasant or very unpleasant for 2 women (9%) in the SSM group and 3 women (18%) in the NSM group (p=0.64). Still, in the NSM group, 12/18 women (67%) reported erection of the nipple to cold and/or touch as weaker to unchanged. In the SSM group, 4 women (19%) were not satisfied with the position of the (reconstructed) NAC versus 10 women (56%) in the NSM group (p=0.02). Of the 4 women in the SSM group who were not satisfied with the position of the NAC, 1 woman found the position of the reconstructed NAC too lateral, 1 too caudal, 1 found it too cranial and 1 did not further specify. Of the 10 women in the NSM group who were not satisfied with the position of the NAC, 6 specified that

the NAC position was too lateral, 2 that it was too caudal, 1 found it too cranial and 1 did not further specify. If they could choose again, 18/22 women (82%) in the SSM group would opt for this procedure again, as compared with 17/18 women (94%) in the NSM group (p=0.36). In the SSM group, 16/22 women (73%) would advise an SSM followed by a NAC reconstruction to other women and in the NSM group 15/18 women (83%) would recommend to undergo an NSM to other women (p=0.48).

DISCUSSION

We assessed various patient reported outcomes after SSM and NSM as performed in our clinic in a high breast cancer risk population who chose prophylactic mastectomy for risk reduction. In addition, sensitivity of the NAC was compared with NAC sensitivity in a healthy control group. In line with the conclusion of Didier et al. in 2009 we hypothesized that prophylactic NSM would result in higher patient satisfaction as compared with SSM followed by NAC reconstruction[8].

Body image did not differ significantly between the SSM and NSM groups. Body image scale (BIS) scores were low in most patients, representing relatively low levels of body image related distress, which may have been due to the fact that all patients underwent immediate breast reconstruction and therefore feelings of mutilation may have been limited[22]. Earlier studies, however, have reported a strong negative psychological impact of prophylactic mastectomy even after immediate breast reconstruction[23, 24]. In contrast, a study on delayed breast reconstruction reported improved body image[25], likely because breast reconstruction was an improvement compared with the post-mastectomy situation. Patient satisfaction and QoL as assessed by Breast-Q scales was high[26, 27] and did not differ between groups after adjustment for the longer follow-up in the SSM group. Therefore, trends for lower 'satisfaction with breasts' and 'satisfaction with outcome' in the NSM group in univariable analysis were probably attributable to a short follow up, causing incomplete recovery and adaptation at the time of examination[26]. Other possible factors of influence may have been the higher rates of necrotic complications and reoperations in the NSM group (Table 1). The SSM group, on the other hand, comprised higher comorbidity rates, higher exposure rates to smoking, chemotherapy and radiation and more infectious complications. Despite these unfavorable factors, body image, patient satisfaction and QoL were similar in both groups.

In line with previous studies, subjective NAC sensitivity was described as minimal or absent by all women with NAC reconstruction and by most women after NSM[28, 29]. Two women after NSM reported an unpleasant hypersensitivity of the spared NAC to cold and/or touch. No differences in the role of the breast or NAC for sexuality were found. Sexual pleasure decreased similarly after SSM and NSM. This is in line with previous studies on sexuality after NAC reconstruction and NSM[8, 29] and contradicts the argument that

NSM may protect from sexual problems. In total, 36% had a history of prophylactic oophorectomy, which may have further aggravated sexual problems after mastectomy[30]. In the SSM group, some patients complained about the loss of projection of the NAC reconstruction or too much projection causing visibility of the NAC when dressed. In the NSM group on the other hand, significantly more patients were unsatisfied with the position of the spared NACs. Loss of projection of a NAC reconstruction after SSM and malposition of a spared NAC after NSM both are frequently reported challenges[6, 16].

To more objectively quantify residual or regained sensitivity we tested NAC and skin flap sensitivity after NSM and - lacking preoperative sensitivity measurements - compared it with sensitivity in a non-operated control group. Sensitivity of all nine tested sites was low after NSM, and significantly higher in the control group (Figure 5). Remarkably, in the NSM group sensitivity of the NAC and surrounding skin flap was lowest at laterocaudal sites, which corresponds with the inferolateral periareolar incisional site in the majority of NSMs (Table 1). Therefore, although the Semmes Weinstein test is only validated for quantification of limb sensitivity[31, 32], its discriminatory ability seemed also adequate for our purpose. Sensitivity was similarly low in 5 women who underwent NSM through an inframammary incision (data not shown). Despite the low sensitivity as tested and reported by women in the NAC-specific questionnaire, nipple erection as a reaction to touch and/or cold was reported present in 67% of women after NSM. This was also observed during the Semmes Weinstein sensitivity tests (data not shown). Previously, nipple erection has been used as an instrument to measure sensitivity of the NAC after NSM and was found to be present in all cases after NSM[19]; our data show that by these means sensitivity after NSM is most likely overrated. Nipple erection is most likely innervated by the autonomous nerve system and is therefore not the same as NAC sensation through somatosensory nerves. Nonetheless, an intact nipple erection may contribute to a woman's feeling of a more natural NAC after NSM than after NAC reconstruction[28]. The expectation of preserving NAC sensitivity should not be a motivation for choosing NSM over SSM and NAC reconstruction.

The value of a spared or reconstructed NAC for psychological adjustment after breast surgery has been described before[8, 9]. In this study we did not specifically evaluate motivations for choosing SSM or NSM. As reported postoperatively, there were no preoperative differences between groups in importance of the breast or NAC for sexuality. However, some women in the NSM group explained that their breast reconstruction felt more natural because of the spared NAC. As a contrast, in the SSM group, several women commented that their breasts had always represented a high cancer risk and that they had felt detached from their breasts since long before mastectomy. Remarkably, in the NSM group 5 women underwent reoperations for dissatisfaction with aesthetic outcome compared with 0 in the SSM group. Although this may be due to different aesthetic outcomes after NSM versus SSM, higher or distinct preoperative expectations

may have played a role[33]. Interestingly, a recent study summarized the psychological advantage of a spared NAC over a reconstructed NAC as follows: NSM is a 'conservative' procedure while NAC reconstruction after SSM is mainly a 'restorative' procedure[33]. Possibly these differences result in distinct motivations for choosing SSM or NSM, since for some patients a spared NAC may unpleasantly remind them of their own breast and the concurrent breast cancer risk. In this study for example, in the NSM group 4 women did not have a proven *BRCA1/2* gene mutation versus 0 in the SSM group. We speculate that the population at high breast cancer risk, especially with a *BRCA1/2* gene mutation, is distinct with respect to some of their motivations for choosing prophylactic NSM or SSM with NAC reconstruction, as compared with the sporadic breast cancer population undergoing prophylactic surgery. A qualitative study regarding the motivations to choose for prophylactic NSM should be performed to explore this hypothesis.

To our knowledge, this is the first study evaluating sensitivity of the NAC in combination with patient satisfaction specifically in women with high breast cancer risk undergoing NSM compared with prophylactic SSM and NAC reconstruction. Another important strength was the use of the Breast-Q and the BIS, two validated and widely used questionnaires. However, there are several limitations. First, because of the small groups we may have missed differences in outcomes between the groups. This is mainly attributable to the select population of *BRCA1/2* gene mutation carriers and other women at high breast cancer risk that were eligible for this study. Second, as addressed above the NSM group had a significantly shorter follow-up which may have resulted in an incomplete coping and/or recovery process at the time of examination. This is partly attributable to the cross-sectional design of this study and the observation that the frequency of NSM has increased in the past years in our institute. Finally, lacking a validated questionnaire for satisfaction and sexuality with respect to a spared or reconstructed NAC, specifically, we designed a NAC specific questionnaire that did not undergo formal psychometric evaluation.

In conclusion, in this population of women at high breast cancer risk patient satisfaction and body image was similar after SSM and NSM. Both reported and measured NAC sensitivity was low after NSM and therefore NSM should not be recommended for preservation of NAC sensitivity. SSM followed by NAC reconstruction and NSM led to comparable results in satisfaction with the NAC. Therefore, SSM is a balanced alternative for NSM in a high breast cancer risk population. Motivations and preferences of patients may differ and should be explored before choosing either SSM or NSM as a risk reducing option.

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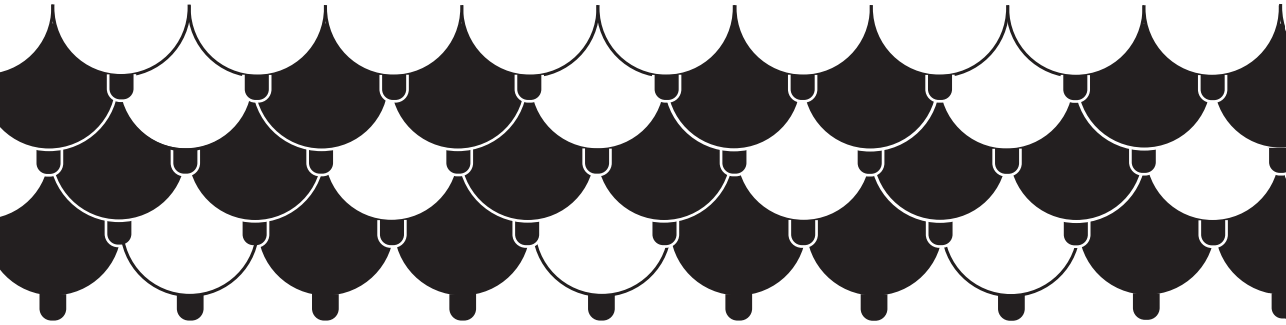

CHAPTER 10

Long-term patient satisfaction and aesthetic outcome after bilateral mastectomy and direct-to-implant breast reconstruction in *BRCA1/2* gene mutation carriers and women at high breast cancer risk

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Submitted

ABSTRACT

Background Challenges of direct-to-implant breast reconstruction are to achieve sufficient implant coverage and lower pole projection. We assessed reoperation rates, long-term patient satisfaction and aesthetic outcome after direct-to-implant BR without acellular dermal matrix (ADM) in women with high breast cancer risk.

Methods Women who underwent bilateral mastectomy and immediate direct-to-implant BR between 1994-2006 completed a survey on reoperations and the Breast-Q Reconstruction questionnaire. Photographs taken during follow-up were rated for long-term aesthetic outcome (scale 1-10) by five plastic surgeons. Outcomes were compared between women who never underwent unanticipated reoperations after immediate BR and women who underwent one or more reoperations, adjusted for potential confounders using multivariable linear regression.

Results Seventy women (49%) were never reoperated and 73 (51%) had undergone reoperations. Median follow-up was 12 years in both groups (range 7-17 and 6-19 years, respectively). Baseline characteristics were comparable except for history of prophylactic oophorectomy with 81% in the no-reoperations group versus 66% in the reoperated group (P=0.03). Breast-Q scores were 59.7±17.3 vs. 58.0±17.8 (P=0.67) for 'satisfaction with breasts' and 71.1±20.3 vs. 68.1±22.9 (P=0.47) for 'satisfaction with outcome' in the no-reoperation versus reoperation group, respectively. Aesthetic outcome was scored 5.8±1.1 in the no-reoperation group versus 5.3±1.3 in the reoperation group (P=0.01).

Conclusion The single-stage intent did not prevent unanticipated surgical reinterventions in 51% of the patients. Long-term patient satisfaction was reasonable and not affected by reoperations. Aesthetic outcome, however, was only poor to reasonable and scores were significantly lower in the reoperated group.

INTRODUCTION

BRCA1/2 gene mutation carriers and other women at high breast cancer risk may choose to undergo risk-reducing mastectomy (RRM) followed by immediate breast reconstruction[1]. Bilateral mastectomy reduces breast cancer risk by 90-100% after 3-13 years of follow-up[2-6]. The various BR options after mastectomy are typically grouped in autologous BR, implant-based BR or a combination of both. Compared with implant-based techniques, autologous BRs are associated with a superior, more natural result, at the expense of more complex surgical procedures with higher risk of more severe complications and additional donor site morbidity[7, 8]. Modern implant-based BR techniques typically consist of expanding of the subpectoral pocket using a tissue expander (TE), followed by placement of a permanent implant in a second stage. Implant-based BR is indicated in women with small to moderate breast volume requirements without previous breast irradiation and in women unwilling or physically unsuitable to undergo autologous BR[7, 8].

To avoid the additional operation for permanent implant placement after TE, interest in single-stage direct-to-implant BRs has been increasing. The challenge of single-stage procedures is that implant coverage needs to be achieved by the unexpanded pectoral muscle to protect the implant and avoid complications including mastectomy skin flap thinning, skin flap necrosis, infection, implant loss and capsular contracture[7-10]. Furthermore, breast asymmetry is a frequent complication after single-stage subpectoral implant placement[11]. Alternatively, acellular dermal matrix (ADM) products have gained interest to provide a better inframammary fold (IMF) definition, better lower pole projection as well as coverage of the implant in combination with the pectoral muscle[1 12]. Because of higher early complication rates and costs compared to submuscular implant techniques[12, 13], the use of ADM products is not undisputed. To our knowledge, long-term outcomes of direct-to-implant reconstruction without ADM have never been reported and patient-reported outcomes are unknown.

Between 1994 and 2006, direct-to-implant BRs were frequently performed at our centre in *BRCA1/2* mutation carriers and other women at high breast cancer risk opting for risk reducing mastectomy. The aims of the current study were 1) to assess long-term aesthetic outcome, quality of life (QoL) and patient satisfaction after bilateral direct-to-implant BR, and 2) to investigate the impact of complications and re-operations. Therefore, long-term aesthetic outcome, QoL and patient satisfaction were compared between patients who did not undergo reoperations and patients who underwent one or more reoperations. In addition, to assess long-term aesthetic outcome photographs were scored by an expert panel.

PATIENTS AND METHODS

Patients

Between 1994 and 2006, all female *BRCA1/2* mutation carriers and other women at increased breast cancer risk ≥21 years who opted for RRM were prospectively included in a follow-up database after written informed consent. Photographs were taken at regular time points during follow-up after BR.

Patients with bilateral skin-sparing or nipple-sparing mastectomy and immediate direct-to-implant BR were selected from the database. Patients with unilateral therapeutic mastectomy without BR followed by secondary contralateral RRM and bilateral BR were also eligible. All BRs were performed by a single plastic and reconstructive surgeon. The implant was placed underneath the pectoralis major muscle, which was opened in the muscle fibre course or lifted at the inferior edge. Implant size largely depended on the limited submuscular space that could be created, which was preoperatively discussed with the patient.

Follow-up started at BR or, if applicable, at nipple-areola complex (NAC) reconstruction. Exclusion criteria were a body mass index (BMI) ≥30 kg/m^2 at BR - based on current national guidelines that women with a BMI ≥30 are at increased risk for complications and poor outcome after implant-based BR, BR using tissue expanders, an autologous flap or an autologous flap-implant combination. The institutional ethics committee approved the study.

Data on complications and reoperations were collected from medical records, taking into account that women may have consulted another clinic for follow-up or reoperations, and by means of a telephonic patient survey. Women who were eligible received a written notification and were called within two weeks for completion of the survey. Patients who did not complete the telephonic survey were excluded from analyses. After completing the telephonic survey patients were mailed the Breast-Q Reconstruction questionnaire and a paid return envelope, if necessary followed by one telephonic and one written reminder.

Patient groups

We identified two groups. Group 1 consisted of patients without any reoperation since bilateral implant BR ± NAC reconstruction. Patients who had undergone a single reoperation because of hematoma at the surgical site within 24 hours from BR were also included in this group. Group 2 consisted of patients who had undergone ≥1 reoperations >24 hours after mastectomy and BR, who either still had subpectoral breast implants at the time of patient survey or who had undergone a tertiary revision BR over time. Tertiary reconstructions were defined as tissue expander-assisted BR's, implant reconstructions in combination with an autologous flap and autologous reconstructions.

Primary outcomes

Reoperations

Between March 2013 and November 2013 patients were interviewed by telephone regarding primary BR, experienced complications and unanticipated reoperations (date, indication and type of reoperation), history of breast cancer and comorbidity. Information from the telephonic patient survey was validated and complemented with information from the follow-up database and medical records. An unanticipated reoperation was defined as a reoperation ± implant revision. NAC reconstruction was not considered an unanticipated reoperation.

Patient reported outcomes

The Breast-Q Reconstruction module was used to assess BR-related quality of life (QoL) and patient satisfaction. The Breast-Q was developed by Pusic et al with good reported validity and reliability[14]. After completion of the telephonic survey, Breast-Q questionnaires were sent by mail. For the current study, six of the 19 independent Breast-Q scales were used: (1) 'satisfaction with breasts', measuring satisfaction with breast appearance, shape and symmetry; (2) 'satisfaction with outcome', assessing whether patients' expectations were met;[15] 'psychosocial well-being', rating social confidence and feelings of normalcy and equality; (4) 'sexual well-being', addressing the impact of breast surgery and reconstruction on sexuality; (5) 'satisfaction with nipple reconstruction', measuring satisfaction with the appearance and symmetry of the NAC reconstruction and (6) 'physical well-being', assessing the frequency of pain or discomfort in the breast area and the upper body. Breast-Q scores of the independent scales were calculated using the Q-Score Scoring Software, which provides a total score per scale that ranges from 0-100 with a higher score representing greater satisfaction or better quality of life. Missing or non-unique answers were invalid.

Secondary outcomes

Panel evaluation of aesthetic result

During follow-up, all women underwent photographic assessment of the BR at various time points resulting in ≥1 photograph per patient. To objectively evaluate aesthetic outcome an expert panel of five plastic breast surgeons (three male, two female) assessed the most recent photograph that was taken at least 12 months after the last surgical intervention (BR, NAC reconstruction or reoperation). Evaluated parameters were symmetry, shape, size, position of the IMF, definition of IMF, NAC reconstruction, appearance of the scar and overall aesthetic result[16]. One (mean) score was given for both breasts. Outcomes were measured using 5-point Likert Scales with '1' indicating a

very poor outcome and '5' indicating an excellent outcome. Overall aesthetic outcome was rated ranging from '1' (very poor) to '10' (excellent). To avoid observer bias, photographs were anonymised. To assess intra-observer variability, 20 random photographs were added in twofold and spread over the set.

Statistical analysis

Continuous variables were analysed using Mann-Whitney U tests and categorical variables using Chi-Square tests or Fisher's Exact tests. The cumulative per cent of women undergoing a first reoperation over time was illustrated by a Kaplan-Meier survival curve. Outcomes of Breast-Q scales and panel scores were analysed as continuous variables. Impact of differences in baseline characteristics with $P \leq 0.10$ on outcomes was tested by univariate linear regression analysis and, if $P \leq 0.10$, adjusted for by multivariable linear regression analysis. Inter- and intra-observer variance of the mean of panel scores and of the overall aesthetic result was assessed using intraclass correlation coefficients (ICCs). All P-values were two-sided and $\alpha \leq 0.05$ was considered statistically significant. SPSS (version 21.0) was used for statistical analyses.

RESULTS

Patients

In total, 182 women with bilateral mastectomy followed by direct-to-implant BR between 1994 and 2006 were identified from the database. Thirteen had a BMI $\geq$ 30 kg/m^2, 22 did not respond, two did not want to participate, one could not be reached due to emigration and one had died. Therefore, 143 (79%) were eligible for analyses. As shown in Table 1, 70 patients (49%) did not undergo any reoperation (Group 1). Seventy-three patients (51%) underwent $\geq$1 unanticipated surgical intervention after BR $\pm$ NAC reconstruction (Group 2). Baseline characteristics at immediate BR, follow-up, oncological history and comorbidity were comparable between the two groups. History of risk-reducing oophorectomy differed with 81% in the no-reoperation group vs. 66% in the reoperated group (P=0.03).

Table 1. Demographics, follow-up, clinical and primary breast reconstruction characteristics

	Group 1: No reoperations	Group 2: ≥ 1 reoperations	P
	n (%)	n (%)	
Total number of patients	70 (49%)	73 (51%)	
Response with			
Breast-Q	61 (87%)	60 (82%)	
Interview only	9 (13%)	13 (18%)	
Follow-up			
BR- Time to Breast-Q, years (median, range)	12.0 (7.0-17.0)	12.0 (6.0-19.0)	0.41*
Patient characteristics			
Age at BR years (median, range)	38.5 (23.0-55.0)	36.0 (24.0-58.0)	0.33*
Period of BR year (median, range)	2001 (1996-2006)	2000 (1994-2006)	0.41*
Body Mass Index at time of BR kg/m^2 (mean, ± SD)	22.5 (± 2.4)	23.1 (± 2.8)	0.32*
Prophylactic oophorectomy prior to/ after BR			
Yes	57 (81%)	48 (66%)	0.03**
No	13 (19%)	25 (34%)	
Oncological history			
Indication prophylactic mastectomy			
BRCA1 mutation	51(73%)	45 (62%)	0.29**
BRCA2 mutation	10 (14%)	12 (16%)	
Non-*BRCA* familial breast cancer risk	9 (13%)	16 (22%)	
History of, or breast cancer at time of mastectomy			
Yes	17 (24%)	22 (30%)	0.43**
No	53 (76%)	51 (70%)	
Mastectomy			
Bilateral prophylactic	58 (83%)	57 (78%)	0.47**
Unilateral therapeutic, unilateral prophylactic	12 (17%)	16 (22%)	
Oncological history, other than breast cancer			
Yes	4 (6%)	4 (6%)	1.00**
No	66 (94%)	69 (94%)	
Risk factors			
Smoking at time of BR*			
Yes	15 (28%)	20 (33%)	0.56**
No	39 (72%)	41 (67%)	
Unknown	16	12	
Diabetes at time of BR[1]			
Yes	1 (2%)	1 (2%)	1.00**

Table 1. Demographics, follow-up, clinical and primary breast reconstruction characteristics (continued)

	Group 1: No reoperations	Group 2: ≥ 1 reoperations	P
	n (%)	n (%)	
No	63 (98%)	63 (98%)	
Unknown	6	9	
Chemotherapy for breast cancer prior to/ after BR			
Yes	12 (17%)	12 (16%)	0.91**
No	58 (83%)	61 (84%)	
Radiation history prior to/ after BR[1]			
Yes	4 (6%)	10 (11%)	0.26**
No	66 (94%)	65 (89%)	
Primary BR			
Implant size cc (mean ± SD)	339 (± 68.5)	342 (± 78.6)	0.88*
Nipple-areola complex (NAC)			
Reconstruction[1]	53 (76%)	60 (82%)	0.57**
Nipple-areola sparing	2 (3%)	4 (6%)	
No reconstruction or sparing of the NAC	15 (21%)	9 (12%)	

BR: direct-to-implant breast reconstruction; SD: standard deviation; cc: cubic centimeter
[1]Nipple-areola complex (NAC) reconstruction by dermal graft or skin flap and/or intradermal tattoo
*Mann-Whitney U Test
**Chi-square Test
***Fisher's Exact Test

Reoperations and indications in Group 2

Mean number of reoperations per patient was 2.2 (±1.9; Table 2). Most indications for reoperations concerned implant related issues, with capsular contracture being the most frequent indication (n=32, 44%). Nine of 17 women with a tertiary reconstruction received a latissimus dorsi flap (LD). Median time to first reoperation was 20.5 months (range 0-204), meaning that approximately 50% of the first reoperations were performed in the first 24 months after primary BR.

Table 2. Indications for reoperations and tertiary reconstructions

	N (% of 73 reoperated patients)
Number of reoperations Total per patient (mean, ± SD)	2.2 (±1.9)
Postoperative complications (> 24 hours postoperative)	
Surgical site infection	6 (8%)
Wound dehiscence	1 (1%)
Seroma	1 (1%)
Implant related issues	
Capsular contracture	32 (45%)
Implant size related	24 (33%)
Implant position related	17 (24%)
Implant rupture	14 (20%)
Mastectomy skin flap necrosis	3 (4%)
Passed implant expiration date	1 (1%)
Unknown	1 (1%)
Local recurrence of breast cancer	3 (4%)
Pain	10 (14%)
Breast contour and scar related issues*	12 (17%)
Tertiary reconstructions	17 (23%)
Latissimus dorsi flap	9 (12%)
Tissue expander and breast implant	5 (7%)
DIEP flap	3 (4%)

Multiple complications per patient and per operation were possible.
SD: standard deviation; DIEP flap: Deep Inferior Epigastric artery Perforator flap
[1]i.e. correction of dogears, scar, skin excess

Patient satisfaction and quality of life (QoL)

Breast-Q Reconstruction questionnaires were completed and returned by 61 patients (86%) in Group 1, and by 60 patients (82%) in Group 2 (Table 3). There were neither significant differences between both groups in 'satisfaction with breasts' or 'satisfaction with overall outcome', nor in the QoL scales regarding 'psychosocial', 'sexual' and 'physical well-being', nor in 'satisfaction with nipples'. Differences between groups regarding 'risk-reducing oophorectomy' did not affect Breast-Q scores as tested by univariate linear regression (data not shown). A subgroup analysis in women who underwent tertiary BR showed patient satisfaction and QoL comparable to the group without reoperations (Table 4).

Table 3. Patient satisfaction and quality of life assessed by Breast-Q Reconstruction scales

	Group 1: No reoperations		Group 2: ≥ 1 reoperations			
Number of patients	71		85			
Number of Breast-Q's (% of group)	61 (86%)		70 (82%)			
Breast-Q scales	Mean	± SD	Mean	± SD	Mean score difference	P-value[1]
Satisfaction with breasts	59.7	±17.3	58.0	± 17.8	1.7	0.67
Satisfaction with overall outcome	71.1	± 20.3	68.1	± 22.9	3.0	0.47
Psychosocial well-being	70.8	±17.8	70.1	± 20.0	0.6	0.69
Sexual well-being	59.8	± 22.2	57.7	± 21.9	2.1	0.45
Physical well-being	78.6	± 15.1	72.9	± 20.0	5.7	0.14

Breast-Q scores ranged from 0 (minimal satisfaction or quality of life) to 100 (maximum satisfaction or quality of life)
[1]Mann-Whitney U Test

Table 4. Patient satisfaction and quality of life after tertiary reconstruction assessed versus without reoperations by Breast-Q Reconstruction scales

	Group 1: No reoperations		Tertiary reconstruction			
Number of patients	71		17			
Number of Breast-Qs (% of group)	61 (86%)		12 (74%)			
Breast-Q scales	Mean	± SD	Mean	± SD	Mean score difference	P-value[1]
Satisfaction with breasts	59.7	± 17.3	61.8	± 17.4	-2.2	0.86
Satisfaction with overall outcome	71.1	± 20.3	64.2	± 22.2	6.9	0.32
Psychosocial well-being	70.8	± 17.8	63.5	± 17.6	7.3	0.14
Sexual well-being	59.8	± 22.2	59.2	± 20.8	0.6	0.97
Physical well-being	78.6	± 15.1	70.2	± 21.6	8.4	0.14

Breast-Q scores ranged from 0 (minimal satisfaction or quality of life) to 100 (maximum satisfaction or quality of life)
[1]Mann-Whitney U Test

Panel evaluation of photographs

Photographs of 69 women in Group 1 and of 68 in Group 2 were suitable for panel evaluation (Table 5; Figures 1-4). Median follow-up since BR was 7.0 years (range 1.0-11.0) and 6.5 years (range 1.0-17.0), respectively. In Group 2, 40 patients (28%) underwent reoperations (mean 1.0 ±1.6) after the photograph used for panel evaluation. Panel scores were significantly favourable in Group 1 vs. Group 2 for the items 'symmetry' (3.17 ±0.57 vs. 2.96 ±0.70; P=0.02), 'shape' (3.02 ±0.57 vs. 2.78 ±0.69; p=0.02), 'definition of

IMF' (3.02 ±0.63 vs. 2.78 ±0.68; p=0.03), 'NAC reconstruction' (3.42 ±0.57 vs. 3.17 ±0.67; p=0.05). Overall aesthetic result (scale 1-10) was 5.82 ±1.14 in Group 1 vs. 5.31 ±1.31 in Group 2 (P=0.01). 'Risk-reducing oophorectomy' did not affect panel outcomes as tested by univariate linear regression, except for the item 'position of IMF' with B=0.22 (P=0.06) in univariate and B=0.19 (P=0.11) in multivariable linear regression analyses (data not shown). Consequently, outcome of 'position IMF' was adjusted for 'risk-reducing oophorectomy'. Inter-observer variability was satisfactory for the mean of scored scales (ICC=0.66) and for the overall aesthetic result (ICC=0.68). Intra-observer variability ranged from satisfactory (0.63) to excellent (0.94) (Table 6).

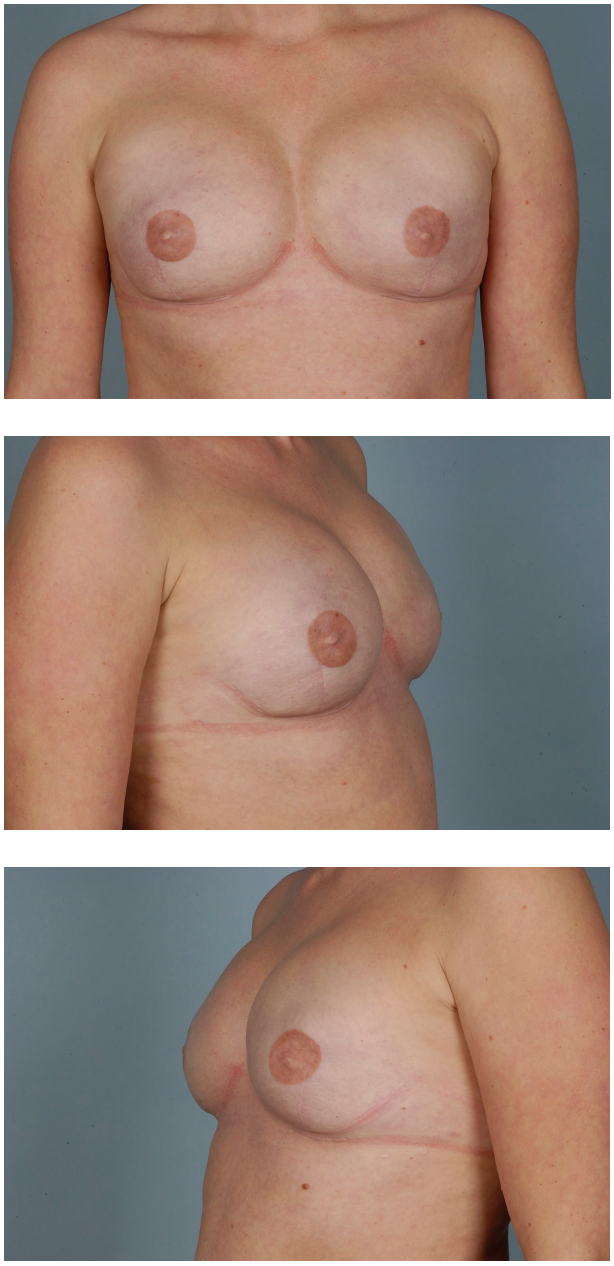

Figure 1. Panel photographs 1 year after direct-to-implant breast reconstruction. No reoperations had been performed before panel photograph but four years after panel photograph the patient underwent capsulotomy and revision of the implants to larger implants. Photographs were rated by the panel 7.8/10 points for overall aesthetic outcome (ranging from 1: very poor to 10: excellent aesthetic outcome). **A** Frontal **B** and **C** oblique views.

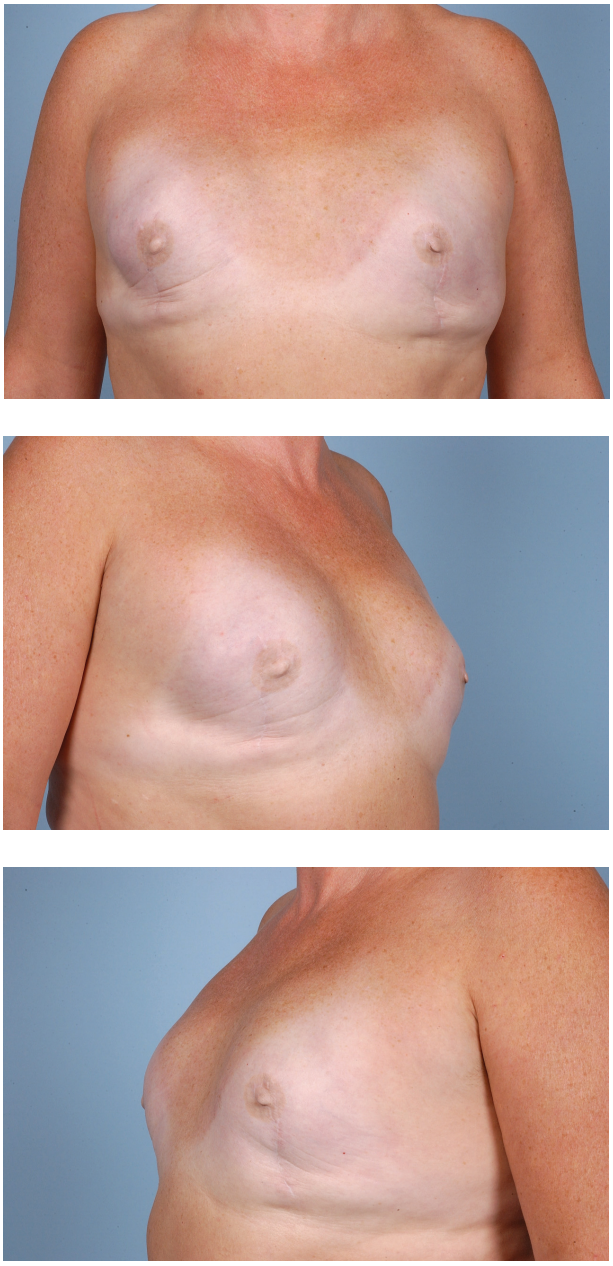

Figure 2. Panel photographs 6 years after direct-to-implant breast reconstruction. No reoperations had been performed. Photographs were rated by the panel with a mean of 2.8/10 points for overall aesthetic (ranging from 1: very poor to 10: excellent aesthetic outcome). **A** Frontal **B** and **C** oblique views.

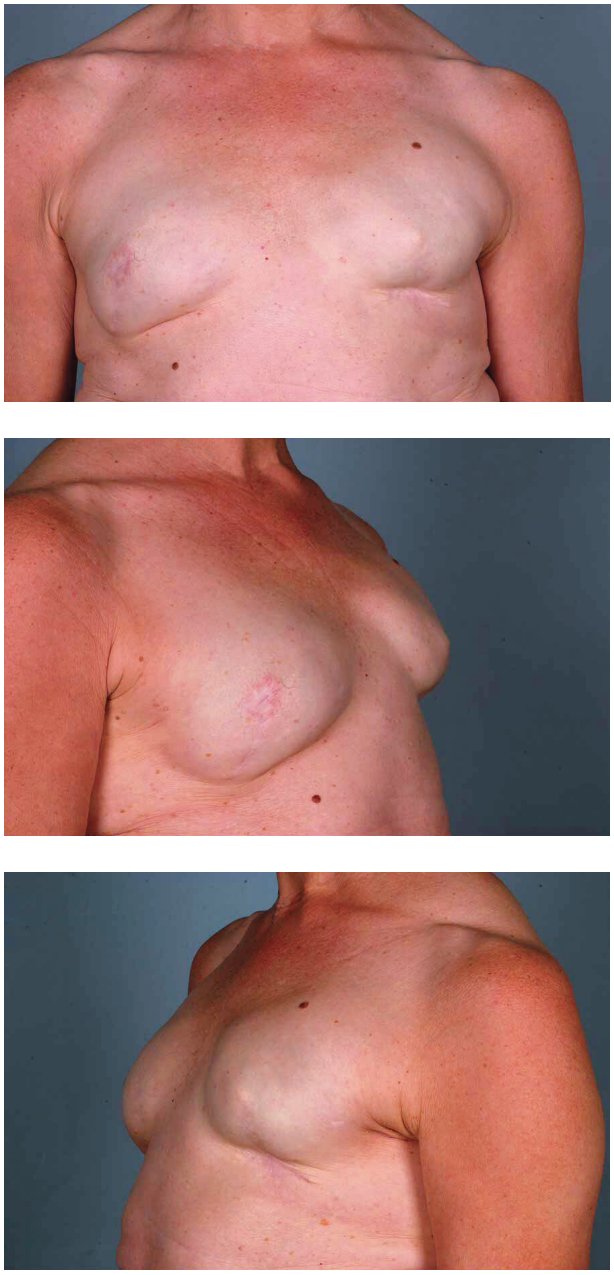

Figure 3. Panel photographs 4 years after direct-to-implant breast reconstruction. The patient needed excision of the scar due to breast cancer recurrence. Photographs were rated by the panel with a mean of 2.6/10 points for overall aesthetic outcome (ranging from 1: very poor to 10: excellent aesthetic outcome). **A** Frontal **B** and **C** oblique views.

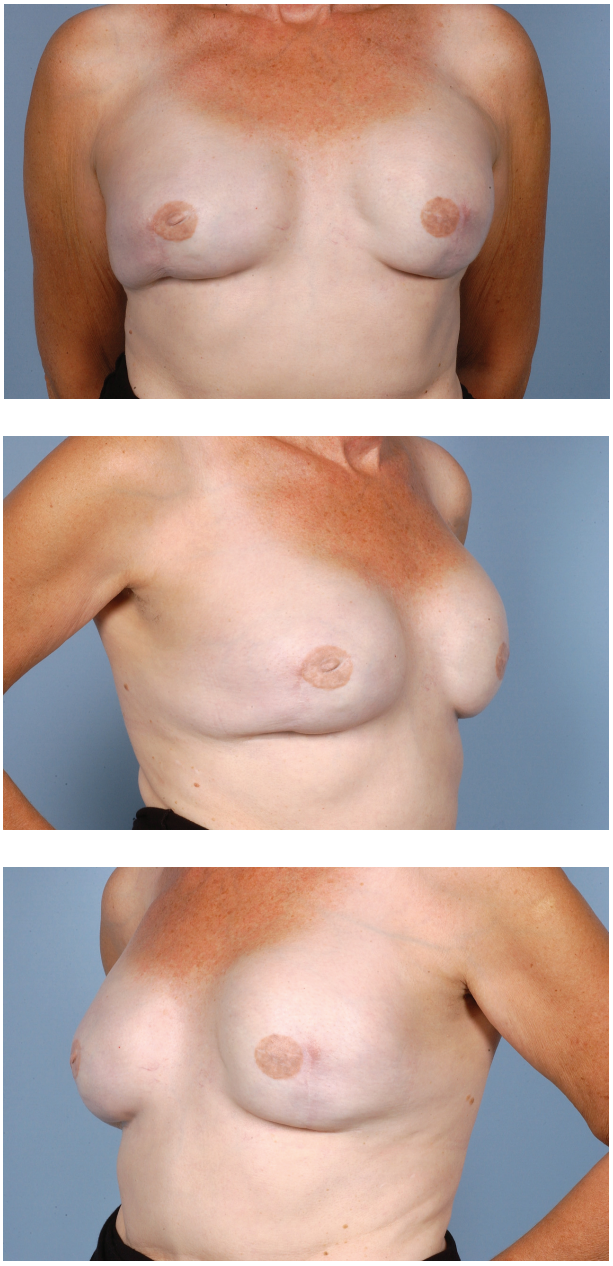

Figure 4. Panel photographs 7 years after direct-to-implant breast reconstruction. The patient was reoperated once due to capsular contraction 4 years after BR. Photographs were rated by the panel with a mean of 6.0/10 points for overall aesthetic outcome (ranging from 1: very poor to 10: excellent aesthetic outcome). **A** Frontal **B** and **C** oblique views.

Table 5. Panel evaluation of postoperative photographs

	Group 1: No reoperations		Group 2: ≥ 1 reoperations		P-value[3]
Number of patients	70		73		
Number of photographs (% of group)	69 (99%)		68 (88%)		
Years BR – panel photograph (median, range)	7.0	(1.0-11.0)	6.5	(1.0-17.0)	0.82
Satisfaction with[1]	Mean	± SD	Mean	± SD	
Symmetry	3.17	± 0.57	2.96	± 0.70	0.02
Shape	3.02	± 0.57	2.78	± 0.69	0.02
Size	3.22	± 0.95	2.99	± 0.72	0.14
Position IMF	3.24	± 0.57	3.05	± 0.65	0.14*
Definition IMF	3.02	± 0.63	2.78	± 0.68	0.03
NAC reconstruction	3.42	± 0.57	3.17	± 0.67	0.05
Scar	3.11	± 0.44	3.05	± 0.45	0.43
Overall aesthetic result[2]	5.82	± 1.14	5.31	± 1.30	0.01

BR: breast reconstruction; SD: standard deviation; IMF: inframammary fold;
[1]Items scored on a Likert scale ranging from 1 (very poor result) to 5 (excellent result)
[2]Overall aesthetic result scored on a 10-point scale ranging from 1 (very poor result) to 10 (excellent result)
[3]Mann-Whitney U Test
*P-value adjusted for 'prophylactic oophorectomy' using linear regression. Potential confounding of 'prophylactic oophorectomy' on outcome of 'position IMF' was found with B=0.22 (P=0.06) in univariate and B=0.19 (P=0.11) in multivariable analyses.

Table 6. Inter-observer and intra-observer variability of five panel members

	Inter-observer	Intra-observer variability[3]				
	5 panel members	1	2	3	4	5
Satisfaction with	ICC scores	ICC scores	ICC scores	ICC scores	ICC scores	ICC scores
Symmetry[1]	0.54	0.88	0.66	0.54	0.61	0.83
Shape[1]	0.55	0.86	0.70	0.51	0.73	0.76
Size[1]	0.18	0.75	0.47	0.31	0.69	0.44
Position IMF[1]	0.51	0.76	0.70	0.43	0.42	0.54
Definition IMF[1]	0.55	0.87	0.71	0.79	0.71	0.43
NAC reconstruction[1]	0.44	0.81	0.47	0.66	0.82	0.61
Scar[1]	0.30	0.71	0.41	0.47	0.69	0.67
Overall aesthetic result[2]	0.67	0.94	0.82	0.63	0.84	0.90

ICC: Intraclass correlation coefficient (0= no agreement, 1= perfect agreement), IMF: inframammary fold
[1]Scored on a Likert scale ranging from 1 (very poor result) to 5 (excellent result)
[2]Scored on a 10-point scale ranging from 1 (very poor result) to 10 (excellent result)
[3]Intra-observer variability based on 20 random panel photographs that were added in twofold and spread over the set

DISCUSSION

Interest in direct-to-implant BR has been increasing since the introduction of ADM products for implant coverage. For women who chose BR after mastectomy, direct-to-implant BR without the use of ADM used to be standard of care at our centre until the expander/implant technique was introduced in 2006 because of promising results. This prompted us to evaluate reoperation rates, long-term patient satisfaction and aesthetic outcomes by use of a prospectively collected database between 1994 and 2006.

Strikingly, despite the single-stage intent, over time 51% needed ≥1 unanticipated reoperations. Indications for reoperations could be medical, such as surgical site infections or capsular contractures or for aesthetic reasons, such as dissatisfaction with size or symmetry. Reoperations for surgical site infections or capsular contractures may compromise aesthetic outcome, while reoperations for purely aesthetic dissatisfaction should increase aesthetic outcome. In the current study, most reoperations were performed for a combination of indications. Capsular contracture was reported most frequently as a reason for reoperation (45% of reoperations), in line with expander/implant BR[17]. Various studies have evaluated reoperations after implant BR[15, 17-21] and found that direct-to-implant BR is associated with significantly more early complications (≤30 days after BR) due to implant failure compared with expander/implant BR[15, 19]. Although a 51% reoperation rate is high, comparable percentages have been reported after TE/implant and autologous BR in other studies[20, 22].

A panel of five plastic surgeons assessed aesthetic outcome. Photographs had been taken during the postoperative course with a median follow-up of 7 years. Scores on symmetry, shape, definition of IMF and NAC reconstruction were significantly lower in the reoperated group. Specifically, scores on size and shape were low in both groups ranging between 2.80 and 3.04 out of 5. The low scores on size possibly reflect the difficulty to achieve enough breast volume in direct-to-implant BR due to the limited non-expanded subpectoral space. Overall aesthetic outcome was scored poor to reasonable in both groups, but significantly lower in the reoperations group compared with the no-reoperation group (Table 5). The panel outcomes were not in line with Breast-Q outcomes, which show significant differences between both groups (Table 3). Previous studies reported that satisfaction of plastic surgeons with aesthetic outcome is significantly lower than patient satisfaction[16, 23]. It is hypothesized that due to their professional expectations plastic and reconstructive surgeons are trained to focus on technical imperfections that could easily be refined by additional surgery, while patients compare the overall result with the preoperative situation[16].

To our knowledge, the present study is the first to use the Breast-Q Reconstruction questionnaire after direct-to-implant BR. After a median follow-up of 12 years since BR, we found no differences in patient satisfaction or QoL between patients with and without reoperations (Table 3). In both groups, scores on satisfaction with breasts (Breast-Q

scores 59.7 in the non-operated and 58.0 in the operated group) and overall outcome (Breast-Q scores, 71.1 and 68.1, respectively) were comparable with studies that used the Breast-Q after expander/implant BR (satisfaction with breasts 52.5-67.8; satisfaction with outcome 65.8-74.8)[24-27]. Considering that long-term satisfaction with implant BR may further decrease with time[27, 28] and the studies referred to had much shorter follow-up periods than our study, Breast-Q scores in the present study were very reasonable. This may implicate that patient satisfaction does not necessarily decrease as much as expected when considering the rather moderate panel outcomes and the large amount of reoperations needed.

The finding that undergoing one or more unanticipated reoperations did not significantly affect patient satisfaction or QoL was unexpected. Satisfaction after complicated BR has previously been assessed with conflicting outcomes. Three relatively old and small studies did not find any association between satisfaction and surgical complications after expander/implant BR[29-31], while more recent, larger studies found decreased satisfaction, and even increase of anxiety and depressive symptoms after complicated expander/implant BR[17, 32]. These studies, however, had a shorter median follow-up of less than three years. Other studies with comparable long-term follow-up are not available. After 12 years follow-up, as in the present study, adequate coping of patients with a poor result or following adverse events may have caused a relatively mild rating of outcome. Of all patients, 17 (12%) had chosen to undergo a tertiary reconstruction over time and 12 of them completed and returned the Breast-Q. A subgroup analysis showed lower outcomes on satisfaction and QoL compared with the no-reoperation group, except for a higher 'satisfaction with breasts' (Table 4). None of these differences, however, were statistically significant and the group was too small and heterogeneous regarding tertiary BR methods to draw conclusions from. An earlier analysis by Visser et al. of 42 women from our centre with tertiary reconstructions partly covered our study population and did show higher patient satisfaction after tertiary autologous reconstruction using free flaps[16].

An important strength of this study was the long follow-up period. To our knowledge no other studies have described patient reported outcomes of direct-to-implant reconstruction after comparable follow-up. Response rates were high. To collect as much information as possible patients had to complete a telephonic survey on complications and reoperations. Recall-bias was minimized by validating survey information with information from the follow-up database and medical charts. Another strength of the current analysis was the use of the Breast-Q. Patient reported outcomes are important indicators of health care quality and the Breast-Q Reconstruction module is a frequently used instrument. The most important limitation of the present study was that 28% still needed an additional reoperation after the panel photograph. Therefore, the correlation between long-term Breast-Q outcomes and panel evaluation was limited. Being

a standardized questionnaire for breast reconstruction, the Breast-Q may have missed subtleties specific for the single-stage procedure including the fact that 49% had to undergo only one operation and (a-)symmetry of a bilateral reconstruction. Since panel scores on symmetry differed significantly between the groups it would be interesting to have a patient reported outcome on symmetry as well. Further, the group consisted of women who underwent bilateral RRM and of women with a unilateral risk-reducing and unilateral therapeutic mastectomy. The latter subgroup may be at greater risk for an asymmetric outcome. A subgroup analysis, however, in the 115 women who underwent bilateral RRM showed results similar to the overall group (data not shown).

In conclusion, the single-stage intent of a direct-to-implant BR did not prevent surgical reinterventions in a majority of patients. Long-term aesthetic panel outcome was poor to reasonable in both groups and significantly lower in the reoperated group. However, long-term patient satisfaction and QoL were good and did not differ between the groups, suggesting reoperations after direct-to-implant BR do not affect long-term patient satisfaction and QoL. Prospective follow-up studies are urgently needed that assess long-term patient satisfaction and aesthetic outcome of modern direct-to implant BR compared with expander/implant BR.

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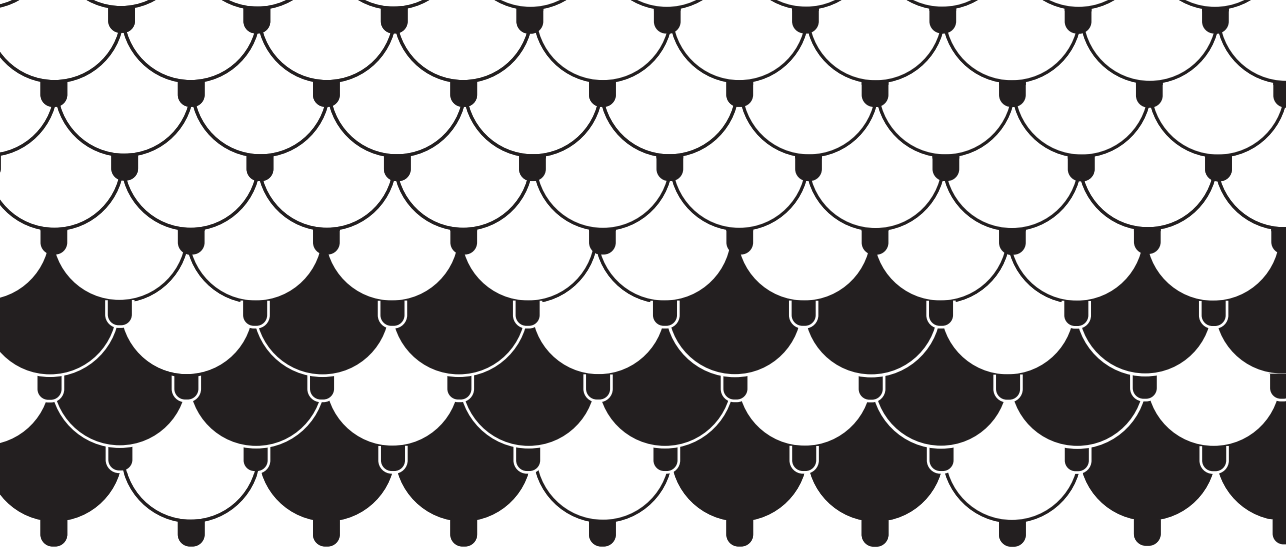
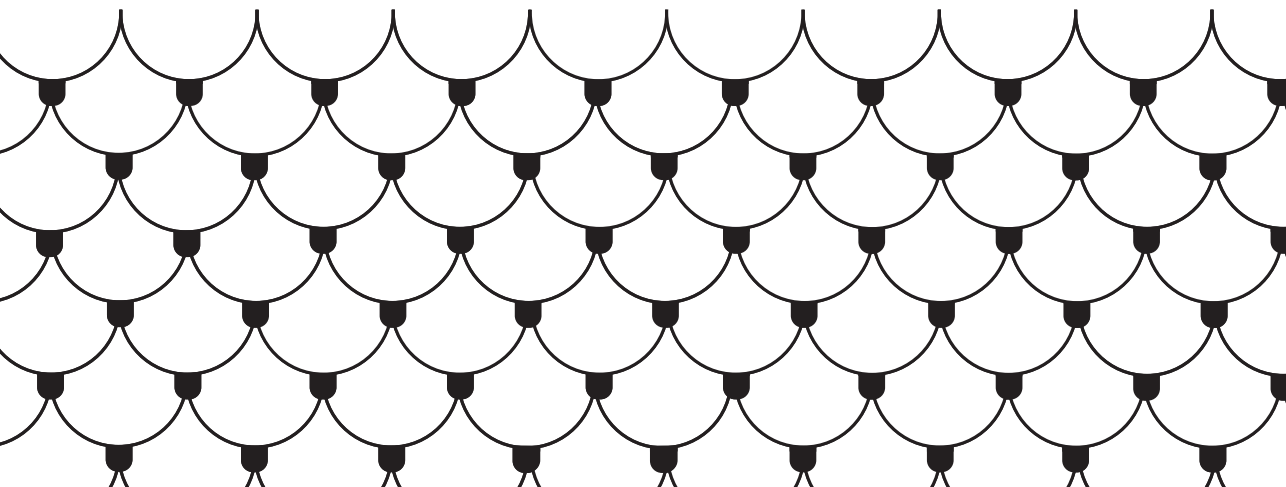
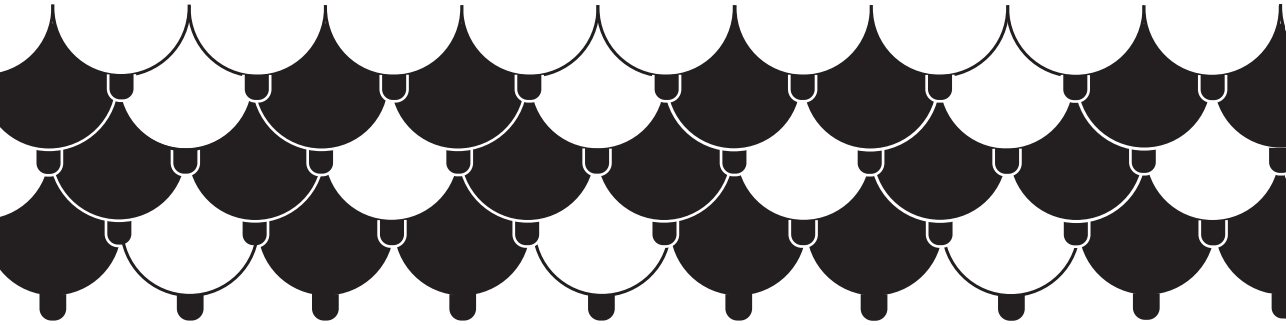
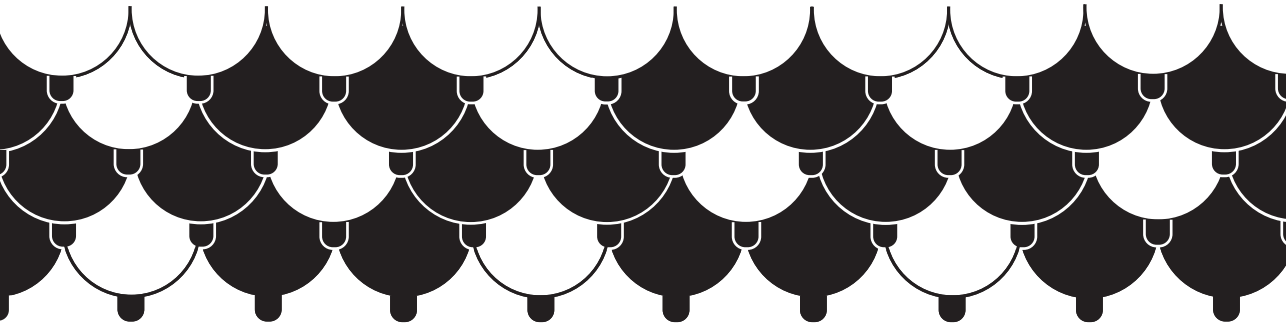




SUMMARY AND DISCUSSION





CHAPTER 11

Summary

SUMMARY

This thesis focuses on breast surgery in *BRCA1/2* gene mutation carriers. We studied a broad variety of subjects ranging from molecular and prognostic tumor characteristics in breast cancer, to safety, aesthetics and patient reported outcomes of prophylactic breast surgery.

In **Chapter 2** we review the literature on safety of prophylactic mastectomy in *BRCA1/2* gene mutation carriers. To allow for immediate breast reconstruction after bilateral mastectomy the skin envelope is left in situ with or without the nipple-areola complex (NAC). Any residual breast tissue with the skin flap of with the NAC represents a remaining breast cancer risk after prophylactic surgery. Anatomical studies on prophylactic mastectomy find residual breast glandular tissue behind the skin flap or the spared NAC in up to 60% mastectomies. The associated remaining breast cancer risk seems low. However, when pooling all reviewed data on *BRCA1/2* gene mutation carriers, over time approximately 1 in 140 will develop a primary breast cancer after risk-reducing mastectomy.

Chapter 3 studies the diminishing effect of risk-reducing salpingo-oophorectomy (RRSO) on the incidence of post-RRSO breast cancers in *BRCA1/2* gene mutation carriers. The hypothesis is that breast cancers developing after RRSO have a less aggressive character compared to breast cancers that develop before RRSO. From a cohort of *BRCA1/2* gene mutation carriers with screen detected breast cancers, women who developed their first breast cancer < 12 months after RRSO were age-matched with women with breast cancer before RRSO. Breast cancers that developed after RRSO indeed had a lower mitotic count (12 versus 22 mitotic counts/2mm^2) and were smaller at detection (11 vs. 17 mm diameter). Median tumor volume doubling times were assessed on serial mammographies or MRIs and were non-significantly longer after RRSO (124 days vs. 93 days). These data suggest a less aggressive biological phenotype of breast cancers that develop after RRSO, which after being confirmed in larger series in the future may have consequences for the intensity of breast cancer screening after RRSO. The inhibiting mechanism of estrogen depletion on mainly estrogen receptor negative tumors, as typically found in *BRCA1* gene mutation carriers remains unclear.

Chapter 4 continues to focus on tumor characteristics. Due to intensive breast cancer screening protocols, most *BRCA1/2*-associated breast cancers are detected in an early stage - e.g. small and lymph node negative – poorly differentiated and in case of a *BRCA1* gene mutation triple-negative. The use of histologic characteristics for risk stratification is therefore limited, as are targeted therapy options in the triple-negative subgroup. In a Rotterdam cohort of 138 *BRCA1* and 37 *BRCA2* gene mutation carriers (breast cancer diagnosis 1982-2008) several histologic breast cancer characteristics that have earlier been investigated in sporadic breast cancers were evaluated in relation to recurrence-free survival (RFS), corrected for established prognostic factors. Tumor-associated

inflammation was the strongest independent favorable factor (HR 0.18 for moderate/marked vs. absent/mild; 95%CI 0.05-0.61). Other prognostic factors for RFS were tumor size (HR 2.47 for >2 cm vs. ≤2 cm; 95%CI 1.10-5.57) and intra-tumor necrosis (HR 2.60 for presence vs. absence; 95%CI 1.12-6.05). Nodal status and differentiation grade were not significantly related to RFS.

Since *BRCA1/2* gene mutation carriers have high risks of developing breast cancer, contralateral breast cancer as well as ovarian cancer, over time multiple tumors may be prevalent in one patient with or without concurrent metastases. Metastatic patterns may be atypical. However, knowing the tumor origins and clonal relationships between different tumors has consequences for therapy and prognosis and is therefore important. In **Chapter 5** we evaluate the value of molecular analysis in the form of Next Generation Sequencing (NGS) in a cohort of 42 female *BRCA1/2* gene mutation carriers who have multiple tumor localizations. In 4 of the 42 patients tumor origins could not conclusively be determined with tumor morphology and immunohistochemistry alone (cases) and therefore targeted NGS was performed. Another 10 patients with conclusive histopathology outcomes served as controls. In all 4 cases, the intra-patient clonal relationships between the tumor localizations could be unequivocally identified by molecular analysis. In all controls, molecular outcomes unequivocally matched the conventional histopathological results. Therefore we conclude that in case of inconclusive conventional histology results molecular analyses using NGS can reliably determine clonal relationships between tumors.

Following the results of Chapter 4, **Chapter 6** assesses the value of another potential prognostic marker in *BRCA1/2*-associated breast cancers. Nuclear β-catenin expression as a marker of Wnt activity has been studied in various cancers, and it is most significantly expressed in colorectal cancers. In sporadic breast cancer, the role of the Wnt/β-catenin pathway is thought to vary depending on the molecular subtype, being a prognostic factor especially in basal-like breast cancer, which suggests a role in *BRCA*-associated tumorigenesis. The Rotterdam cohort as described in Chapter 4 was extended to 2014. Since paraffin blocks of tumor material were needed for β-catenin stainings only 120 *BRCA1/2* gene mutation carriers were analyzed. When corrected for established prognostic markers, the only significant prognostic factor associated with RFS was strongly positive membrane-associated β-catenin expression (HR 0.19; 95%CI 0.05-0.73). Nuclear β-catenin expression was found in 38% of breast cancers, suggesting a potential role of Wnt activity in *BRCA1/2*-associated breast cancers. However in most cases nuclear β-catenin staining was confined to one or two nucleuses per tumor slide, and of no prognostic influence. Membranous β-catenin is also involved in cell-cell adhesion. The loss of membranous β-catenin expression may lead to epithelial-mesenchymal transition of cancer cells and therefore is probably not merely a marker of Wnt activity. Further, nuclear and membranous β-catenin expressions were not clearly (inversely)

correlated in this study. The prognostic value of membranous β-catenin expression has to be further elucidated and it would be useful to identify target genes as markers of Wnt activity.

Chapter 7 describes an anatomical study on the numbers of terminal duct lobular units (TDLUs) - being a quantification of the amount of breast glandular tissue - remaining with skin flap and the NAC after nipple-sparing mastectomy (NSM). In conventional mastectomies the NAC and an adjacent skin island were dissected as if it were an NSM and sectioned perpendicularly to the skin. Slides were scanned and slide surface areas (cm^2) were measured using Photoshop. TDLUs were counted microscopically and reported as numbers of TDLUs per cm^2 slide surface area. In total 105 NACs and skin islands of 90 women were analyzed pairwise. Sixty-four NACs (61%) vs. 25 skin islands (24%) contained ≥1 TDLUs. TDLU density was higher in NACs as compared to skin islands. Independent risk factors for presence of TDLUs in the NAC were younger age and parity (versus nulliparity). The finding of higher TDLU density behind the NAC as compared to the skin flap suggests that sparing the NAC in prophylactic NSM in high-risk patients possibly may increase postoperative breast cancer risk as compared to prophylactic SSM.

A frequently used scale to assess body image after cancer surgery is the Body Image Scale (BIS). In 2000 the BIS was developed and validated in breast cancer patients by Hopwood and colleagues. It assesses affective, behavioral and cognitive changes that may result from a cancer treatment. Because of its conciseness it may be broadly applied in clinical practice. In **Chapter 8** we describe the validation of the Dutch translated version of the BIS, which was performed in 150 patients who underwent mastectomy and 150 after breast conserving treatment. Psychometric evaluation of the Dutch BIS showed consistent results that were comparable to those of the original version.

In **Chapter 9**, the Dutch translated BIS was concurrently used for assessment of body image after prophylactic mastectomy. Studies in breast cancer populations have shown that patient satisfaction after prophylactic NSM is higher as compared with SSM. In succession to Chapter 7 considering the possible extra risk of residual TDLUs with the spared NAC, the question raised whether, according to women who choose prophylactic mastectomy, NSM is superior to SSM followed by reconstruction of the NAC using local skin flaps and intradermal tattooing. We administered the Breast-Q Reconstruction questionnaire and the Dutch BIS to 25 patients who underwent prophylactic SSM and 20 patients after prophylactic NSM. Additionally we developed a questionnaire that specifically focused on satisfaction with the spared or reconstructed NAC, including NAC sensitivity and the role of the NAC and the reconstructed breast in sexuality. One open-ended question was added to further explore patient's motivations for choosing either NSM or SSM, their opinion on the breast reconstruction, the spared or reconstructed NAC and about changes in sexuality. Univariably, Breast-Q scores were favorable in the SSM group for 'satisfaction with breasts' and 'satisfaction with outcome'. However, when

corrected for follow-up BIS and Breast-Q scores did not differ significantly between SSM and NSM. Also satisfaction with the reconstructed NAC was similar to satisfaction with the spared NAC. The sensitivity of the spared NAC was tested using monofilaments and compared to a healthy, non-operated control group, and was significantly lower after NSM. The results from this study suggest that SSM with NAC reconstruction is a balanced alternative to NSM. Motivations of women to choose either operation varied and therefore should be explored when choosing prophylactic NSM or SSM.

From 1994-2006 women in the Erasmus MC Cancer Center who underwent bilateral mastectomy for high breast cancer risk (bilateral prophylactic or unilateral therapeutic, unilateral prophylactic) followed by direct-to-implant breast reconstruction were photographed during follow-up. For the study described in **Chapter 10** we asked these patients to complete a survey on re-operations and complications, and to complete the Breast-Q Reconstruction questionnaire, to assess long-term patient satisfaction with direct-to-implant reconstruction. A panel consisting of reconstructive surgeons analyzed the patient photographs for aesthetic outcome. Although the direct-to-implant reconstruction is a single-stage reconstruction, half of the patients had undergone additional surgery over the years for complications of aesthetic dissatisfactory results. The aesthetic outcome as scored by the panel was only poor to reasonable in the overall group, and significantly lower in the group who underwent reoperations. Breast-Q scores on 'satisfaction with breasts' and 'satisfaction with overall outcome' were comparable to scores reported in the literature after a two-stage implant reconstruction with tissue expansion, and not significantly lower in the group who underwent reoperations. This suggests that reoperations do not significantly impact patient satisfaction on the long-term. Whether direct-to-implant breast reconstruction is a viable alternative for two-stage implant reconstruction after previous tissue expanding is unknown, but does not seem likely based on the aesthetic outcome scores in this study.

SAMENVATTING

Dit proefschrift gaat over mammachirurgie in *BRCA1/2*-genmutatiedraagsters. De onderwerpen die aan bod komen variëren van moleculaire en prognostische tumorkarakteristieken in *BRCA*-geassocieerde borstkanker tot veiligheid, cosmetische resultaten en patient reported outcomes van preventieve mammachirurgie.

Hoofdstuk 2 bevat een review van literatuur over veiligheid van de preventieve ablatio bij *BRCA1/2*-genmutatiedraagsters. Wanneer na een mastectomie een directe reconstructie volgt wordt de huid in situ gelaten, waarbij ook de tepel-areola gespaard kan worden. Er is een kans dat borstklierweefsel achterblijft met de huid en met de gespaarde tepel-areola, wat mogelijk een risico vormt op het ontwikkelen van mammacarcinoom na de preventieve ablatio. Anatomische studies vonden resterend borstklierweefsel na 60% van de profylactische mastectomieën. Uit studies blijkt dat hierin slechts zeer incidenteel nog een mammacarcinoom ontstaat, maar na bundelen van de data blijkt het toch te gaan om 1 op de 140 vrouwen die een mammacarcinoom ontwikkelen na preventieve ablatio.

In **hoofdstuk 3** bestuderen we het verlagende effect van de risico-reducerende salpingo-ovariëctomie (RRSO) op de incidentie van mammacarcinomen die ontstaan na RRSO bij *BRCA1/2*-genmutatiedraagsters. De hypothese is dat mammacarcinomen die na RRSO ontstaan minder agressieve tumorkenmerken hebben dan de mammacarcinomen die ontstaan bij vrouwen die (nog) geen RRSO hebben ondergaan. Uit een cohort van *BRCA1/2*-genmutatiedraagsters selecteerden we alle vrouwen die hun eerste mammacarcinoom kregen tenminste 12 maanden na RRSO en vergeleken hen met op leeftijd (ten tijden van mammacarcinoom) gematchte vrouwen die hun eerste mammacarcinoom voor RRSO kregen. Borstkankers die ontstonden na RRSO hadden inderdaad een lagere mitotische index (12 versus 22 mitosen/2mm^2) en waren kleiner op het moment van diagnose (11 versus 17 mm diameter). De tumorverdubbelingstijd werd gemeten op mammografieën en MRI's, en was (niet-significant) langer na RRSO (124 dagen vs. 93 dagen). Deze data suggereren dat mammacarcinoom dat ontstaat na RRSO een minder agressief fenotype heeft dan mammacarcinoom zonder RRSO. Uiteindelijk kan deze bevinding consequenties hebben voor de frequentie van borstkankerscreening bij *BRCA1/2*-genmutatiedraagsters die een RRSO hebben ondergaan. Het is echter nog onduidelijk hoe oestrogeenverlaging een vertragende invloed kan hebben op de ontwikkeling van oestrogeenreceptor-negatieve tumoren zoals bij *BRCA1*-genmutatiedraagsters vaak worden aangetroffen.

Ook in **hoofdstuk 4** staan tumorkarakteristieken centraal. Dankzij intensieve screeningsprotocollen bij vrouwen met een *BRCA1/2*-genmutatie worden veel *BRCA1/2*-mammacarcinomen in een vroeg stadium gediagnosticeerd. Hierdoor is er meestal sprake van een kleine, hooggradige tumor zonder lymfekliermetastasen en – passend bij *BRCA1*-genmutaties – vaak een triple-negatieve status voor oestrogeen-, progesteron-

en Her2-receptoren. De toegevoegde waarde van gebruikelijke histologische tumorkenmerken voor risicostratificatie is daarom beperkt, net als de opties voor doelgerichte therapieën. Van een cohort van *BRCA1/2*-genmutatiedraagsters met een mammacarcinoom gediagnosticeerd tussen 1982 en 2008 reviseerden we de tumoren. Verschillende histologische tumorkenmerken die in eerdere studies bij sporadisch mammacarcinoom van potentieel prognostische waarde leken, correleerden we met de ziektevrije overleving, gecorrigeerd voor bekende prognostische markers. Tumor-geassocieerde inflammatie was de sterkste onafhankelijke prognostische marker in deze serie (hazard ratio; HR 0.18 voor matig tot sterk aanwezige versus milde of afwezige tumor-geassocieerde inflammatie; 95% confidence interval; CI 0.05-0.61). Andere onafhankelijke factoren van invloed op de prognose waren tumorgrootte (HR 2.47 voor > 2 cm versus ≤2 cm; 95%CI 1.10-5.57) en intra-tumor necrose (HR 2.60 voor necrose versus geen necrose; 95%CI 1.12-6.05). Lymfeklierstatus en differentiatiegraad waren geen van beide significant gecorreleerd met ziektevrije overleving.

Omdat *BRCA1/2*-genmutatiedraagsters naast een hoog risico op mammacarcinoom en contralateraal mammacarcinoom ook een hoog risico op het ontwikkelen van ovariumcarcinoom hebben, kunnen in de loop van de tijd verschillende tumoren voorkomen in een patiënt, met daarbij eventueel metastasen op afstand. Hierbij is het op basis van klinische en histopathologische kenmerken niet altijd mogelijk goed onderscheid te maken tussen de verschillende tumoren en origines van de metastasen. In **hoofdstuk 5** onderzoeken we de waarde van clonaliteitsanalyse middels Next Generation Sequencing (NGS) in een cohort van 42 *BRCA1/2*-genmutatiedraagsters met meerdere tumorlokalisaties. Twee pathologen reviseerden het beschikbare tumormateriaal van deze patiënten en deden op indicatie extra immunohistochemische analyses. Bij 4 van de 42 patiënten konden de tumor-origines niet met zekerheid bepaald worden en was NGS geïndiceerd (cases). Bij nog eens 10 patiënten met een zekere histopathologische uitslag werd ook NGS uitgevoerd (controles). In alle cases kon de clonaliteit van de verschillende tumoren met zekerheid worden aangetoond of uitgesloten. In alle controles kwamen de uitkomsten van de clonaliteitsanalyse overeen met de histopathologische uitkomsten bevestigd middels NGS. Daarom concluderen we dat bij een inconclusieve histopathologie-uitslag NGS een zinvolle bijdrage kan leveren om met zekerheid de clonale relaties tussen verschillende tumoren vast te stellen.

Als vervolg op de resultaten van hoofdstuk 4, bekijken we in **hoofdstuk 6** opnieuw de waarde van een potentiële prognostische marker in het *BRCA1/2*-geassocieerde mammacarcinoom. Nucleaire β-catenine aankleuring als marker van Wnt-activiteit werd al in meerdere soorten kanker bestudeerd en is het meest duidelijk aanwezig in colorectaal carcinoom. In sporadisch mammacarcinoom hangt de rol van de Wnt/β-catenine pathway mogelijk deels af van het moleculaire subtype van het mammacarcinoom. Mogelijk speelt de Wnt/β-catenine pathway een rol in basal-like mammacarcinoom en

daarom mogelijk in *BRCA*-geassocieerde tumorgenese. Het Rotterdamse cohort zoals beschreven in hoofdstuk 4 werd voor deze studie uitgebreid naar 2014. Omdat voor de β-catenine kleuringen paraffineblokjes van tumoren nodig waren konden in deze studie maar 120 *BRCA1/2*-genmutatiedraagsters geanalyseerd worden. Gecorrigeerd voor bekende prognostische markers was de enige onafhankelijke prognostische factor die geassocieerd was met ziektevrije overleving sterk positieve expressie van membraangeassocieerde β-catenine (HR 0.19; 95%CI 0.05-0.73). Nucleaire β-catenine expressie werd gezien in 38% van de tumoren, wat een mogelijke rol van Wnt activiteit in *BRCA1/2*-mammacarcinoom suggereert. In de meeste tumoren was nucleaire β-catenine aankleuring echter slechts zichtbaar in een paar celkernen per tumor. Nucleaire expressie van β-catenine was dan ook niet van prognostische waarde. Membraneuze β-catenine speelt ook een rol in cel-cel adhesie. Het verlies van β-catenine op de celmembraan kan een teken zijn van epithelial-mesenchymal transition van tumorcellen en is daarom waarschijnlijk niet alleen maar een marker van Wnt-activatie. Bovendien waren nucleaire en membraneuze β-catenine expressie niet omgekeerd evenredig met elkaar gecorreleerd. De prognostische waarde van membraneuze β-catenine expressie behoeft meer onderzoek terwijl het nuttig zou zijn om target genen te identificeren als specifieke markers van Wnt activiteit.

Hoofdstuk 7 beschrijft een anatomische studie naar de hoeveelheid terminal duct lobular units (TDLU's) die een kwantificatie zijn van de hoeveelheid borstklierweefsel dat achterblijft met de huid en de tepel-areola na tepelsparende mastectomie. In conventionele en in huidsparende mastectomieën werden tepel-areola en een daarnaast gelegen huideiland geprepareerd alsof het een sparende mastectomie betrof, en daarna uitgenomen en loodrecht op de huid gelamelleerd voor histologisch onderzoek. De coupes van borst en huideiland werden ingescand en de oppervlaktes van de coupes (cm^2) werden opgemeten met behulp van Photoshopâ. De TDLU's werden geteld onder de microscoop en gedocumenteerd als hoeveelheid TDLU's/cm^2 coupe-oppervlakte. In totaal werden 105 tepel-areola's en huideilanden gepaard geanalyseerd. Vierenzestig tepel-areola's (61%) en 25 huideilanden (24%) bevatten TDLU's. Vergeleken met de huideilanden was de TDLU-dichtheid (/cm^2) hoger in tepel-areola's. Onafhankelijke risicofactoren voor het vinden van TDLU's in een tepel-areola waren een jongere leeftijd en pariteit (versus nulliparitеit). Een hogere TDLU-dichtheid achter de tepel-areola vergeleken met de huid suggereert dat het sparen van de tepel-areola bij een preventieve mastectomie het postoperatieve risico op het krijgen van borstkanker verhoogt ten opzichte van het risico na een huidsparende mastectomie.

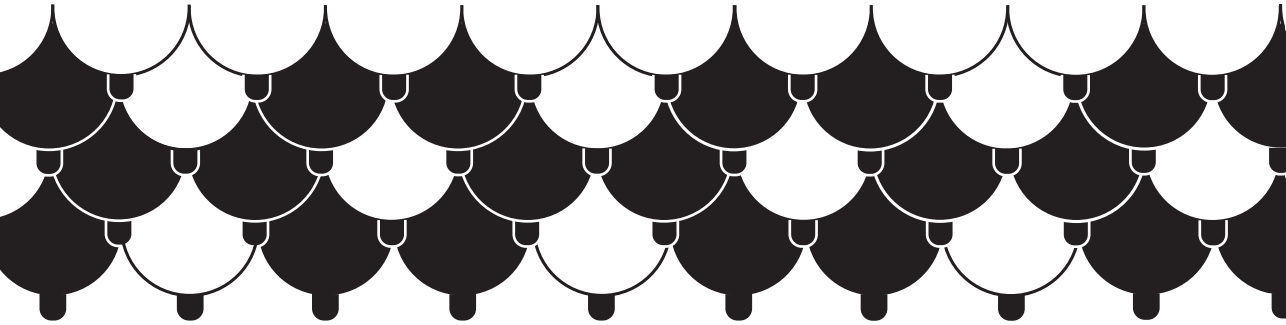
Om de body image van een patiënt te beoordelen na een operatie voor kanker wordt vaak de Body Image Scale (BIS) gebruikt. De BIS is ontwikkeld in 2000 in het Verenigd Koninkrijk door Hopwood en beoordeelt affectieve, gedragsmatige en cognitieve veranderingen die kunnen resulteren na een behandeling voor kanker. Omdat het een

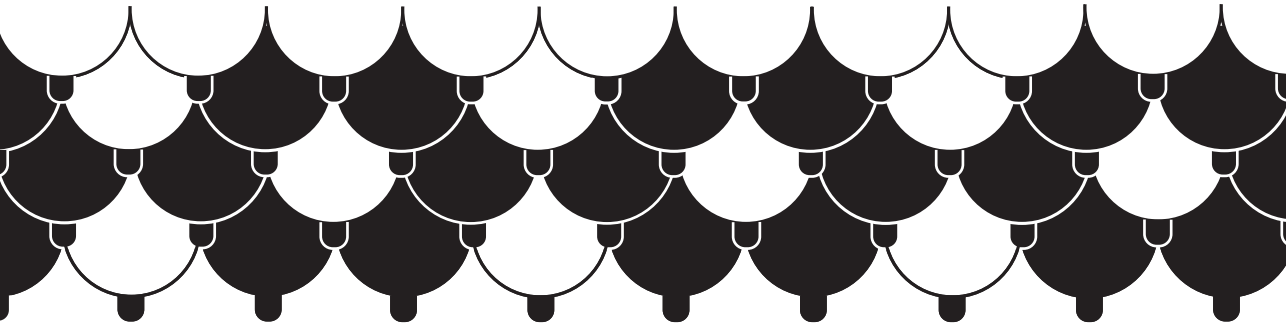
korte vragenlijst is, is het een aantrekkelijke vragenlijst voor gebruik in de kliniek. In **hoofdstuk 8** beschrijven we het validatieproces van de Nederlandse vertaling van de BIS. De validatie werd uitgevoerd in 150 vrouwen na mammasparende therapie en 150 vrouwen na mastectomie in verband met borstkanker. Psychometrische evaluatie van de Nederlandse BIS toonde consistente uitkomsten die vergelijkbaar waren met de oorspronkelijke versie van de BIS.

In **hoofdstuk 9** gebruiken we de BIS vervolgens om body image uit te vragen na een profylactische mastectomie. Studies bij borstkankerpatiënten laten zien dat patiënttevredenheid na een tepelsparende mastectomie mogelijk hoger is dan na een huidsparende mastectomie. Als vervolg op hoofdstuk 7 vragen we ons af of, gezien het additionele risico wat er mogelijk gelopen wordt door het behoud van de tepel-areola, een preventieve tepelsparende ablatio inderdaad superieur is aan een huidsparende ablatio, gevolgd door een reconstructie van de tepel-areola met behulp van een lokaal huidflapje en intradermale tatoeage. We gaven de Breast-Q Reconstructie vragenlijst en de Nederlandse BIS aan 25 patiënten die een preventieve tepelsparende ablatio ondergingen en aan 20 patiënten na een preventieve huidsparende ablatio. Daarnaast ontwikkelden we een vragenlijst om specifiek de tevredenheid met de gespaarde of gereconstrueerde tepel, de sensibiliteit van de tepel-areola en de rol van de tepel-areola bij de seksualiteit uit te vragen. Hieraan was een open vraag toegevoegd om de motivatie van de patiënt te achterhalen om voor een tepelsparende danwel een huidsparende ingreep te kiezen. Univariaat waren de Breast-Q scores beter in de huidsparende mastectomie groep dan in de tepelsparende mastectomiegroep voor de onderdelen 'tevredenheid met borsten' en 'tevredenheid met gehele resultaat'. Echter, gecorrigeerd voor follow-up verschilden de BIS en Breast-Q scores niet significant van elkaar. Ook de tevredenheid met de tepelreconstructie was niet significant verschillend van de tevredenheid met de gespaarde tepel-areola. De sensibiliteit van de gespaarde tepel-areola werd getest met monofilamenten en vergeleken met een niet-geopereerde controlegroep, en was significant lager na tepelsparende ablatio. De resultaten uit deze studie laten zien dat een preventieve huidsparende mastectomie gevolgd door tepelreconstructie een goed alternatief is voor een preventieve tepelsparende mastectomie. Het is daarom belangrijk om de motivaties en voorkeuren van vrouwen die een preventieve tepelsparende danwel huidsparende mastectomie overwegen goed uit te vragen.

Tussen 1994 en 2006 werden vrouwen die in het Erasmus MC Kankerinstituut een bilaterale mastectomie ondergingen gevolgd door een directe, 1-etappe, implantaatreconstructie in verband met een hoog borstkankerrisico (bilateraal preventief of unilateraal therapeutisch en unilateraal preventief) gefotografeerd tijdens de follow-up. Voor de studie beschreven in **hoofdstuk 10** vroegen wij deze vrouwen om een vragenlijst over re-operaties en complicaties in te vullen die gedurende de jaren daarna hadden plaatsgevonden. Wij vroegen hen de Breast-Q Reconstructie vragenlijst in

te vullen voor evaluatie van de lange-termijn patiënttevredenheid met de directe implantaatreconstructie. Een panel van vijf plastisch chirurgen beoordeelde de foto's op cosmetisch resultaat. Ondanks het feit dat het in principe om een 1-etappe reconstructie ging bleek de helft van de patiënten een of meerdere re-operaties te hebben ondergaan in verband met complicaties of ontevredenheid over het cosmetisch resultaat. Het cosmetische resultaten werd als slecht tot redelijk beoordeeld door het panel in de hele groep, maar was significant slechter in de gere-opereerde groep. De Breast-Q scores ten aanzien van 'tevredenheid met borsten' en 'tevredenheid met hele resultaat' waren vergelijkbaar met de scores die in de literatuur beschreven zijn na een 2-etappe reconstructie middels tissue-expander gevolgd door implantaatreconstructie, en niet significant lager in de gere-opereerde groep. Deze resultaten suggereren dat re-operaties niet de lange-termijn tevredenheid van de patiënt beïnvloeden. Of een 1-etappe directe implantaatreconstructie een waardig alternatief is voor een 2-etappe reconstructie met tissue-expander gevolgd door implantaat is onbekend, maar lijkt op basis van deze gegevens onwaarschijnlijk.





CHAPTER 12

General discussion and future perspectives

IS RISK-REDUCING MASTECTOMY INDICATED IN ALL FEMALE *BRCA1/2* GENE MUTATION CARRIERS?

For ethical reasons, no randomized controlled trials exist that compare the efficacy of breast cancer screening versus risk-reducing mastectomy (RRM) in *BRCA1/2* gene mutation carriers. Alternatively, Kurian et al developed a Monte Carlo model with input of cancer risks and survival data as a simulation of various risk-reducing options to estimate their efficacies on mortality [1]. According to this model, women with a *BRCA1* gene mutation who do not undergo breast cancer screening or risk-reducing surgery (e.g. risk-reducing salpingo-oophorectomy; RRSO or RRM) have a 53% survival by the time they reach the age of 70. *BRCA2* gene mutation carriers have a 71% survival. To compare; survival probability in the general US population is 84% at 70 years. The most effective risk-reducing combination strategy for *BRCA1* gene mutation carriers was RRM at age 25 combined with RRSO at 40 years, yielding a survival gain of 26% resulting in 79% survival at 70 yrs. The same strategy seemed the most effective in *BRCA2* gene mutation carriers yielding a survival gain of 12%, resulting in 83% survival at 70 years. According to the model, breast cancer screening until their 40th year followed by RRM combined with RRSO at 40 years reduced the survival gain by 2% (from 79% to 77%) in *BRCA1* and by 1% (83% to 82%) in *BRCA2* gene mutation carriers. When RRM was eliminated completely, a breast cancer-screening schedule (yearly mammography and MRI from 25-69 years) combined with RRSO at 40 years, decreased the survival probability at 70 years with another 3% in *BRCA1* gene mutation carriers to 74% and with 3% to 80% in *BRCA2* gene mutation carriers. These model-derived survival rates do show a survival benefit of RRM in *BRCA1/2* gene mutation carriers when compared with intensive breast cancer screening, albeit very small. The survival advantage of RRM of 3% for both *BRCA1* and *BRCA2* gene mutation carriers decreases even more when RRM is performed at the age of 40 instead of 25 years [1].

As compared with breast cancer screening, up to date no clinical studies have been able to reproduce this survival benefit of RRM in *BRCA1/2* gene mutation carriers. Heemskerk-Gerritsen et al. studied a large prospective database of a non-randomized series (the MRISC study population) with women who at inclusion had both breasts and no history of breast cancer [2]. Breast cancer incidence was, logically, higher in the screening group. However, breast cancer-specific survival was only non-significantly lower in the RRM group compared with the screening group (HR 0.29; 95% CI 0.03-2.61). Ingham et al. found a clear overall mortality reduction of RRSO compared with no risk-reducing surgery (HR 0.22; 95%CI 0.08-0.61), but found no survival benefit of RRM (HR 0.25; 95% CI 0.03-1.81) [3]. Another large multicenter cohort study of 2,482 female *BRCA1/2* gene mutation carriers (257 underwent RRM and 992 underwent RRSO) shows a survival benefit of RRSO on overall survival and even on breast cancer specific survival (HR 0.44; 95%CI 0.26-0.76). The impact of RRM on survival was not studied [4]. Overall, there is plenty of

evidence that RRM reduces breast cancer risk in *BRCA1/2* gene mutation carriers, but no clinical study has convincingly established an actual survival benefit [3-7].

Two notes are of importance concerning the efficacy of breast cancer screening. First, sensitivity and efficacy of breast cancer screening have spectacularly increased by adding MRI to screening protocols [8, 9]. MRI is probably essential to reach survival rates of women who choose breast cancer screening that are comparable to women who choose RRM. A recent study showed survival benefit of MRI added to mammography-only breast cancer screening in *BRCA1/2* gene mutation carriers [10].

Second, without subsequent RRSO, RRM and breast cancer screening both are much less effective, especially in *BRCA1* gene mutation carriers, as is shown by the model of Kurian et al [1]. Following RRSO at the age of 40, overall survival in breast cancer-screened *BRCA1* and *BRCA2* gene mutation carriers rises by 15% and 5%, respectively. In *BRCA1* gene mutation carriers RRSO should be performed at 40 years; when performed at 50 years RRSO only yields about half the survival benefit [1].

Almost as important as the discussion about the impact of breast cancer screening and RRM on breast cancer survival, is the question whether we will be able to better guide *BRCA1/2* gene mutation carriers while choosing either RRM or screening. Breast cancer screening allows one to keep ones own breasts, while breast surgery and its complications can be postponed or even avoided at all. Downsides are the recurrent, frequent outpatient clinic visits, burdensome radiological examinations and biopsies and anxiety towards to examination results. However, a study from 2004 (n= 334) showed that quality of life did not further decrease after starting breast cancer screening, and was even better as compared with the general population [11]. As an alternative, RRM minimizes breast cancer risk and, after a satisfying aesthetic result of the reconstruction is obtained, it minimizes hospital visits too. On the other side, the surgery may feel very mutilating, may compromise sexual functioning (in up to 70%) and body image as also described in Chapter 9 [12, 13]. In Chapter 10 we show that surgical complications may delay and even compromise the final aesthetic result and enlarge the psychological burden, which also has been reported by others [14]. Complications – major and minor – of the surgery and the reconstruction occur in up to 50% and surgical reinterventions are eventually needed also in about half of the patients (Chapters 9 and 10) [12]. The impact on quality of life, body image and sexual functioning of a – after all previously healthy – *BRCA1/2* gene mutation carrier of both screening and RRM should be further investigated. Certain types of personalities may help to benefit more from RRM or breast cancer screening in terms of quality of life. It would be of interest to assess personality traits of women who choose RRM versus those who choose breast cancer screening and correlate them to quality of life results. Regarding cancer surveillance programs, risk factors that have been associated with a negative impact on quality of life during cancer surveillance are female gender, a personal history of cancer, having a first degree relative who was

diagnosed with cancer and/or died from it, a high perceived risk of developing cancer, negative illness perceptions, a passive or pessimistic coping style and having little social support [15]. Even more, possibly women who exhibit predominant cancer anxiety may benefit more from a cognitive behavioral intervention with concurrent breast cancer screening, whether or not followed by a delayed RRM, than from RRM alone [16, 17]. Assessment of possibly subclinical psychological disorders is common in the intake of newly diagnosed *BRCA1/2* gene mutation carriers but tools that can predict quality of life after RRM or the start of breast cancer screening are lacking [15].

Finally, in choosing screening versus RRM it would be also helpful to be able to more precisely predict the exact breast cancer risk of the individual patient. As mentioned before, reported breast cancer risks of *BRCA1/2* gene mutation carriers vary strongly. This is due to the type and the exact location of the mutation, but also may be subject to unrecognized environmental factors that influence genotype-phenotype translation [18]. More insight in these potential risk modifiers would help to better counsel women towards RRM or breast cancer screening. Using data on risk-reduction and survival benefit in combination with a patient's personalized risk estimate, medical history, personality traits, coping style and personal preferences a decision model could be developed.

DOES RISK-REDUCING SALPINGO-OOPHORECTOMY DECREASE BREAST CANCER INCIDENCE?

As compared with cancer screening, the only surgical risk-reducing option that has been proved to reduce mortality in *BRCA1/2* gene mutation carriers is risk-reducing salpingo-oophorectomy (RRSO). This is partly due to the limited role of gynaecological screening, which does not contribute to the early detection of ovarian cancer [19, 20]. Ovarian cancer-related mortality is reduced by RRSO, but the effects of RRSO on breast cancer incidence and breast cancer related mortality are still subject of discussion. In daily practice it is common to communicate a breast cancer risk reduction of about 50% after RRSO. These risk reduction rates are derived from large clinical studies [4, 21-23]. Unfortunately, again due to ethical considerations, these studies were not randomized and therefore subject to various types of bias. Recently, risk reduction of RRSO was analyzed in a large Dutch cohort. Included were female *BRCA1/2* gene mutation carriers without a history of a previous cancer or of risk reducing surgery [24]. The previously mentioned studies were replicated in the Dutch cohort copying their methods and not adjusting for bias, yielding hazard ratios of about 0.5 that were comparable to the results of the replicated studies. Additionally, analyses were repeated while adjusting for several types of bias: the cumulative breast cancer incidence did not differ between the RRSO-group and the non-RRSO group. The authors therefore concluded that the risk-reducing effect of RRSO found in previous studies was most likely attributable to various forms of bias, and that

the true breast cancer risk reduction of RRSO is probably much smaller than commonly thought. In Chapter 3, we studied characteristics of breast cancers that originated after RRSO. The finding of fewer mitotic counts suggested a less aggressive biological phenotype of breast cancers that develop after RRSO [25]. The biological mechanism of estrogen depletion as accomplished with RRSO on the development of frequently estrogen-receptor negative breast cancers still has not been clarified. Future studies may be able to elucidate the effect of RRSO on breast cancer development.

IS NIPPLE-SPARING MASTECTOMY (NSM) SUPERIOR TO SKIN-SPARING MASTECTOMY (SSM)?

To minimize the residual breast cancer risk after prophylactic nipple-sparing mastectomy (NSM), as much breast glandular tissue as possible should be removed behind the nipple-areola complex (NAC) and skin. Anatomically, the NAC is the center of the breast gland, while the peripheral skin-flap is divided from the breast glandular tissue by subcutaneous fat. According to this concept, the risk of residual breast tissue behind the NAC should be higher. In a pathological study using mastectomy specimen (Chapter 7) we have established that after NSM indeed more terminal duct lobular units (TDLUs) are left in situ behind the NAC as compared to the skin [26]. However, the true impact of microscopic amounts of residual breast tissue on breast cancer risk is difficult to estimate. Further, (microscopic amounts of) breast glandular tissue may be left elsewhere, for example in the axilla [27, 28]. In Chapter 2, studies on residual breast glandular tissue and concurrent breast cancer risk after prophylactic mastectomy are reviewed [29].

More importantly, it is necessary to balance any remaining oncological risk versus the desired aesthetic outcome. It has been reported that NSM leads to a more natural and thus more desirable aesthetic outcome and possibly increases patient satisfaction. However, studies that report higher patient satisfaction after NSM as compared with SSM have been conducted in breast cancer populations [30-32]. Likely, women undergoing mastectomy for prophylaxis because of a *BRCA1/2* gene mutation cannot be compared to a (sporadic) breast cancer population in terms of pre-operative expectations and postoperative patient satisfaction [33, 34]. Obviously, especially in this population due to the prophylactic character of the mastectomy and the young age at the time of the surgery a natural aesthetic outcome should be pursued. On the other hand, many *BRCA1/2* gene mutation carriers who were interviewed for the studies on patient satisfaction and quality of life after prophylactic surgery (Chapters 9 and 10) remarked that they felt that aesthetic outcome was clearly secondary to the oncologic risk reduction. Some wrote that the high breast cancer rates in their family in combination with their own high breast cancer risk made them feel unattached to their breasts. Others were very content with the natural look of their NAC preservation after NSM. Unfortunately, these are just single

observations. It would be informative to gain more insight for example by a prospective study that preoperatively assesses motivations that determine the type of surgery and breast reconstruction of choice. Lastly, current techniques of NAC reconstruction consist of a small skin flap and specialized tattoo techniques. When performed well, the reconstructed NAC can hardly be distinguished from the native NAC. SSM followed by NAC reconstruction may even have a superior aesthetic outcome compared with NSM since NAC reconstruction routinely is delayed until the final postoperative breast reconstruction shape is achieved. Then, the position and shape of the NAC is adjusted. In Chapter 9, we show that women were equally satisfied with a reconstructed NAC as compared with a spared NAC. There were no differences in Breast-Q scores and Body Image Scale (BIS) scores after reconstruction, when adjusted for follow-up. Unadjusted Breast-Q scores were even slightly in favor of the SSM group for 'satisfaction with breasts' (66.2 vs. 56.6; P=0.06) and 'satisfaction with outcome' (76.1 vs. 61.5; P=0.09) [12].

IS IT ACHIEVABLE TO FURTHER PERSONALIZE MEDICAL TREATMENT OF *BRCA1/2*-ASSOCIATED BREAST CANCER PATIENTS?

Breast cancers in *BRCA1* and *BRCA2* gene mutation carriers are marked by some distinct tumor characteristics as compared with sporadic breast cancers. *BRCA1*-associated breast cancers are triple-negative (with negative status for estrogen receptor; ER, progesterone receptor; PR and HER2) in about 70%, and frequently have a medullary-like morphology. *BRCA2*-associated breast cancers more frequently are ER positive. Both *BRCA1/2*-associated breast cancers typically are of high grade and have a young age of onset [35]. Alike in all forms of cancer, the concept of personalized medicine is gaining interest in this subgroup of breast cancers, especially for *BRCA1*-associated breast cancer since a triple-negative status is common and regular targeted therapies are not applicable most of the time. According to the current guidelines, many *BRCA1/2*-associated breast cancer patients are candidates for adjuvant chemotherapy albeit having small tumors (node-negative Tis or T1 in 75%), due to their young age and a Bloom Richardson grade 3 [2, 36]. In sporadic breast cancer patients, several tests – for example Oncotype DX® and Mammaprint® - help identify patients at high recurrence risk who may benefit from adjuvant chemotherapy [37]. However, these tests are mainly used for ER positive breast cancer and have not been validated for use in the *BRCA1/2* gene mutation subgroup. In the present thesis, we present two studies that investigate potential prognostic markers (Chapter 4 and 6). Despite some promising and hypothesis-generating results, we could not draw firm conclusions from our results [38]. This is mainly due to the relatively small numbers of *BRCA1/*2-associated breast cancers, in combination with high breast cancer survival rates, in *BRCA1/2* gene mutation carriers alike in sporadic breast cancer patients. Of course, this is not a local limitation. To be able to identify prognostic markers and

potential therapeutic targets in *BRCA1/2*-associated breast cancer it is mandatory to extend study populations to nationwide and even international cohorts, comparable with already existing genetic and epidemiologic databases. This way, large series of breast cancer material can be analyzed by pathologists or biologists and linked to clinical data in order to identify novel markers that have impact on breast cancer survival.

SHOULD NEXT GENERATION SEQUENCING (NGS) BE APPLIED MORE BROADLY IN DAILY CLINICAL PRACTICE?

Lately, next generation sequencing (NGS) enables us to perform sequencing of previously selected genes or parts of genes that are known - when mutated - to be pathogenic or of other clinical consequence in certain types of cancer. NGS is a cheaper and less time-consuming technique than whole-genome sequencing and we showed in Chapter 5 that in certain cases NGS can be of clinical consequence [39]. Further, molecular characteristics of breast cancers help to increasingly personalize breast cancer treatment (for example gene expression profiles such as Oncotype DX® and Mammaprint® in sporadic breast cancer). In regular breast cancer workup, to perform such a relatively quick, non-invasive test that provides a great amount of information is tempting. However, as long as it is not possible to translate the genetic information found to a clinical outcome, NGS should be reserved for selected clinical and research purposes with a clear hypothesis that can be answered using NGS. Collecting genetic information in a more haphazard manner may lead to random associations of unclear clinical significance.

SHOULD WE EXTENT TESTING FOR GERM LINE *BRCA1/2* GENE MUTATIONS TO IMPROVE SURVIVAL IN *BRCA1/2* GENE MUTATION CARRIERS?

In the Netherlands, genetic analyses for *BRCA1/2* gene mutations and for other breast cancer susceptibility genes are currently reserved for women and their families who develop breast cancer at a very young age, who develop triple negative breast cancer at young age and with prevalence of (multiple) breast and ovarian cancers in patients and/or families [40]. Previously to being tested, women who visit the clinical genetic practice are being informed of the consequences of being tested, and of the diagnosis of a germ line *BRCA1/2* gene mutation.

As we know that, as compared to no intervention, breast cancer screening or RRM, and RRSO improve survival in *BRCA1/2* gene mutation carriers even after a first breast cancer [1, 41], it is tempting to extent screening of *BRCA1/2* mutations to the sporadic breast cancer population. It is plausible that NGS will gradually be more routinely used for diagnostic purposes and risk stratification and therefore molecular information of the tumor will become more readily available. This makes it possible to more routinely screen for *BRCA1* and

BRCA2 gene mutations in breast cancers. A mutation in the *BRCA1* or *BRCA2* gene found in the tumor may be a somatic as well as germ line mutation. To confirm the presence of a germ line mutation the analyses only has to be repeated in healthy tissue.

The downside of screening for *BRCA1/2* gene mutations is that probably not all *BRCA1/2* gene mutations are equally pathogenic, while the participants of the studies that demonstrated survival benefit of risk-reducing surgery and breast cancer screening were selected for *BRCA1/2* testing due to high familial breast- and/or ovarian cancer risks [1, 9, 41]. Routine diagnosis of *BRCA1/2* mutations of unknown significance for an underlying breast- and ovarian cancer risk may lead to overdiagnosis and therefore overtreatment of patients and their families. The focus of research should therefore lie on the treatability of *BRCA1/2*-associated breast cancer and on identifying targets that can be used for risk stratification, instead of trying to identify as many *BRCA1/2* gene mutation carriers as possible.

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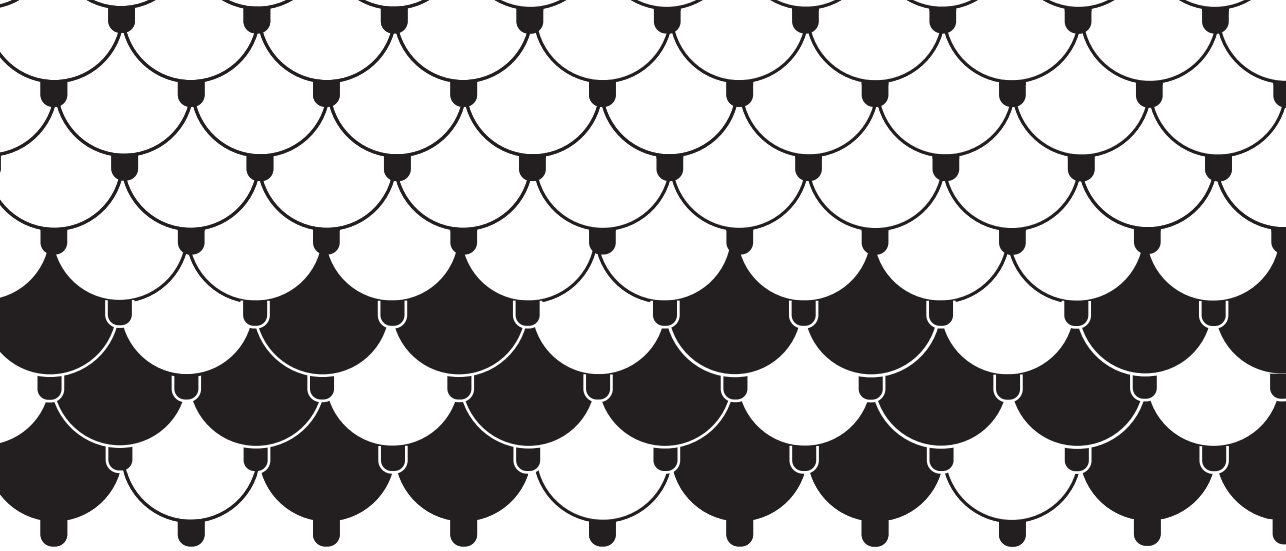
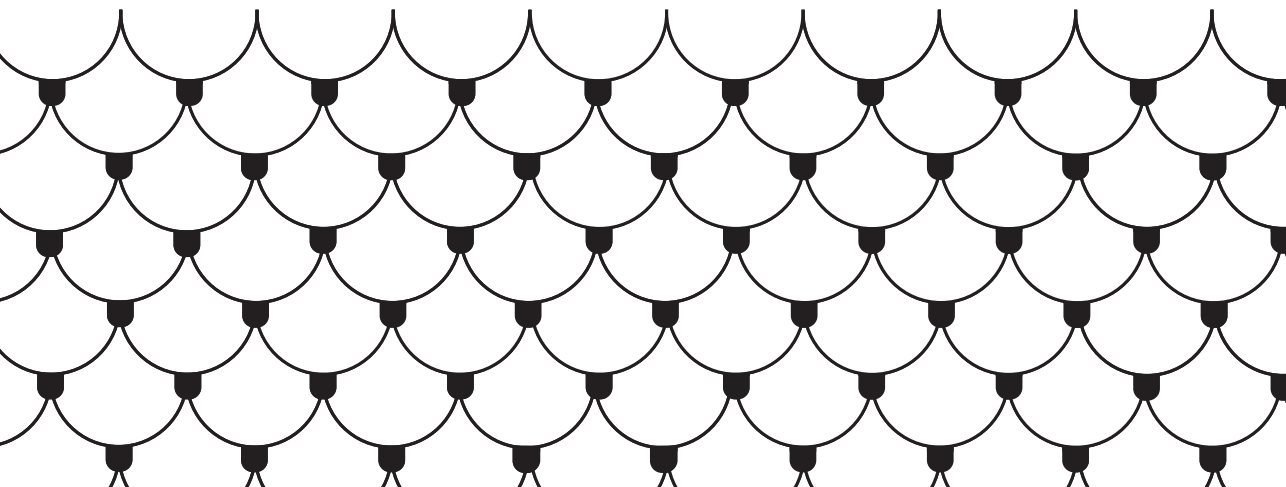
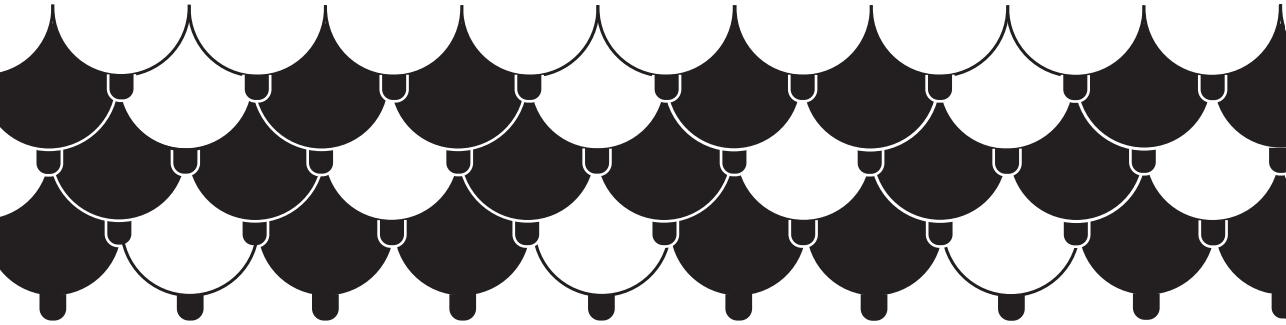
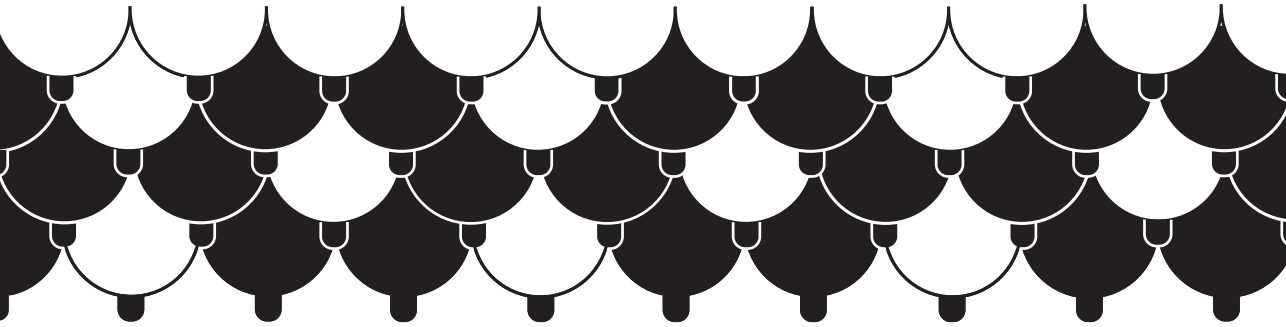




APPENDICES





PhD Portfolio

Name PhD student: V.M.T. van Verschuer PhD period: 2012-2017

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Department of Surgical Oncology Supervisors: Dr. L.B. Koppert
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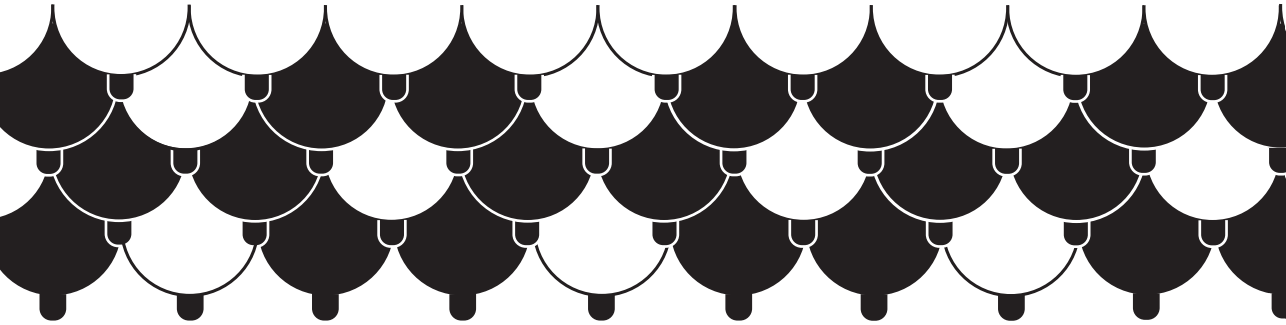
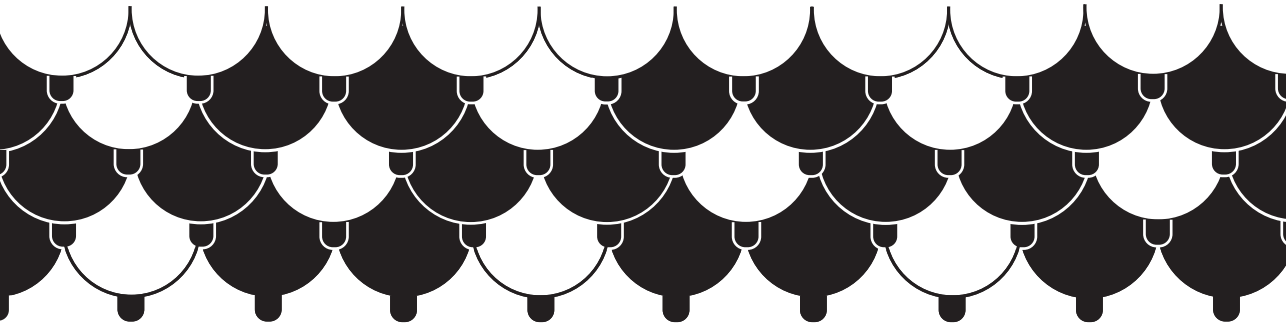
1. PhD training

	Year	Workload (ECTS)
General courses		
- Biostatistical Methods I: Basic Principles, NIHES	2012	5.7
- Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK)	2012	1.5
Seminars and workshops		
- Research meetings Erasmus MC Surgical Oncology	2012-2014	1
- Erasmus MC Werkgroep Erfelijke Tumoren	2012-2014	1
- Hereditair Borst- en Eierstokkanker Onderzoek Nederland (HEBON)	2012-2014	1
Presentations		
- Erasmus MC Chirurgie Stafdag	2013	1
- HEBON Research meeting	2013	1
- Erasmus MC Pathology Research Meeting	2014	1
- Wetenschapsdag Erasmus MC Interne Geneeskunde	2014	1
- NVvH Chirurgendagen	2014	1
- NVvH Najaarsdag	2014	1
International conferences		
- European Breast Cancer Conference, Vienna, Austria (poster presentation)	2012 2013	1 1
- 17th European Cancer Congress, Amsterdam (poster presentation)		

2. Teaching

	Year	Workload (ECTS)
Teaching medical students	2012-2014	1
Supervising master students	2013-2014	4





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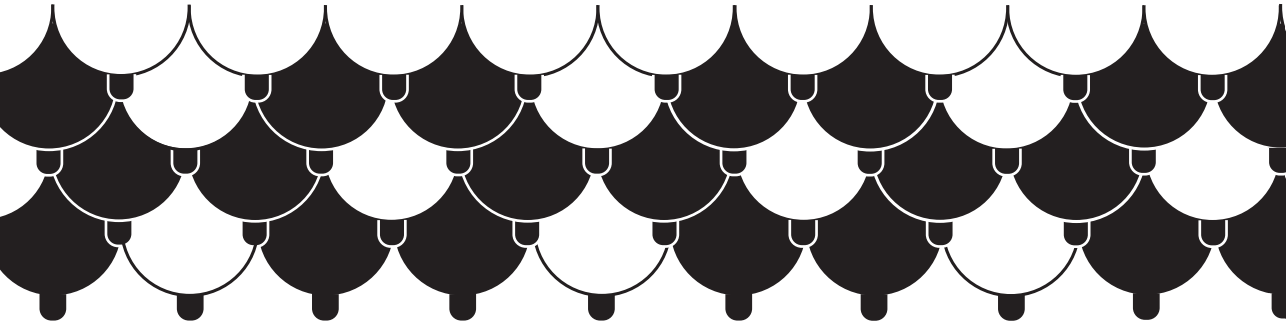

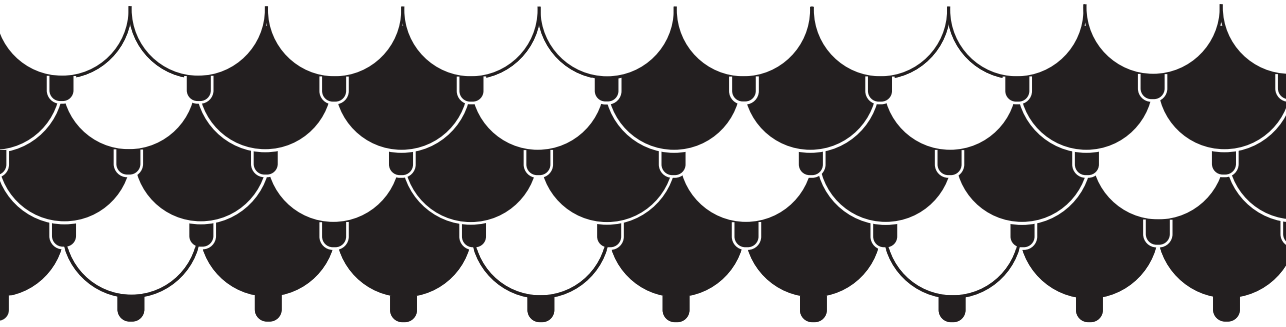

List of publications

Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast and/or ovarian cancer patients is clinically relevant. **V.M.T. van Verschuer**, W.H. Geurts, C.H.M. van Deurzen, P.J. van Diest, R. Pedrosa, M. Collée, C. Seynaeve, L.B. Koppert, W. Dinjens. *Mod Pathol. 2017 Jan;30(1):15-25*

Patient satisfaction and nipple-areola sensitivity after bilateral prophylactic mastectomy and immediate implant breast reconstruction in a high breast cancer risk population: nipple-sparing mastectomy versus skin-sparing mastectomy. **V.M.T. van Verschuer**, M.A.M. Mureau, J.P. Gopie, E.L. Vos, C. Verhoef, M.B.E. Menke-Pluijmers, L.B. Koppert. *Ann Plast Surg. 2016 Aug;77(2):145-52.*

Oncological safety of prophylactic breast surgery: skin-sparing and nipple-sparing versus total mastectomy. **V.M.T. van Verschuer**, M.C. Maijers, C.H.M. van Deurzen, L.B. Koppert. *Gland Surg. 2015 Dec; 4(6):467-75*

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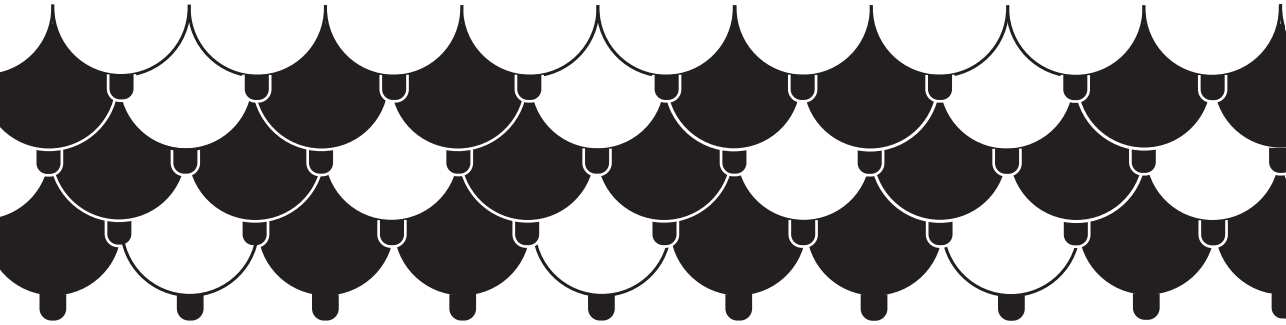
Preoperative prediction of cosmetic results in breast conserving surgery. E.L. Vos, A.H. Koning, I.M. Obdeijn, **V.M.T. van Verschuer**, C. Verhoef, P.J. van der Spek, M.B.E. Menke-Pluijmers, L.B. Koppert. *J Surg Oncol. 2015 Feb;111(2):178-84.*

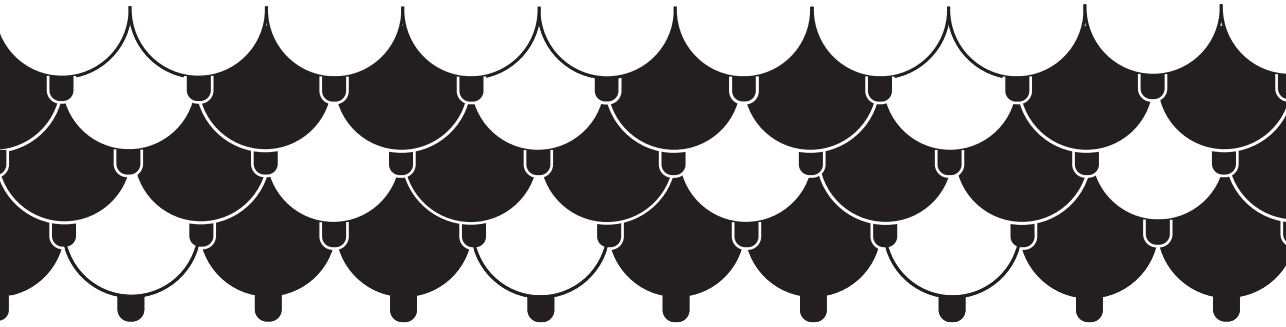
Tumor-associated inflammation as a potential prognostic tool in *BRCA1/2*-associated breast cancer. **V.M.T. van Verschuer**, M.J. Hooning, R.D. van Baare-Georgieva, A. Hollestelle, A.M. Timmermans, L.B. Koppert, L.C. Verhoog, J.W. Martens, C. Seynaeve, C.H.M. van Deurzen. *Hum Pathol. 2015 Feb;46(2):182-90*

Prophylactic nipple-sparing mastectomy leaves more terminal duct lobular units in situ as compared with skin-sparing mastectomy. **V.M.T. van Verschuer**, C.H.M. van Deurzen, P.J. Westenend, J. Rothbarth, C. Verhoef, E.J. Luiten, M.B.E. Menke-Pluijmers, L.B. Koppert. *Am J Surg Pathol 2014 May; 38(5):706-12*

Lower mitotic activity in *BRCA1/2*-associated primary breast cancers occurring after risk-reducing salpingo-oophorectomy. **V.M.T. van Verschuer**, B.A.M. Heemskerk-Gerritsen, C.H.M. van Deurzen, I.M. Obdeijn, M.M.A. Tilanus-Linthorst, C. Verhoef, M.K. Schmidt, L.B. Koppert, M.J. Hooning, C. Seynaeve. *Cancer Biol Ther 2014 Apr; 15(4):371-9*

No effect of atorvastatin and simvastatin on oxidative stress in patients at high risk for cardiovascular disease. P.G. Scheffer, R.K. Schindhelm. **V.M.T. van Verschuer**, M. Groenemeijer, S. Simsek, Y.M. Smulders, P.W.B. Nanayakkara. *Neth J Med. 2013 Sep;71(7):359-65.*





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Allereerst dank ik de deelnemers aan de onderzoeken voor het invullen en terugsturen van de vragenlijsten, en voor de eerlijke antwoorden op de soms persoonlijke vragen.

Mijn promotor, prof.dr. C. Verhoef. Beste Kees, met de avonturen op A1 kan ik pagina's vullen: bowlen op Zuid, curling, steengrillen, satésaus, chique diners met veel wijn, zo vaak mogelijk barbecueën op het dak, liefst boven het ventilatiekanaal van de pacu. Veel met de gemeenschappelijke deler 'rookoverlast' nu ik er over nadenk. De afgelopen jaren heb ik je leren kennen als een scherpe onderzoeker en een toegewijd afdelingshoofd en arts. Dank je wel voor je inspiratie, de titel, je humor en bovenal je vertrouwen. Ik verheug me zeer op een klinisch vervolg.

Geachte leden van de leescommissie, prof.dr. F.J. van Kemenade, prof.dr. S. Sleijfer en prof.dr. E.J.Th. Rutgers, dank u wel voor het lezen en het beoordelen van dit proefschrift.

Geachte copromotoren, dr. L.B. Koppert en dr. C.H.M. van Deurzen,

Beste Linetta, bij jouw aanstelling als kersverse chirurg in de Daniel kreeg je naast alle nieuwe verantwoordelijkheden – verrassing! – Elvira en mij er gratis bij. Ik maakte een tijdsplanning volgens welke ik binnen anderhalf jaar zou promoveren en bestookte jou in de eerste maand met zes onderzoeksvoorstellen, niet gehinderd door veel kennis van zaken. Jij deelde mijn enthousiasme en stuurde me bij. Verder liet je me vrij in tijdsindeling en richting. Daarvoor ben ik je dankbaar. Bijzonder waren de avonden bij jou en Niels thuis, waar het behalve over onderzoek veel over familie, het vak chirurgie en het leven in het algemeen ging. Ik ken weinig mensen met zo een groot hart als jij, en dat hart heb je voor de 'zaak', voor de patiënten, je familie en je collega's. Het is ongelooflijk hoeveel avonden je daarnaast ook nog in dit proefschrift hebt gestoken. Je bent een voorbeeld.

Beste Carolien, voor de PBSO- en TDLU-studies bekeken we honderden coupes onder de microscoop. Dagen bracht ik door op het JNI om coupes over te trekken en in te scannen. Toen je er achter kwam dat ik er zowaar wat van begon te begrijpen volgden er nog drie projecten. Voor de laatste studie gingen we op uitje naar Utrecht, in mijn Peugeot. Dat de as wat kraakte wanneer we een bocht scherper dan 30 graden maakten kon geen kwaad, verzekerde ik je. De garage heeft mij later verteld dat dit niet geheel terecht was. Onze besprekingen op jouw kamer waren altijd erg plezierig: eerst even bijpraten, daarna op efficiënte wijze de nieuwste bottlenecks van onze projecten tackelen. Ik ging altijd weg met een plan, en met frisse moed. Je bent precies en georganiseerd, maar pragmatisch waar nodig. En je hebt een enorme kennis van zaken, ik heb veel van je geleerd.

Dr. Menke, beste Marian. Natuurlijk ben ik aan jou veel dank verschuldigd. Na ons ene gesprekje in jouw kamer in de Daniel over vrouwen in de chirurgie, onderzoek doen en carrière hoefden we alleen nog 'even' die aanvraag te schrijven. Die werd gehonoreerd, zoals alles wat jij doet lukt, heb ik het idee.

De uiteenlopende disciplines die in dit boekje terugkomen maken het rijtje coauteurs divers. Aan Caroline Seynaeve, Maartje Hooning, Ina Geurts, Winand Dinjens en Marc Mureau ben ik bijzonder veel dank verschuldigd.

De afdeling pathologie, in het bijzonder Ian, bedankt voor het verzamelen, bewaren en terugsturen van alle blokjes en coupes.

Chirurgen in de Daniel. Jullie vormen een bijzonder hechte, positieve en ambitieuze groep waar menig afdeling jaloers op zal zijn. Hou dat vast.

Secretaresses en verpleegkundig specialisten in de Daniel. Bedankt voor jullie hulp en voor de gezelligheid op A1. Yfke, dank voor je luisterend oor.

Mede-onderzoekers uit de Daniel, Elvira, Eric, Mareille, Sepideh, Charlotte, Danique en Wijnand. Jullie waren onontbeerlijk voor de koffies en ijsjes, de onderzoekstips and -tricks, de voorbeeldposters en presentaties, de grappen, voor het stoom afblazen op de fiets, de borrels en etentjes, de congresjes en de uitjes (zie boven). Onderzoekers van de centrumlocatie, in het bijzonder Kirstin, Joel, Mirelle, Eelke: idem.

Chirurgen en (oud)arts-assistenten van het Franciscus. Dankzij jullie fiets ik fluitend naar het ziekenhuis. Brechtje en Nienke, dank voor jullie duwtje in de goede richting.

Lieve Johanna, de avond dat wij de kaft gingen ontwerpen begon voor mij het boek pas echt te leven! Wat ben je een goede en creatieve ontwerper.

Lieve vrienden en vriendinnen, Jet, Myrte, Lilian, Lotte, Tinus, almanakkies, groencie, Skulda. De etentjes en de weekendjes weg zorgen voor de nodige afleiding, en voor het niet-medicaliseren. De afgelopen tijd heb ik jullie schromelijk verwaarloosd, dank voor jullie geduld. Lieve tennisvriendinnen, Mirelle, Mareille, Çigdem en Anna, ons wekelijkse tennisuurtje is mij dierbaar! En al die life-events maken het biertje erna extra interessant.

Paranimfen.

Lieve Wijnand, samen deelden wij 2,5 m^2 en als er behang aan de muren had gezeten had ik je daar vaak graag achter geplakt. Optimisme van mijn kant over mijn onderzoek

stuitte slechts op hoon bij jou. De keer dat je parfum op mijn muis had gesprayd. De keren dat mijn scherm 270 graden gekanteld was. De talloze keren dat jij je binnen had opgesloten en ik op de gang 'Het zwangere nijlpaard verlaat het nest' moest roepen voor ik weer naar binnen mocht. Maar ook de terrasjes als het weer lente was, de borrels, het skiën en het tot veel te laat blijven in de Witte Aap, de Blender en de Vrienden van. En als er echt iets was dan was je er.

Lieve Annet. Van vriendinnen tijdens de studie gekoppeld door de studieadviseur, via onze off-route avonturen door China en Uganda, tot nu inmiddels best wel volwassen, vooral jij natuurlijk want jij bent al specialist. Lief en (promotie)leed deelden wij, nu op afstand noodgedwongen vaker telefonisch of op facetime maar bijna dagelijks. Ik ben heel trots op wie je bent geworden en dat je mijn vriendinnetje bent. Jij nog even volhouden, want ze zeggen dat het het waard is.

Lieve Otto, Nynke en Lotje, ik heb de leukste broer en zussen ter wereld.

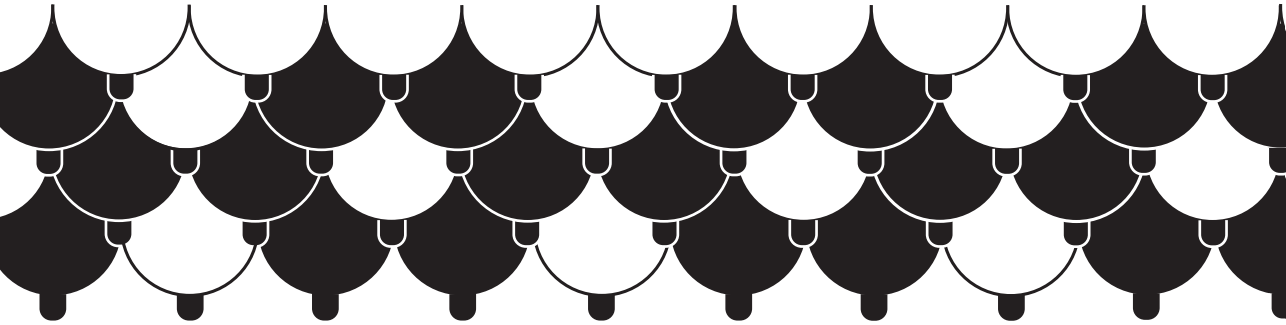
Jullie ideeën omtrent cultuur, stijl en literatuur (Nynke en Lotje), wijn en eten (Otto) en het leven in het algemeen neem ik allemaal ter harte omdat ik weet dat jullie daar stukken beter in zijn dan ik. Ik bewonder jullie allemaal zeer om jullie eigenheid.

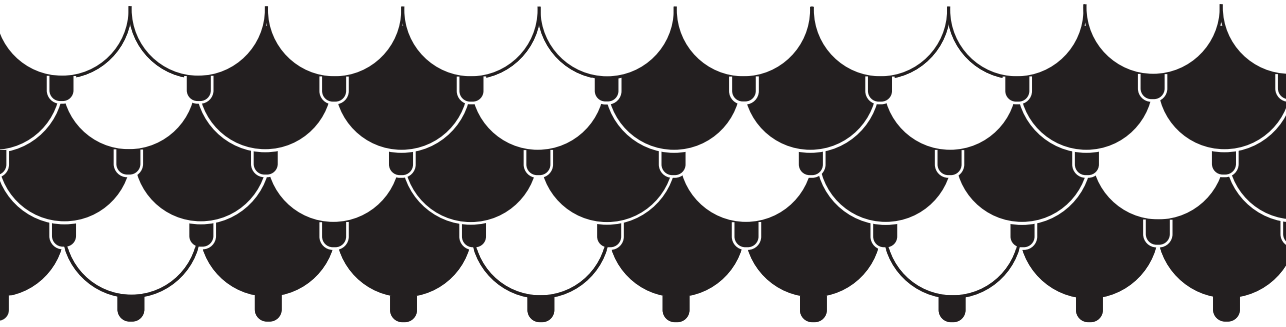
Lieve mama en papa,

Aan jullie ben ik de meeste dank verschuldigd. Jullie hebben mij altijd het vertrouwen gegeven dat ik kon doen en worden wat ik wilde. Jullie hebben allebei een onbevooroordeelde blik en een groot begrip en mededogen voor degenen die daar het meest behoefte aan hebben. In Rotterdam vinden wij elkaar vaak. Mama, jij en ik delen de medische inslag, voor de omgeving waarschijnlijk irritant, maar voor ons ongelooflijk relaxed. Van jou heb ik geleerd dat sommige moeders helemaal niet opfleuren van part-time werken. Papa, wij kunnen soms bijna huilen van het lachen om zaken waar niemand anders de humor van ziet. Na de promotie wordt het weer tijd voor een mamiltourtje naar Noord-Frankrijk – met biefstuk.

Lieve Thomas, Johnny,

Jij en ik hebben zoveel plannen: is het monomane carrière, willen we op avontuur in het buitenland, een hutje op een kitesurfstrand, of misschien toch gewoon de wereld redden… we hebben wel 500 jaar nodig om alles te doen wat we voor ogen hebben. Gelukkig lopen we 'in fase'. Je bent één van de meest authentieke en slimste mensen die ik ken. Je bent er dag en nacht voor mij, je hebt geduld, en je geeft me waar nodig een duwtje. Lief, wat bof ik met jou, ik hou van je.





Curriculum Vitae

Victorine Marie Theresia van Verschuer werd op 11 januari 1984 geboren in 's-Hertogenbosch. De eerste jaren woonde ze met haar ouders in de Betuwe, maar al snel verhuisde het gezin naar Suwâld in Friesland, Sternberg in Duitsland en terug naar Maasland in Nederland. Na haar VWO vertrok ze naar Granada, Spanje voor een cursus Spaans en werkte daarna als vertaler in Madrid. In 2002 startte ze met de studie Geneeskunde aan de VU in Amsterdam. Tijdens haar studie deed ze haar wetenschappelijke stage bij de Interne Geneeskunde aan de VU naar oxidatieve stressverlaging door statines. De interesse voor de chirurgie ontstond tijdens haar coschappen. Keuze-coschappen liep ze in Kinshasa, Democratische Republiek Congo en in Oeganda.

In 2010 werd het weer tijd voor een verhuizing, dit keer naar Rotterdam. Na een jaar als ANIOS chirurgie in het Sint Franciscus Gasthuis kreeg ze de kans om een beursaanvraag te schrijven voor onderzoek naar "terminal duct lobular units" achter de tepel. De honorering van de aanvraag leidde tot een periode als arts-onderzoeker van 2012 tot 2014 in het Erasmus MC-Daniel den Hoed, en uiteindelijk tot dit proefschrift. Sinds 2015 werkt Victorien als arts-assistent in opleiding tot chirurg in het Franciscus Gasthuis en Vlietland. Haar vrije tijd besteedt ze het liefst aan lezen en kitesurfvakanties.