Genotype and phenotype of *in situ* and invasive male breast cancer

Marijn Scheijde-Vermeulen

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General introduction

Male breast cancer

Breast cancer is a well-known and well-studied disease being the leading type of cancer in women worldwide, accounting for approximately 30% of the estimated new cases of cancer in the United States in 2019 [1]. In The Netherlands, approximately 14.000 female patients are diagnosed with invasive breast cancer each year and approximately 2400 patients with *in situ* (non-invasive) breast cancer [2]. In contrast to female breast cancer, male breast cancer is understudied and less well known amongst the public due to its low prevalence. Of all the breast cancers diagnosed in the United States in 2019 only about 1% will occur in the male breast [1]. Of these cases, approximately 5% is diagnosed as *in situ* breast cancer and 95% as invasive breast cancer [3].

Risk factors

Several risk factors for developing male breast cancer have been described. Some factors confer a high risk of developing breast cancer and others confer a low to intermediate risk. Approximately 15-20% of the male breast cancer patients have a positive family history for breast- or ovarian cancer and 10% of the men probably have a genetic predisposition [4]. The most important genetic factor that raises the risk of developing male breast cancer is a germline *BRCA2* mutation. The reported frequency of a *BRCA2* mutation in male breast cancer patients varies between 3.7% and 40%, with an average frequency of approximately 10% [4-7]. High frequencies are the consequence of a strong founder effect in specific populations [8]. Male *BRCA2* mutation carriers have a 6.8% cumulative breast cancer risk at the age of 70 [9]. Other genetic risk factors that harbor a breast cancer risk include *BRCA1* germline mutations, with a 1.2% cumulative breast cancer risk at the age of 70 and *CHEK2* mutation carriers that have a 10-fold increase of developing breast cancer [9, 10]. Because of these genetic risk factors, the American Society of Clinical Oncology (ASCO) recommends that all men with breast cancer should be offered genetic counseling and testing, regardless of the family history [4]. Men that have an altered androgen-to-estrogen ration also seem to have an increased risk of developing breast cancer. An example is Klinefelter's syndrome (47XXY karyotype and primary features of testicular dysgenesis and infertility) that imposes a breast cancer risk that is 20-50 times higher compared to the general male population [11, 12]. Other factors that may contribute to an increased breast cancer risk in men are obesity, liver cirrhosis, race, radiation exposure and gynaecomastia [4].

Clinical features

Male breast cancer occurs in older patients, with an average age of 67 years at time of presentation, compared to 62 years for women [13]. They commonly present with a

painless sub-areolar mass and also nipple retraction, nipple discharge or ulceration can be seen [14, 15]. In about one third of the male breast cancer patients there is a >6 months delay from the first appearance of symptoms to the first contact with a physician [15, 16]. The more advanced stage at presentation, mainly due to lymph node metastases, is very likely to be a result of this delay [13, 15, 17]. Important prognostic factors are age, stage, hormone receptor status, tumor size, nodal status and grade [13, 18].

When cancer is diagnosed in the male breast, it is treated similar to female breast cancer, based on treatment algorithms derived from female breast cancer studies. Treatment options are surgery (with the most common surgical procedure being a modified radical mastectomy) and adjuvant radiotherapy and endocrine therapy [16]. In case of treatment with aromatase inhibitors, treatment is slightly different from female breast cancer concerning the advice of suppressing testicular steroidogenesis [19]. Although this is a step towards optimizing treatment strategies for males, it is just a beginning and more male breast cancer studies are needed for further treatment optimization.

Pathology

The male breast has the same anatomical position as the female breast, extending from the 2nd to 6th anterior ribs. Normal male breast tissue is predominantly composed of fatty tissue, stroma and sparse rudimentary ducts. Unlike female breast tissue there is no formation of lobules.

Invasive breast cancer generally arises from a pre-invasive stage called ductal carcinoma *in situ* (DCIS), where neoplastic cells proliferate, but do not breach the basement membrane of the mammary ducts. DCIS can be graded into 3 groups from well differentiated to poorly differentiated, according to the Holland classification [20]. During breast cancer progression, when the neoplastic cells infiltrate the surrounding stroma, two progression pathways have been suggested in which low grade DCIS progresses to low grade invasive carcinoma and high grade DCIS progresses to high grade invasive carcinoma. Both pathways harbor distinct genomic aberrations with 16q loss being almost exclusively restricted to well and intermediate differentiated DCIS [21, 22]. Progression through grade is rarely observed [22, 23]. In female breast cancer, also columnar cell lesions (CCLs) are regarded as precursor lesions of (low grade) invasive breast cancer. Male breast carcinogenesis is still poorly understood. A previous study, analyzing precursor lesions in male breast cancer (CCLs, atypical lobular neoplasia/lobular carcinoma *in situ*, atypical ductal hyperplasia and DCIS),

found most precursor lesions to be DCIS [24]. Of the 1328 male breast cancer cases, 46.2% had an adjacent precursor lesion in an invasive tumor tissue slide. Of these cases showing a precursor lesion, 97.6% was DCIS and <1% was CCL, atypical ductal hyperplasia or lobular carcinoma *in situ*. Of the 13 cases showing CCLs, 11 cases also contained DCIS. Another finding in this study was that these ducts with CCLs lacked the classical female columnar cell-like morphology. This is in line with a study by Verschuur-Maes et. al., that concluded that in 89 male breast cancer resections no lesions were found with convincing CCL morphology such as cystically dilated acini or the presence of secretions or microcalcifications [25].

Elastosis, describing accumulation of elastic fibers, is a common phenomenon in female breast cancer, especially in estrogen receptor alpha (ERα) positive cancers, but has to the best of our knowledge not been studied in male breast cancer that is almost invariably ERα positive.

Hypoxia is another well studied phenomenon in female breast cancer. Hypoxia occurs when there is a mismatch between oxygen supply and oxygen consumption and tumor cells are capable of adapting to hypoxia in order to survive. Tumor cells use several different signaling pathways in order to achieve this, and the key regulator of the hypoxia response is hypoxia inducible factor-1 (HIF-1) [26]. In female breast cancer, expression of hypoxia-related markers has been described in invasive breast cancer and expression has been correlated with a decreased overall survival, high risk of metastases and higher histologic grade [27-29]. In addition, the presence of a fibrotic focus also seems to have prognostic significance in female breast cancer, concerning a poor survival, high tumor grade, a high mitotic activity and lymph node metastases [27-30]. A fibrotic focus is a scar like lesion composed of a mixture of collagen fibers and fibroblasts that express HIF1-alpha, so it has been related to hypoxia.

In male breast cancer, studies on hypoxia-related phenomena are scarce. Kornegoor et.al. included 134 male breast cancer patients and found that the presence of a fibrotic focus is associated with HIF1-alpha overexpression and found that both are associated with an aggressive tumor phenotype and poor survival [31]. To our knowledge, no studies have been performed that look into hypoxia in male DCIS.

Density of tumor-infiltrating lymphocytes (TILs) is not routinely assessed in daily practice, but has been widely studied in female breast cancer. In female breast cancer, a higher density of TILs has been reported to be associated with inhibition of tumor

progression and to a better response to chemotherapy, especially in triple negative and HER2-positive breast cancer [32-34]. Regarding male breast cancer, data on the presence and significance of TILs is based on a series with 18 male breast cancer patients only, so no definite conclusions could be drawn [35].

Several female breast cancer studies have shown similar levels of gene amplification in DCIS and adjacent invasive carcinoma, indicating that these genes play an early role in breast carcinogenesis, but not in the progression from DCIS to invasive carcinoma [36-38]. Male breast cancer studies aiming at clarifying this event where DCIS becomes invasive carcinoma are sparse.

The majority of the invasive male breast cancers are subtyped as invasive carcinoma of no special type (formerly known as invasive ductal carcinoma) [13, 39, 40]. Lobular carcinomas are rare in male patients, accounting for 1% of all male breast cancers [40, 41]. The majority (>90%) of the tumors express ERα and also progesterone receptor (PgR) positivity is common [15, 39]. Only 9% of the male breast cancers are HER2 positive [39]. In addition to histological subtyping, breast cancer can be subtyped according to surrogate breast cancer subtypes using immunohistochemical surrogates. Compared to female breast cancer the frequency of luminal A subtype, and to a lesser extent luminal B subtype, is higher whilst the frequency of non-luminal HER2+ and basal-like subtypes is lower than those reported for female breast cancer [42-44].

Several studies have been done investigating molecular characteristics. They have for example studied gene expression profiles and epigenetic alterations and have shown that this differs between male and female breast cancer [45-47]. For example, gain of *CCND1* and *EGFR* was more frequent in male breast cancer compared to female breast cancer and amplification of *TRAF4* and *EMSY* was more often observed in female breast cancer in comparison to male breast cancer [45].
In conclusion, studies concerning male breast cancer and in particular male DCIS are sparse and, to our knowledge, no studies concerning breast cancer progression in males have been conducted so far. In this thesis we have conducted several studies in these areas as outlined below.

Study design and thesis outline

We examined a large male breast cancer cohort (the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program) [39, 48]. One study included all enrolled male patients and the other studies included a (Dutch) subgroup of this initial

population. The EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program was conducted as a global effort to retrospectively assess tumor tissue of men diagnosed with breast cancer between 1989 and 2009. Male patients in The Netherlands were identified through the Dutch Cancer Registry. Paraffin embedded male breast cancer tissue was retrospectively collected by the Dutch Breast Cancer Research Group (BOOG).

In this thesis we aim to further characterize male breast cancer by describing and evaluating several histologic features. In addition we aim at enhancing our understanding of male breast carcinogenesis by comparing male DCIS to male invasive carcinoma.

In **chapter 2** we describe and analyze several histologic features including histological subtype, grade, mitotic activity index, presence of a fibrotic focus and density of tumor-infiltrating lymphocytes in 1483 male breast cancers and correlated these with clinical outcome.

In **chapter 3** we describe the frequency of elastosis in 117 ERα positive male breast cancers and correlate degree of elastosis to clinicopathological features and prognosis in comparison with 135 ERα-positive female breast cancer cases.

In **chapter 4** we studied the activated hypoxia response in 76 cases of male DCIS and 58 cases of male invasive carcinoma by conducting immunohistochemical stainings for the hypoxia related proteins HIF-1α, CAIX and Glut-1 in order to assess whether this response is a late bystander or a genuine carcinogenic event.

Breast cancer arises due to genetic and epigenetic aberrations. Oncogene amplification was studied by multiplex ligation-dependent probe amplification (MLPA), targeting 22 breast cancer related genes, in **chapter 5**. In **chapter 6** promotor hypermethylation is described using methylation specific MLPA (MS-MLPA). In both chapters DCIS and invasive carcinoma were studied, giving more insight in the genetic and epigenetic changes during male breast cancer progression.

Chapter 7 provides a summary of the thesis with a general discussion, based on the findings of the previous mentioned studies which form this thesis, followed by a Dutch summary in **chapter 8**.

1

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;**69**;7-34.
2. National institute for public health and the environment. *Breast cancer in the Netherlands*, 2019.
3. Pappo I, Wasserman I, Halevy A. Ductal carcinoma in situ of the breast in men: A review. *Clinical breast cancer* 2005;**6**;310-314.
4. Korde LA, Zujewski JA, Kamin L *et al.* Multidisciplinary meeting on male breast cancer: Summary and research recommendations. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010;**28**;2114-2122.
5. Abdelwahab Yousef AJ. Male breast cancer: Epidemiology and risk factors. *Seminars in oncology* 2017;**44**;267-272.
6. Liede A, Karlan BY, Narod SA. Cancer risks for male carriers of germline mutations in brca1 or brca2: A review of the literature. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2004;**22**;735-742.
7. Lecarpentier J, Silvestri V, Kuchenbaecker KB *et al.* Prediction of breast and prostate cancer risks in male brca1 and brca2 mutation carriers using polygenic risk scores. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2017;**35**;2240-2250.
8. Thorlacius S, Sigurdsson S, Bjarnadottir H *et al.* Study of a single brca2 mutation with high carrier frequency in a small population. *American journal of human genetics* 1997;**60**;1079-1084.
9. Tai YC, Domchek S, Parmigiani G, Chen S. Breast cancer risk among male brca1 and brca2 mutation carriers. *J Natl Cancer Inst* 2007;**99**;1811-1814.
10. Meijers-Heijboer H, van den Ouweland A, Klijn J *et al.* Low-penetrance susceptibility to breast cancer due to chek2(*)1100delc in noncarriers of brca1 or brca2 mutations. *Nature genetics* 2002;**31**;55-59.
11. Brinton LA. Breast cancer risk among patients with klinefelter syndrome. *Acta paediatrica (Oslo, Norway : 1992)* 2011;**100**;814-818.
12. Fentiman IS. The endocrinology of male breast cancer. *Endocr Relat Cancer* 2018;**25**;R365-r373.
13. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
14. Mathew J, Perkins GH, Stephens T, Middleton LP, Yang WT. Primary breast cancer in men: Clinical, imaging, and pathologic findings in 57 patients. *AJR. American journal of roentgenology* 2008;**191**;1631-1639.
15. Goss PE, Reid C, Pintilie M, Lim R, Miller N. Male breast carcinoma: A review of 229 patients who presented to the princess margaret hospital during 40 years: 1955-1996. *Cancer* 1999;**85**;629-639.
16. Ruddy KJ, Winer EP. Male breast cancer: Risk factors, biology, diagnosis, treatment, and survivorship. *Annals of oncology : official journal of the European Society for Medical Oncology* 2013;**24**;1434-1443.
17. Fentiman I. Male breast cancer: A review. *Ecancermedicalscience* 2009;**3**;140.
18. Serdy KM, Leone JP, Dabbs DJ, Bhargava R. Male breast cancer. *American journal of clinical pathology* 2017;**147**;110-119.
19. Losurdo A, Rota S, Gullo G *et al.* Controversies in clinicopathological characteristics and treatment strategies of male breast cancer: A review of the literature. *Critical reviews in oncology/hematology* 2017;**113**;283-291.

20. Holland R, Peterse JL, Millis RR *et al.* Ductal carcinoma in situ: A proposal for a new classification. *Seminars in diagnostic pathology* 1994;**11**;167-180.
21. Buerger H, Otterbach F, Simon R *et al.* Comparative genomic hybridization of ductal carcinoma in situ of the breast-evidence of multiple genetic pathways. *The Journal of pathology* 1999;**187**;396-402.
22. Hwang ES, DeVries S, Chew KL *et al.* Patterns of chromosomal alterations in breast ductal carcinoma in situ. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004;**10**;5160-5167.
23. Schymik B, Buerger H, Kramer A *et al.* Is there 'progression through grade' in ductal invasive breast cancer? *Breast cancer research and treatment* 2012;**135**;693-703.
24. Doebar SC, Slaets L, Cardoso F *et al.* Male breast cancer precursor lesions: Analysis of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2017;**30**;509-518.
25. Verschuur-Maes AH, Kornegoor R, de Bruin PC, Oudejans JJ, van Diest PJ. Do columnar cell lesions exist in the male breast? *Histopathology* 2014;**64**;818-825.
26. Walsh JC, Lebedev A, Aten E, Madsen K, Marciano L, Kolb HC. The clinical importance of assessing tumor hypoxia: Relationship of tumor hypoxia to prognosis and therapeutic opportunities. *Antioxid Redox Signal* 2014;**21**;1516-1554.
27. Chia SK, Wykoff CC, Watson PH *et al.* Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase ix, in invasive breast carcinoma. *J Clin Oncol* 2001;**19**;3660-3668.
28. Bos R, Zhong H, Hanrahan CF *et al.* Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001;**93**;309-314.
29. Dales JP, Garcia S, Meunier-Carpentier S *et al.* Overexpression of hypoxia-inducible factor hif-1alpha predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. *Int J Cancer* 2005;**116**;734-739.
30. Mujtaba SS, Ni YB, Tsang JY *et al.* Fibrotic focus in breast carcinomas: Relationship with prognostic parameters and biomarkers. *Annals of surgical oncology* 2013;**20**;2842-2849.
31. Kornegoor R, Verschuur-Maes AH, Buerger H *et al.* Fibrotic focus and hypoxia in male breast cancer. *Mod Pathol* 2012;**25**;1397-1404.
32. Matsumoto H, Koo SL, Dent R, Tan PH, Iqbal J. Role of inflammatory infiltrates in triple negative breast cancer. *Journal of clinical pathology* 2015;**68**;506-510.
33. Salgado R, Denkert C, Demaria S *et al.* The evaluation of tumor-infiltrating lymphocytes (tils) in breast cancer: Recommendations by an international tils working group 2014. *Annals of oncology : official journal of the European Society for Medical Oncology* 2015;**26**;259-271.
34. Adams S, Gray RJ, Demaria S *et al.* Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase iii randomized adjuvant breast cancer trials: Ecog 2197 and ecog 1199. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2014;**32**;2959-2966.
35. Avisar E, McParland E, Dicostanzo D, Axelrod D. Prognostic factors in node-negative male breast cancer. *Clinical breast cancer* 2006;**7**;331-335.
36. Moelans CB, de Weger RA, Monsuur HN, Maes AH, van Diest PJ. Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: A multiplex ligation-dependent probe amplification study. *Analytical cellular pathology (Amsterdam)* 2010;**33**;165-173.
37. Burkhardt L, Grob TJ, Hermann I *et al.* Gene amplification in ductal carcinoma in situ of the breast. *Breast cancer research and treatment* 2010;**123**;757-765.

38. Aubele M, Mattis A, Zitzelsberger H *et al.* Extensive ductal carcinoma in situ with small foci of invasive ductal carcinoma: Evidence of genetic resemblance by cgh. *Int J Cancer* 2000;**85**;82-86.
39. Cardoso F, Bartlett JMS, Slaets L *et al.* Characterization of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018;**29**;405-417.
40. Giordano SH. Breast cancer in men. *The New England journal of medicine* 2018;**378**;2311-2320.
41. Hill TD, Khamis HJ, Tyczynski JE, Berkel HJ. Comparison of male and female breast cancer incidence trends, tumor characteristics, and survival. *Annals of epidemiology* 2005;**15**;773-780.
42. Kornegoor R, Verschuur-Maes AH, Buerger H *et al.* Molecular subtyping of male breast cancer by immunohistochemistry. *Mod Pathol* 2012;**25**;398-404.
43. Johansson I, Killander F, Linderholm B, Hedenfalk I. Molecular profiling of male breast cancer - lost in translation? *The international journal of biochemistry & cell biology* 2014;**53**;526-535.
44. Shaaban AM, Ball GR, Brannan RA *et al.* A comparative biomarker study of 514 matched cases of male and female breast cancer reveals gender-specific biological differences. *Breast cancer research and treatment* 2012;**133**;949-958.
45. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Oncogene amplification in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research and treatment* 2012;**135**;49-58.
46. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Promoter hypermethylation in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research : BCR* 2012;**14**;R101.
47. Johansson I, Ringner M, Hedenfalk I. The landscape of candidate driver genes differs between male and female breast cancer. *PloS one* 2013;**8**;e78299.
48. Vermeulen MA, Slaets L, Cardoso F *et al.* Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England : 1990)* 2017.

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Pathological characterisation of male breast cancer: Results of the EORTC 10085/TBCRC/BIG/ NABG International Male Breast Cancer Program

Marijn A. Vermeulen , Leen Slaets, Fatima Cardoso, Sharon H. Giordano, Konstantinos Tryfonidis, Paul J. van Diest, Nizet H. Dijkstra, Carolien P. Schröder, Christi J. van Asperen, Barbro Linderholm, Kim Benstead, Renee Foekens , John W.M. Martens, John M.S. Bartlett, Carolien H.M. van Deurzen

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Abstract

Aim: Several prognostic histological features have been established in female breast cancer (BC), but it is unknown whether these can be extrapolated to male BC patients. The aim of this study was to evaluate the prognostic value of several histological features in a large series of male BC.

Methods: Central pathology review was performed for 1483 male BCs collected through part 1 of the European Organisation for Research and Treatment of Cancer (EORTC) International Male BC Program. Pathology review included histological subtype, grade, mitotic activity index (MAI), presence of a fibrotic focus and density of tumour-infiltrating lymphocytes (TILs). These features were correlated with clinical outcome. The relationship between these features and surrogate molecular subtypes using immunohistochemistry was also assessed.

Results: Median follow-up for overall survival (OS) was 7.1 years. Overall histological grade was not significantly associated with OS (p=0.129). MAI, the presence of a fibrotic focus and a low TILs density however were correlated with unfavourable OS (p=0.023, p=0.004 and p=0.011, respectively). BC subtype correlated with TILs density (p=0.015), as we observed a higher density for human epidermal growth factor receptor type 2 (HER2) positive BC compared to luminal HER2-negative subtype. No association was observed between subtype and fibrotic focus.

Conclusions: Histological grade was not significantly correlated with clinical outcome in this series, unlike what is seen in female patients. These results contribute to our understanding of male BC and indicate the importance of further research on the optimisation of risk stratification and treatment decisions for male BC patients.

Introduction

Male breast cancer (BC) is uncommon, accounting for less than 1% of all BCs [1]. Because of this low prevalence, characterising this disease has been tremendously challenging and current management is largely following female BC treatment algorithms. However, although male and female BC share several similarities, they also have many differences. For example, male BC occurs at a higher age and men usually present with a more advanced clinical stage [2, 3]. In addition, several studies reported that male and female BC differ regarding molecular characteristics, including gene expression profiles, epigenetic alterations and the distribution of surrogate BC subtypes based on immunohistochemical surrogates [4-6]. In male BC, the frequency of luminal A subtype and to a lesser extent luminal B subtype is higher, whilst the frequency of non-luminal human epidermal growth factor receptor type 2 (HER2) positive and basal-like subtypes is lower than those reported for female BC [7-9].

The majority of male BCs are ductal carcinomas while lobular carcinomas seem to be rare in male BC patients, accounting for 1% of all male BCs [10]. However, current data are limited as they are mainly based on small series without central pathology review. Besides, data regarding several other histological features in male BC, including Mitotic Activity Index (MAI), lymphovascular invasion, presence of a fibrotic focus and density of tumour-infiltrating lymphocytes (TILs) are scarce [11-15].

In female BC, histological grade is a well-established prognostic feature [16-18]. In male BC, there is no consensus regarding the prognostic impact of histological grade, so the identification of additional histological features with a potential prognostic value is warranted [3, 19].

Density of TILs and the presence of a fibrotic focus are not routinely assessed in daily practice, but have been widely studied in female BC and other solid tumours [20-23]. In female BC, a higher density of TILs has been reported to be associated with inhibition of tumour progression and to a better response to chemotherapy, especially in triple negative and HER2-positive BC [24-26]. Regarding male BC, data regarding the presence and significance of TILs is based on a series with 18 male BC patients only, so no definite conclusions could be drawn [27]. The presence of a fibrotic focus has been reported in 25% of male BC cases [28].

In summary, with only a few, mainly small single-center studies available regarding histological features in male BC, this is a field that needs further research in order to improve risk stratification and patient management. Our study population, which includes 1483 male BC patients, is the largest male BC population with central pathology review studied so far, which enables further characterisation of male BC.

Materials and Methods

Patients

Clinicopathological data were obtained from the retrospective part of the EORTC 10085/TBCRC/BIG/NABG International Male BC Program: a large international joint analysis of clinical and biological data of male BC patients, diagnosed between 1990 and 2010; European Organisation for Research and Treatment of Cancer (EORTC); Translational Breast Cancer Research Consortium (TBCRC); Breast International Group (BIG); North American Breast Cancer Groups (NABCG).

Details of this study have been described previously [29]. Briefly, invasive BC in males above 18 years of age at the time of diagnosis was included if a formalin-fixed paraffin-embedded (FFPE) tissue sample of the excision specimen was accessible for central pathology review and if enough follow-up existed. In this study we adhered to the Declaration of Helsinki and the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmwv.nl). When applicable in the site, informed consent from patients according to the International Conference of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use/ Good Clinical Practices guidelines (ICH/GCP) and applicable national laws was obtained.

Central pathology review

Out of the 1800 eligible male BC patients enrolled in the main study, only patients with available central lab assessments were analysed (N=1483) for the present substudy. For each patient, one tumour tissue block was centrally collected. A tissue microarray (TMA) was constructed for central immunohistochemical (IHC) staining assessment of oestrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki67. ER and PgR were scored according to the Allred scoring system[30]. HER2 status was scored according to the American Society of Clinical Oncology (ASCO)-College of American Pathologists (CAP) guidelines [31]. In one of the European central labs (The Netherlands), central review of histological features was performed by a dedicated breast pathologist (CvD or PvD), based on a haematoxylin and eosin (H&E) stained whole tissue slide (N=1203 out of 1483; the remaining cases were included in the TMA, but whole slides were not available for review). This included histological subtyping (according to the World Health Organization (WHO) classification), grading (according to the modified Bloom and Richardson score), presence of a fibrotic focus, density of TILs and lymphovascular invasion [16]. In some cases, additional immunohistochemical stainings were performed (e.g. E-cadherin) to improve subtyping. A fibrotic focus was defined according to the

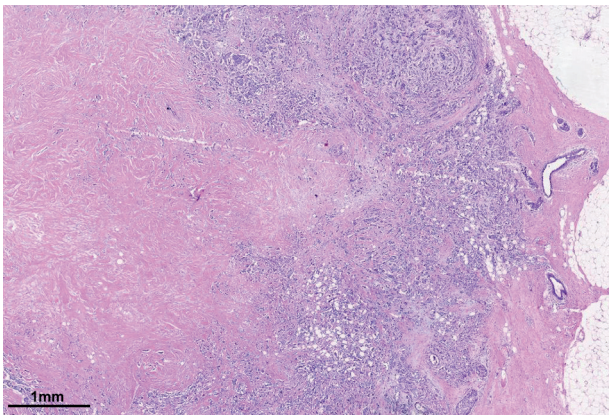


Figure 1. Example of an invasive ductal carcinoma with a fibrotic focus (left) surrounded by a cellular zone of infiltrating tumour cells at the periphery.

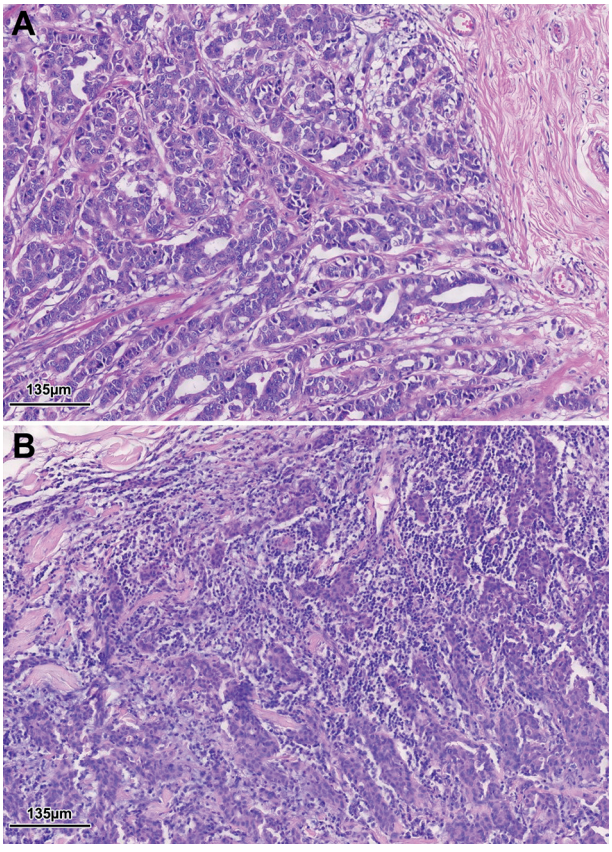


Figure 2. Examples of male BC cases with a minimal (A) and severe (B) density of TILs.

criteria described by van den Eynden et al [32]. Figure 1 provides an example of a male BC with a fibrotic focus. Density of TILs was scored in four categories (minimal, mild, moderate and severe) according to Lee et al [13], as illustrated in Figure 2. The presence of lymphovascular invasion outside the invasive component was recorded as absent or present. IHC-based surrogate BC subtypes were defined according to the 2013 St. Gallen consensus guidelines (referred to as surrogate BC subtypes) [33].

Statistics

The analyses that correlate histological features with clinical outcome were restricted to patients with non-metastatic (M0) disease at diagnosis. These survival analyses are summarised by the Kaplan-Meier curves, hazard ratios (HRs) and their associated 95% Wald confidence intervals and the score test p-value in the univariate Cox Model. Relapse-free survival (RFS) was defined as the time until locoregional recurrence, distant progression or death due to any cause. Overall Survival (OS) was defined as the time until death due to any cause. Patients without an event for the above end-points were censored at the last date known to be alive. The end-points were calculated from the time of first diagnosis. Additional analyses include the assessment of association between histological features (fibrotic focus and TILs) and surrogate BC subtype, for all patients with known subtype, irrespective of the metastatic status. In the analysis with surrogate BC subtype, a Fisher exact test was used for fibrotic focus and a trend test (in the proportional odds model) for TILs.

Results

General patient- and tumour characteristics

Clinicopathological features of 1483 male BC patients are shown in Table 1 and 2. Patients' age ranged from 25 to 98 years (median age: 68.4 years). The majority of M0 patients (95.9%) were treated with a mastectomy, the remaining cases (4.0%) were treated with breast conserving surgery or received no surgery (0.1%). Median follow-up was 6 years for RFS and 7.1 years for OS. The most frequent histological subtype was invasive ductal carcinoma not otherwise specified (86.6%). Invasive lobular carcinomas (classic or a variant) were rare and only seen in 1.4% of cases. Carcinomas were graded as grade 1 (21.8%), grade 2 (50.1%) or grade 3 (28.1%). Based on immunohistochemistry, the majority of carcinomas were classified as luminal A (41.9%) or luminal B (57.1%). Non-luminal HER2-positive and basal subtypes were infrequent (0.1% and 1% respectively).

Table 1 Baseline patient- and tumour characteristics of 1483 male BC patients. Reported percentages were calculated after exclusion of missing data.

Characteristics	**No (%, excluding missing data)**	
Age at diagnosis		
Median	68.4 (yrs)	
Metastasis-status		
M0	1054	(71.1)
M1	57	(3.8)
Mx	372	(25.1)
T-stadium (M0-patients)		
T1	511	(48.7)
T2	402	(38.3)
T3	21	(2.0)
T3	116	(11.0)
Missing	4	
N-stadium (M0-patients)		
N0	592	(59.4)
N1	321	(32.2)
N2	53	(5.3)
N3	30	(3.0)
Missing	58	
(Neo-) adjuvant treatment for M0 patients		
Adjuvant radiotherapy		
No	422	(51.5)
Yes	397	(48.5)
Missing	235	
(Neo-) adjuvant chemotherapy		
No	576	(70.2)
Yes	245	(29.8)
Missing	233	
Adjuvant endocrine therapy		
No	189	(23.2)
Yes	627	(76.8)
Missing	238	
Adjuvant Trastuzumab treatment for Her2 positive cases		
No	5	(25.0)
Yes	15	(75.0)
Missing	12	
Metastases-sites for M1 patients		
Bone	10	(22.2)
Lung	6	(13.3)
Soft tissue	1	(2.2)
Distant lymphnodes	3	(6.7)
Skin/subcutaneous tissue	2	(4.2)
Other	1	(2.2)
Combination of sites	22	(48.9)
Missing	12	

Table 2 Baseline pathological features of 1483 male BC patients.
Reported percentages were calculated after exclusion of missing data.

Pathological features	**No (%, excluding missing data)**	
Histological subtype		
Ductal NOS	1019	(86.6)
Lobular classic	9	(0.8)
Lobular variant	7	(0.6)
Mixed	70	(5.9)
Micropapillary	32	(2.7)
Invasive papillary	4	(0.3)
Mucinous	15	(1.3)
Cribriform	7	(0.6)
Tubular	4	(0.3)
Metaplastic	2	(0.2)
Adenoid cystic	5	(0.4)
Other	6	(0.5)
- *Secretory*	*2*	*(0.2)*
- *Apocrine*	*3*	*(0.3)*
- *Clear cell*	*1*	*(0.1)*
Missing	303	
Histological grade		
1	260	(21.8)
2	598	(50.1)
3	336	(28.1)
Missing	289	
Surrogate BC subtype		
Luminal A	585	(41.9)
Luminal B, HER2-negative	687	(49.2)
Luminal B, HER2-positive	107	(7.9)
Non-luminal HER2-positive	2	(0.1)
Basal	13	(1.0)
Not defined	2	(0.1)
Missing	87	
Fibrotic focus		
Present	385	(32.2)
Absent	811	(67.8)
Missing	287	
Density of TILs		
Minimal	304	(25.4)
Mild	721	(60.3)
Moderate	149	(12.5)
Severe	22	(1.8)
Missing	287	
Lymphovascular invasion		
Present	250	(20.9)
Absent	947	(79.1)
Missing	286	

Histological grade

Overall histological grade was not significantly correlated with RFS (p=0.099, HR=1.19, 95% CI 0.85-1.67 for grade 2 versus grade 1 and HR=1.5, 95% CI 1.02-2.20 for grade 3 versus grade 1) or OS (p=0.129, HR=1.27, 95% CI 0.95-1.70 for grade 2 versus grade 1 and HR=1.39, 95% CI 1.00-1.93 for grade 3 versus grade 1)[29]. However, analysis of MAI showed a significant association between MAI and outcome. The median RFS was 10.3 years for patients with 0-7 mitoses/2mm^2, 7.4 years for patients with 8-12 mitoses/2mm^2 and 6.5 years for patients with ≥13 mitoses/2mm^2 (p=0.024, HR=1.41, 95% CI 1.03-1.94 for MAI 8-12 versus MAI 0-7, and HR=1.45, 95% CI 1.06-1.96 for MAI≥13 versus MAI 0-7; Figure 3a). OS showed a similar trend: median OS equals 11.8 years, 8.4 years and 8.4 years, respectively (p=0.023 for trend, HR=1.39, 95% CI 1.07-1.82 for MAI 8-12 versus MAI 0-7 and HR=1.31, 95% CI 1.00-1.72 for MAI≥13 versus MAI 0-7; Figure 3b).

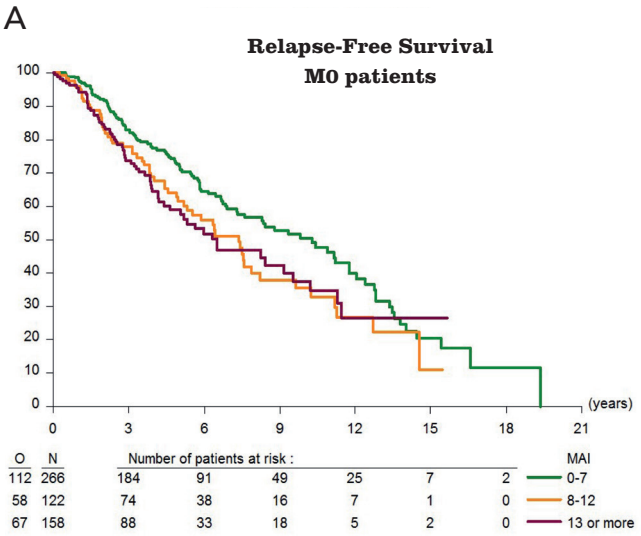
Fibrotic focus

Thirty-two percent of cases showed a fibrotic focus, which was associated with reduced RFS and OS. The median RFS was 5.6 years for patients with a fibrotic focus compared to 10.2 for patients lacking a fibrotic focus (p<0.001, HR 1.67, 95% CI 1.29-2.18; Figure 4a). The median OS was 8.2 years for patients with a fibrotic focus compared to 11.5 years for patients lacking a fibrotic focus (p=0.004, HR 1.39, 95% CI 1.11-1.74; Figure 4b). No significant association was observed between the presence of a fibrotic focus and surrogate BC subtype (p=0.059).

Density of TILs

The majority of patients had either a minimal or mild density of TILs (25.4% and 60.3%, respectively). Remaining cases were scored as moderate (12.5%) or severe (1.8%). Density of TILs was associated with RFS (p=0.020, HR=0.69, 95% CI 0.52-0.90 for mild versus minimal, HR=0.59, 95% CI 0.39-0.90 for moderate versus minimal and HR=0.65, 95% CI 0.24-1.78 for severe versus minimal; Figure 5a) and OS (p=0.011, HR=0.68, 95% CI 0.53-0.87 for mild versus minimal, HR=0.71, 95% CI 0.49-1.03 for moderate versus minimal and HR=0.46, 95% CI 0.19-1.14 for severe versus minimal; Figure 5b). Patients with a minimal density of TILs had the worst RFS and OS when compared to the other groups.

The density of TILs was also associated with surrogate BC subtype as shown in Table 3 (p=0.015). Luminal B HER2-positive subtypes were more often associated with a moderate to severe density of TILs as compared to luminal HER2-negative subtypes. The groups of patients with non-luminal HER2-positive and basal subtypes were too small for conclusions.



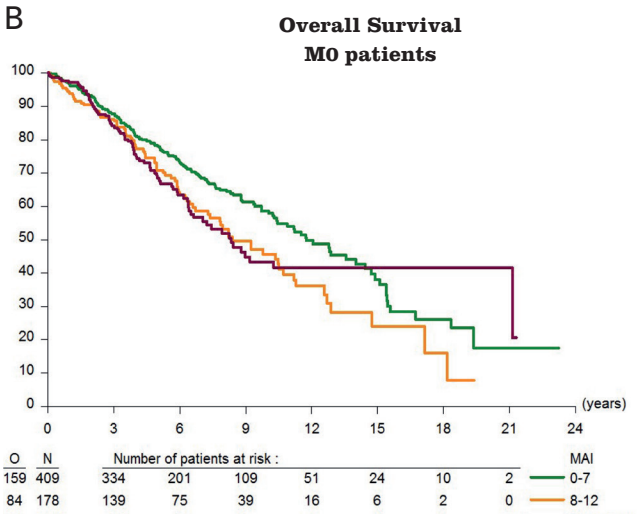
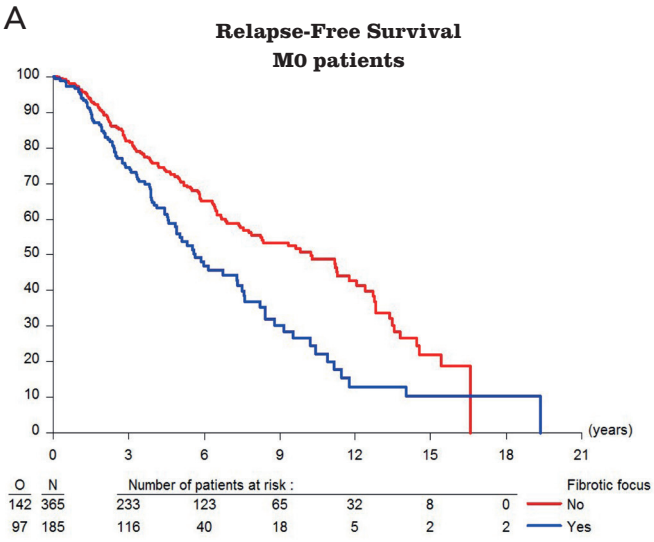


Figure 3a. Relapse free survival for mitotic activity index (MAI) categories 0-7, 8-12 and ≥ 13 mitoses/2mm2 in M0 patients (p=0.024).
Figure 3b. Overall survival for mitotic activity index (MAI) categories 0-7, 8-12 and ≥ 13 mitoses/2mm2 in M0 patients (p=0.023).



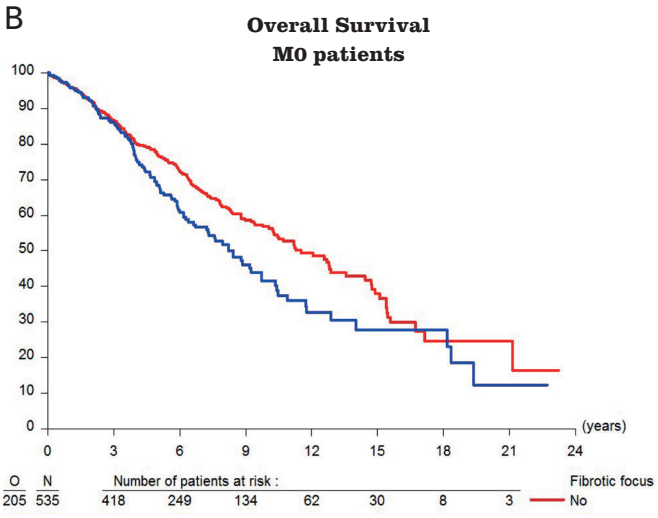
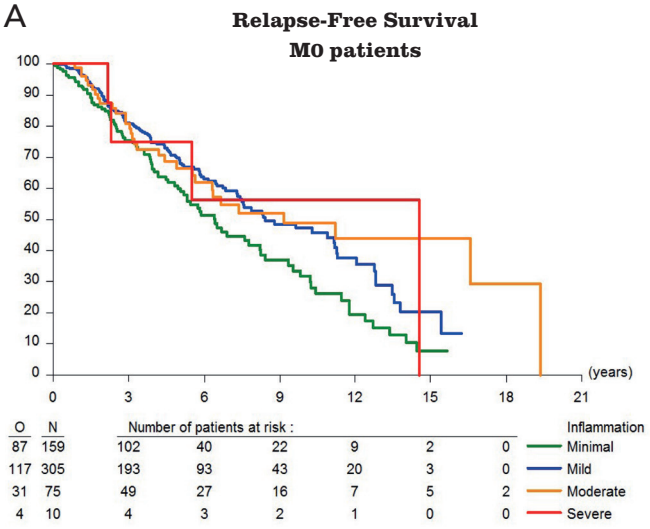


Figure 4a. Relapse free survival for M0 patients with and without a fibrotic focus ($p<0.001$).
Figure 4b. Overall survival for M0 patients with and without a fibrotic focus ($p=0.004$).



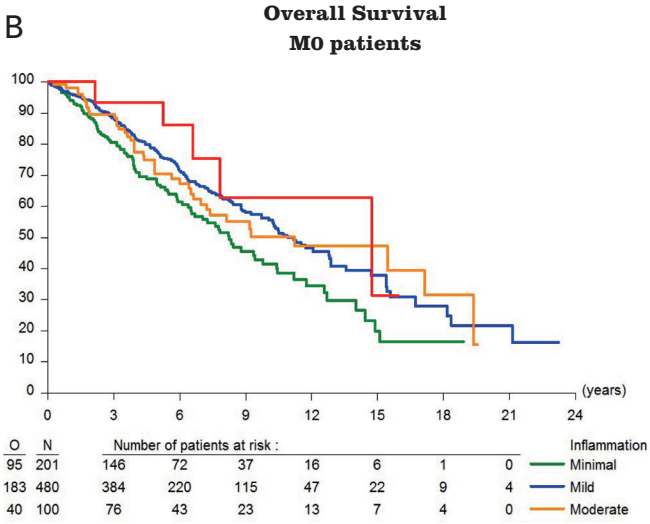


Figure 5a. Relapse free survival for each density-category of tumour infiltrating lymphocytes for M0 patients (p=0.020).
Figure 5b. Overall survival for each density-category of tumour infiltrating lymphocytes for M0 patients (p=0.011).

Lymphovascular invasion

Lymphovascular invasion was detected in 20.9% of cases. The median RFS was 8.2 years for the group without lymphovascular invasion and 7.4 years for the group with lymphovascular invasion, with no difference in outcome between the two (p=0.755, HR=1.05, 95% CI 0.78-1.42) and no association with OS (p=0.684, HR=0.95, 95% CI 0.72-1.24).

Table 3. Relationship between surrogate BC subtype and the different density categories of TILs. Patients with missing data are excluded from the table and association test.

Surrogate BC subtype								
	Luminal A HER2-negative (N=476)	**Luminal B HER2-positive (N=591)**	**Luminal B HER2-positive (N=62)**	**Non-luminal (N=2)**	**Basal (N=13)**	**Not defined (N=2)**	**Total (N=1146)**	**Test for trend**
Density, No (%)								
Minimal	124 (26.1)	159 (26.9)	9 (14.5)	0 (0.0)	1 (7.7)	0 (0.0)	293 (25.6)	
Mild	301 (63.2)	340 (57.5)	38 (61.3)	2 (100)	11 (84.6)	0 (0.0)	692 (60.4)	
Moderate	44 (9.2)	80 (13.5)	13 (21.0)	0 (0.0)	1 (7.7)	2 (100)	140 (12.2)	p=0.015
Severe	7 (1.5)	12 (2.0)	2 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	21 (1.8)	

Discussion

The aim of the present study was to describe and analyse several pathological features in male BC. Our descriptive study is unique as it includes the largest male BC population ever studied using central pathology review and it includes histological features that are not assessed in routine daily practice, including fibrotic focus and density of TILs.

In female BC, the most common histological subtype is ductal carcinoma not otherwise specified (74.3%) and the second most common subtype is a lobular carcinoma (11.8%) [3]. In our study, we reported a relatively high proportion of ductal carcinomas (86.6 %) and a low proportion of lobular carcinomas (1.4%), which is consistent with literature [3, 9, 34].

In female BC, histological grade has prognostic value, with a significantly worse RFS and OS corresponding with a higher grade [16]. In our male BC study population, histological grade did not significantly correspond with RFS or OS. Therefore, the grading system that was developed for females could perhaps not be extrapolated to males. Another potential explanation could be that we used OS and not BC-specific survival. The male BC population is relatively old as compared to the female BC population and a substantial proportion of patients die from other diseases. Also, the adjuvant treatment in this study was not defined by the protocol and not standardised. Therefore, grade could have played a role in the choice of adjuvant treatment and the latter could have affected outcome. A multivariate prognostic factor analysis is foreseen in a pooled analysis after completion of the prospective part of the male BC program.

The majority of carcinomas in our population were classified as grade 2 (50.1%), followed by grade 3 (28.1%), which is similar to the grade-distribution in females [35, 36]. In our series, a high mitotic count (≥8 mitoses/2mm^2) was associated with unfavourable outcome, but further subdivisions did not have additional discriminative value.

In female BC, a fibrotic focus is described in 20-50% of the cases and the presence of a fibrotic focus correlates with a more aggressive tumour behaviour [12, 20, 23]. This is consistent with our series, in which we reported a fibrotic focus in about one-third of cases and a correlation with adverse outcome.

Density of TILs has been described to have both prognostic and predictive implications in female BC [24-26]. Although the composition of the TILs seems to be important, scoring of density of TILs on an H&E-stained slide also has prognostic significance without knowing the details of the subpopulations [26]. In line with these female BC studies, we found an improved outcome in patients with a higher density of TILs. In our study we scored the density of TILs in four categories, according to the definition described by Lee et al [13]. As this is not a generally used scoring method, this is a

limitation of our study. However, at the start of this retrospective male BC program, no uniform scoring methods of TILs were published. Recently, Salgado et al. published recommendations for the evaluation of TILs in order to accomplish a more uniform and reproducible scoring system [25]. This scoring method, in case it becomes generally used in future studies, may contribute to an easier extrapolation of results. The current study demonstrated a significantly higher density of TILs in the luminal HER2-positive subtypes compared to the luminal HER2-negative subtypes, which is in line with the results from previous female BC studies [13, 37].

As previously mentioned, our study is unique in its population size, especially considering the rarity of the disease. But our study also has limitations, some of them are mentioned above. Most important, the treatments that were received by the patients were not highly standardised, which could have confounded the association observed between some pathological markers and outcome. Therefore, analyses correlating baseline factors with outcome should be considered hypothesis generating only and cannot yield definite conclusions regarding their prognostic value. The currently ongoing prospective registry of the Male BC program, which will be a more recent and homogeneous series, allows us to verify these findings prospectively. In addition, there was a relatively high number of missing data regarding the disease-status, and therefore OS results should be considered more reliable than RFS results. Finally, we reviewed only one H&E-stained slide per patient.

In conclusion, our current results demonstrated that overall histological grade was not significantly correlated with outcome, unlike what is known in female BC, although MAI, the presence of a fibrotic focus and density of TILs strongly correlated with survival. This descriptive study contributes to our understanding of male BC and may generate new hypotheses for the optimisation of risk stratifications and treatment decisions.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA: *a cancer journal for clinicians* 2015;**65**;5-29.
2. Anderson WF, Jatoi I, Tse J, Rosenberg PS. Male breast cancer: A population-based comparison with female breast cancer. Journal of clinical oncology : *official journal of the American Society of Clinical Oncology* 2010;**28**;232-239.
3. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: *A population-based study. Cancer* 2004;**101**;51-57.
4. Kornegoor R, Moelans CB, Verschuur-Maes AH et al. Promoter hypermethylation in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research: BCR* 2012;**14**;R101.
5. Lacle MM, Kornegoor R, Moelans CB et al. Analysis of copy number changes on chromosome 16q in male breast cancer by multiplex ligation-dependent probe amplification. Modern pathology: *an official journal of the United States and Canadian Academy of Pathology, Inc* 2013;**26**;1461-1467.
6. Johansson I, Nilsson C, Berglund P et al. High-resolution genomic profiling of male breast cancer reveals differences hidden behind the similarities with female breast cancer. B*reast cancer research and treatment* 2011;**129**;747-760.
7. Kornegoor R, Verschuur-Maes AH, Buerger H et al. Molecular subtyping of male breast cancer by immunohistochemistry. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc* 2012;**25**;398-404.
8. Johansson I, Killander F, Linderholm B, Hedenfalk I. Molecular profiling of male breast cancer - lost in translation? *The international journal of biochemistry & cell biology* 2014;**53**;526-535.
9. Shaaban AM, Ball GR, Brannan RA et al. A comparative biomarker study of 514 matched cases of male and female breast cancer reveals gender-specific biological differences. *Breast cancer research and treatment* 2012;**133**;949-958.
10. Hill TD, Khamis HJ, Tyczynski JE, Berkel HJ. Comparison of male and female breast cancer incidence trends, tumor characteristics, and survival. *Annals of epidemiology* 2005;**15**;773-780.
11. Hasebe T, Tsuda H, Hirohashi S et al. Fibrotic focus in invasive ductal carcinoma: An indicator of high tumor aggressiveness. *Japanese journal of cancer research: Gann* 1996;**87**;385-394.
12. Hasebe T, Tsuda H, Tsubono Y, Imoto S, Mukai K. Fibrotic focus in invasive ductal carcinoma of the breast: A histopathological prognostic parameter for tumor recurrence and tumor death within three years after the initial operation. *Japanese journal of cancer research: Gann* 1997;**88**;590-599.
13. Lee AH, Gillett CE, Ryder K, Fentiman IS, Miles DW, Millis RR. Different patterns of inflammation and prognosis in invasive carcinoma of the breast. *Histopathology* 2006;**48**;692-701.
14. Rakha EA, Martin S, Lee AH *et al.* The prognostic significance of lymphovascular invasion in invasive breast carcinoma. *Cancer* 2012;**118**;3670-3680.
15. Lee AH, Pinder SE, Macmillan RD et al. Prognostic value of lymphovascular invasion in women with lymph node negative invasive breast carcinoma. *European journal of cancer (Oxford, England: 1990)* 2006;**42**;357-362.
16. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;19;403-410.

17. Thomas JS, Kerr GR, Jack WJ et al. Histological grading of invasive breast carcinoma--a simplification of existing methods in a large conservation series with long-term follow-up. *Histopathology* 2009;**55**;724-731.
18. Schwartz AM, Henson DE, Chen D, Rajamarthandan S. Histologic grade remains a prognostic factor for breast cancer regardless of the number of positive lymph nodes and tumor size: A study of 161 708 cases of breast cancer from the seer program. *Archives of pathology & laboratory medicine* 2014;**138**;1048-1052.
19. Cutuli B, Le-Nir CC, Serin D et al. Male breast cancer. Evolution of treatment and prognostic factors. Analysis of 489 cases. *Critical reviews in oncology/hematology* 2010;**73**;246-254.
20. Mujtaba SS, Ni YB, Tsang JY et al. Fibrotic focus in breast carcinomas: Relationship with prognostic parameters and biomarkers. *Annals of surgical oncology* 2013;**20**;2842-2849.
21. Nishimura R, Hasebe T, Tsubono Y *et al*. The fibrotic focus in advanced colorectal carcinoma: A hitherto unrecognized histological predictor for liver metastasis. *Virchows Archiv: an international journal of pathology* 1998;**433**;517-522.
22. Watanabe I, Hasebe T, Sasaki S et al. Advanced pancreatic ductal cancer: Fibrotic focus and beta-catenin expression correlate with outcome. *Pancreas* 2003;**26**;326-333.
23. Hasebe T, Sasaki S, Imoto S, Mukai K, Yokose T, Ochiai A. Prognostic significance of fibrotic focus in invasive ductal carcinoma of the breast: A prospective observational study. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc* 2002;**15**;502-516.
24. Matsumoto H, Koo SL, Dent R, Tan PH, Iqbal J. Role of inflammatory infiltrates in triple negative breast cancer. *Journal of clinical pathology* 2015;**68**;506-510.
25. Salgado R, Denkert C, Demaria S et al. The evaluation of tumor-infiltrating lymphocytes (tils) in breast cancer: Recommendations by an international tils working group 2014. *Annals of oncology : official journal of the European Society for Medical Oncology /* ESMO 2015;**26**;259-271.
26. Adams S, Gray RJ, Demaria S et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase iii randomized adjuvant breast cancer trials: Ecog 2197 and ecog 1199. Journal of clinical oncology: *official journal of the American Society of Clinical Oncology* 2014;**32**;2959-2966.
27. Avisar E, McParland E, Dicostanzo D, Axelrod D. Prognostic factors in node-negative male breast cancer. *Clinical breast cancer* 2006;**7**;331-335.
28. Kornegoor R, Verschuur-Maes AH, Buerger H et al. Fibrotic focus and hypoxia in male breast cancer. Modern pathology: *an official journal of the United States and Canadian Academy of Pathology,* Inc 2012;**25**;1397-1404.
29. Cardoso F, Bartlett J, Slaets L et al. Characterisation of male breast cancer: First results of the eortc10085/tbcrc/big/nabcg international male bc program. *Cancer Research 75(9 Supplement)*:S6-05-S6-05, 2015.
30. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Modern pathology: *an official journal of the United States and Canadian Academy of Pathology,* Inc 1998;**11**;155-168.
31. Wolff AC, Hammond ME, Hicks DG et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of american pathologists clinical practice guideline update. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 2013;**31**;3997-4013.

32. Van den Eynden GG, Colpaert CG, Couvelard A et al. A fibrotic focus is a prognostic factor and a surrogate marker for hypoxia and (lymph)angiogenesis in breast cancer: Review of the literature and proposal on the criteria of evaluation. *Histopathology* 2007;**51**;440-451.
33. Goldhirsch A, Winer EP, Coates AS et al. Personalizing the treatment of women with early breast cancer: Highlights of the st gallen international expert consensus on the primary therapy of early breast cancer 2013. *Annals of oncology: official journal of the European Society for Medical Oncology* / ESMO 2013;**24**;2206-2223.
34. Burga AM, Fadare O, Lininger RA, Tavassoli FA. Invasive carcinomas of the male breast: A morphologic study of the distribution of histologic subtypes and metastatic patterns in 778 cases. *Virchows Archiv: an international journal of pathology* 2006;**449**;507-512.
35. Holm J, Li J, Darabi H et al. Associations of breast cancer risk prediction tools with tumor characteristics and metastasis. Journal of clinical oncology: *official journal of the American Society of Clinical Oncology* 2016;**34**;251-258.
36. Schymik B, Buerger H, Kramer A et al. Is there 'progression through grade' in ductal invasive breast cancer? Breast cancer research and treatment 2012;**135**;693-703.
37. Loi S, Sirtaine N, Piette F et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase iii randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: Big 02-98. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 2013;**31**;860-867.

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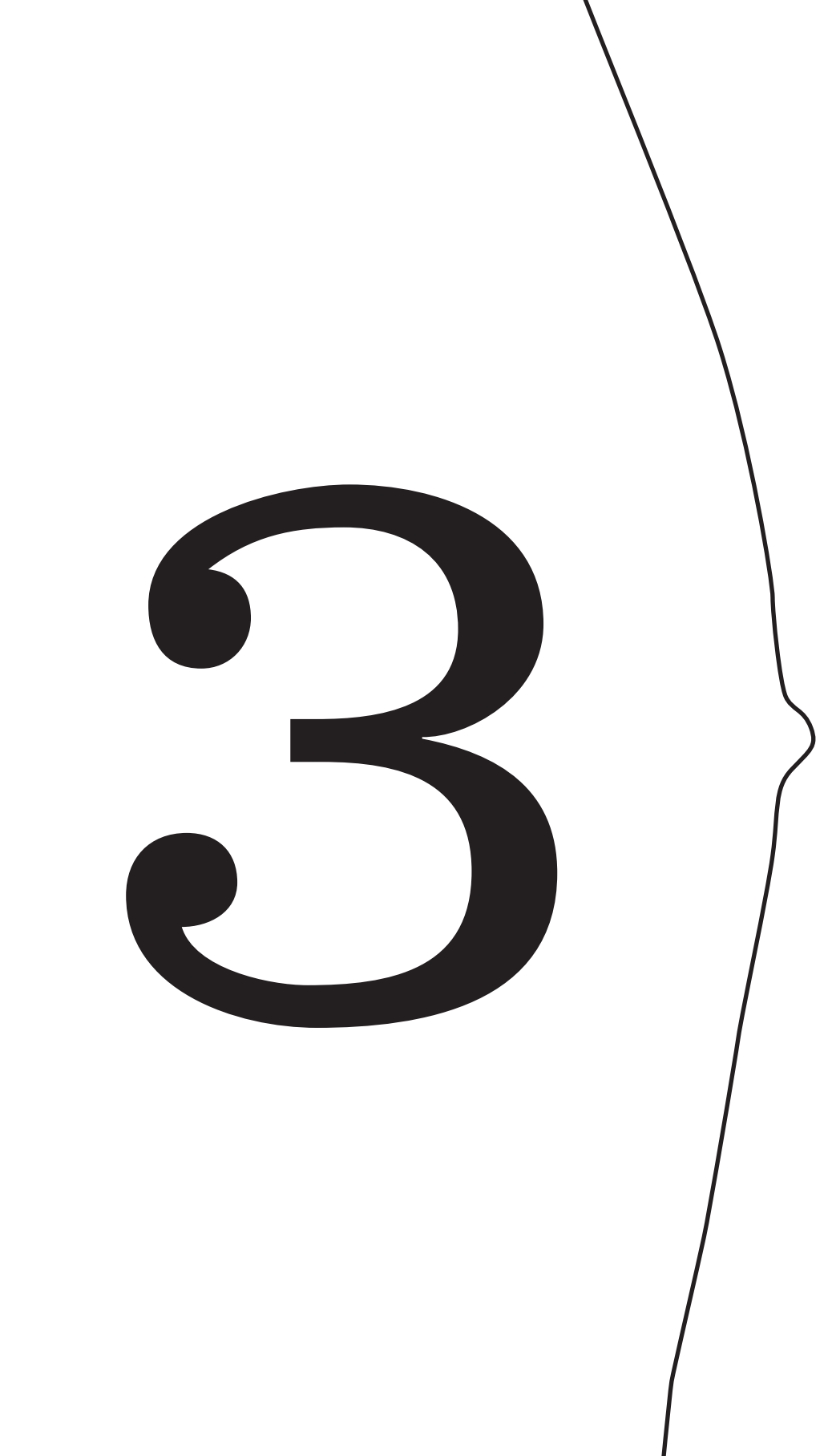

Elastosis in ERα positive male breast cancer

Marijn A Vermeulen, Carolien HM van Deurzen,
Elise van Leeuwen-Stok, Paul J van Diest

Submitted

Abstract

Many breast cancer (BC) studies have shown various differences hidden behind similarities between male and female BC. In female BC, elastosis is strongly related to estrogen receptor alpha (ERα) expression. Male breast cancers almost invariably express ERα, so the aim of this study was to investigate elastosis frequency in invasive male BC as well as clinicopathological correlations, in comparison with females.

177 male BC cases and 135 female BC cases were included, all ERα-positive and invasive carcinoma of No Special Type. Elastosis on H&E stained slides was scored in a 4 tiered system as elastosis grade (EG) 0 (no elastosis) to EG3 (high amount of elastosis). EG scores in male BC were correlated to histopathological characteristics and overall survival, and compared to female BC EG scores.

Male BC showed some degree of elastosis in 26/117 cases (22.2%) with none showing EG3, while female BC cases showed elastosis in 89/135 cases (65.9%) with 21.5% showing EG3 ($p < 0.001$). This difference retained its significance in multivariate logistic regression. In male BC cases no significant correlations were found between the amount of elastosis and age, grade, mitotic activity index, and progesterone receptor (PgR). In addition, no significant prognostic value of elastosis was seen.

In conclusion, despite high ERα expression, male BC showed significantly less elastosis than female BC. Elastosis did not show clinicopathological correlations or prognostic value. Therefore, elastosis seems to be a less useful ERα tissue biomarker with less clinical significance in male BC compared to females, pointing towards important BC sex differences.

Introduction

Elastic fibers are composed of 2 important components; elastin and small microfibrils. The precursor tropoelastin is secreted by fibroblasts, chondrocytes and smooth muscle cells, and this protein is crosslinked by one of the lysyl oxidase family members. The microfibrils are thought to function as a scaffold to facilitate this. Cross-linked aggregates form larger structures and eventually form a functional elastic fiber, providing elastic recoil to several different tissues [1]. Large aggregates of these elastic fibers in breast cancer (BC) are called elastosis.

Elastosis is a well-known phenomenon in female BC, and has been studied for decades. The biological background of elastosis in the breast is not well understood but it is suggested that the elastic fibers are not produced by only fibroblasts, but also by endothelial cells and neoplastic epithelial cells [2]. It can be observed in the periductal and perivascular spaces or diffusely in the tumor stroma. Shivas & Douglas categorized elastosis in 1972 into 4 grades with grade 0 corresponding to no elastosis and grade 3 corresponding to numerous dense aggregates of elastic fibers, and found a favorable survival in female breast cancers showing a high amount of elastosis [3]. This correlation of elastosis with survival or favorable tumor characteristics such as low grade and Ki67 index has later been confirmed by different groups, although other groups could not confirm this [4-6]. Another well-known correlation is that of elastosis with expression of the estrogen receptor alpha (ERα). In ERα-positive tumors a high amount of elastosis can be found, compared to ERα negative tumors that show less elastosis [4, 5, 7]. Nowadays, elastosis is a well recognizable phenomenon for the pathologist that provides a strong clue that the tumor will be ERα positive, thereby serving as a surrogate tissue biomarker for ERα expression.

An estimated 2670 men will develop BC in the United States in 2019, which is almost 1% of the total number of estimated new breast cancer cases, making male BC a rare disease [8]. Previous studies have shown similarities, but certainly also differences between BC in males compared to females. For instance, there is a difference in distribution of histologic as well as molecular subtypes, men tend to present with BC at a higher age and present with more advanced disease at presentation compared to women [9-12]. In addition, important differences at the molecular and epigenetic level have been described [13, 14].

BC in males is almost invariably ERα positive, but because of the important differences between male and female BC, it cannot just be assumed that elastosis in male BC occurs in a similar frequency and shows the same clinicopathologic correlations as in female BC. In the present study our aim was therefore to establish the frequency of elastosis in ERα-positive male BC and to correlate degree of elastosis to clinicopathological features and prognosis in comparison with ERα-positive female BC cases.

Materials and Methods

Patients material

Male patients with ERα-positive invasive BC were selected from the Dutch part of the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program, that was conducted as global effort to retrospectively assess tumor tissue of men diagnosed with breast cancer between 1989 and 2009 [15, 16]. Male patients in The Netherlands were identified through the Dutch Cancer Registry. Paraffin embedded male breast cancer tissue was retrospectively collected by the Dutch Breast Cancer Research Group (BOOG). Archival tissue of all patients was handled according to the Dutch Code for Proper Use of Human Tissue (www.federa.org). A subgroup of this initial population was selected based on at least one available hematoxylin and eosin (H&E) stained slide and known ERα status. All patients were diagnosed with invasive carcinoma (IC) of No Special Type (NST, according to the 2012 WHO), resulting in 117 male patients [17]. Representative H&E stained slides from 135 female patients with ERα -positive IC NST were collected between 2017 and 2018 at the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. The slides were chosen based on the amount of tumor in the slide. Elastosis was not taken into account when choosing the slide. All H&E stained slides contained a whole tumor containing tissue section.

Patient and tumor characteristics including age at diagnosis were recorded and the H&E stained slides were reviewed by two experienced pathologists to confirm the diagnosis and to assess the degree of elastosis. Unfortunately, sufficient data on tumor size, lymph node status and presence of lymphovascular invasion was not available for the male BC cases, so these factors could not be taken into account. The tumors were graded according to the modified Bloom and Richardson score [18]. ERα, progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) were evaluated using immunohistochemistry and scored according to ASCO-CAP guidelines [19]. ERα and PgR were considered positive when >10% of the tumor cells showed positive

staining. Survival data was available for male BC cases but not for female BC cases. Survival outcome was defined as death due to any cause. The average length of follow-up was 8.32 years.

Quantification of elastosis

Elastosis was quantified using a 4-tiered system, according to the degree of elastosis observed on the H&E stained slide. One representative slide per patient was examined. Elastosis can be seen as clumps of elastic fibers that appear as an acellular area, usually surrounding ducts or tumor fields, in H&E stained sections. This is easily distinguished by experienced pathologists from fibrosis or desmoplastic stroma, as elastosis appears as an eosinophilic to greyish area and appears as a well circumscribed area, and not as diffuse changes in the stroma. Elastosis grade (EG) 0 corresponded to no demonstrable elastosis, EG1 corresponded to 1 to 3 single ducts or tumor fields surrounded by elastosis, EG2 to 4-6 single ducts or tumor fields surrounded by elastosis, or 2-3 bigger and confluent fields of elastosis, and EG3 corresponded to > 6 single ducts or tumor fields surrounded by elastosis or > 3 confluent fields of elastosis (Figure 1).

Statistics

Statistical calculations were performed using SPSS for Windows version 25. P-values of <0.05 were regarded as significant. For correlations between categorical variables Pearson χ^2 test (or Fisher's exact test when appropriate) was used. Continuous variables were analyzed using the t-test. Multivariate analysis was done with logistic regression, taking the clinicopathological features that showed significance with univariate analysis into account. Survival analysis was done by plotting a Kaplan-Meier survival curve and assessing significance with logrank test. Multivariate survival analysis was done with Cox regression.

Elastosis in male versus female breast cancer

Table 1 shows EG scores in male and female BC. Using the 4-tiered system of grading elastosis, a significant difference was found between male and female BC. Male BC showed in general a lower amount of elastosis ($p<0.001$). Male BC showed at least some degree of elastosis in 26/117 cases (22.2%) with no cases showing EG3, while female BC cases showed elastosis in 89/135 cases (65.9%) with 21.5% showing EG3 ($p <0.001$). When comparing EG0/1 to EG2/3, significance remained ($p<0.001$).This difference between male and female BC was found in subgroups of histologic grade 1 and grade 2 tumors ($p<0.001$ for both), but not in grade 3 tumors ($p=0.199$).

In logistic regression considering age, MAI, grade, PgR and elastosis (EG0/1 versus EG2/3), elastosis was the highest predictor for gender ($p<0.001$, HR 22.487, 95% CI 8.319-60.782).

Elastosis in male breast cancer: correlation with histopathological features and overall survival

No significant differences were found between the amount of elastosis and grade ($p=0.651$), age (cut-off 55y, $p=0.276$) MAI (cut-off 8, $p=0.613$), PgR ($p=0.834$) or HER2 ($p=0.668$)

In univariate analysis and multivariate analysis there was no significant difference in 10 year survival for any elastosis (EG1/2) versus no elastosis (EG0) in male BC. The Kaplan-Meier survival curve is shown in Figure 2.

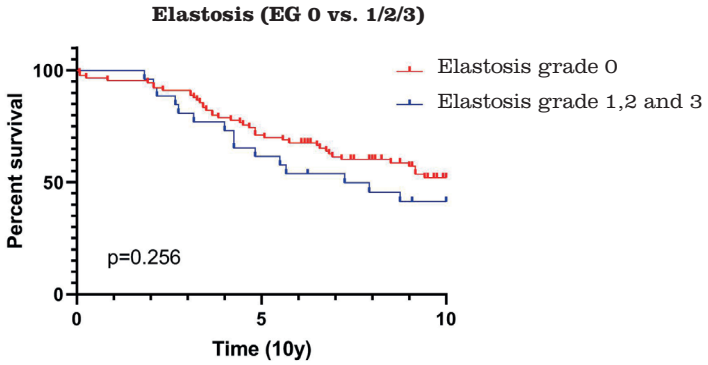

Figure 2 Kaplan-Meier survival curve of invasive male breast cancer according to the amount of elastosis.

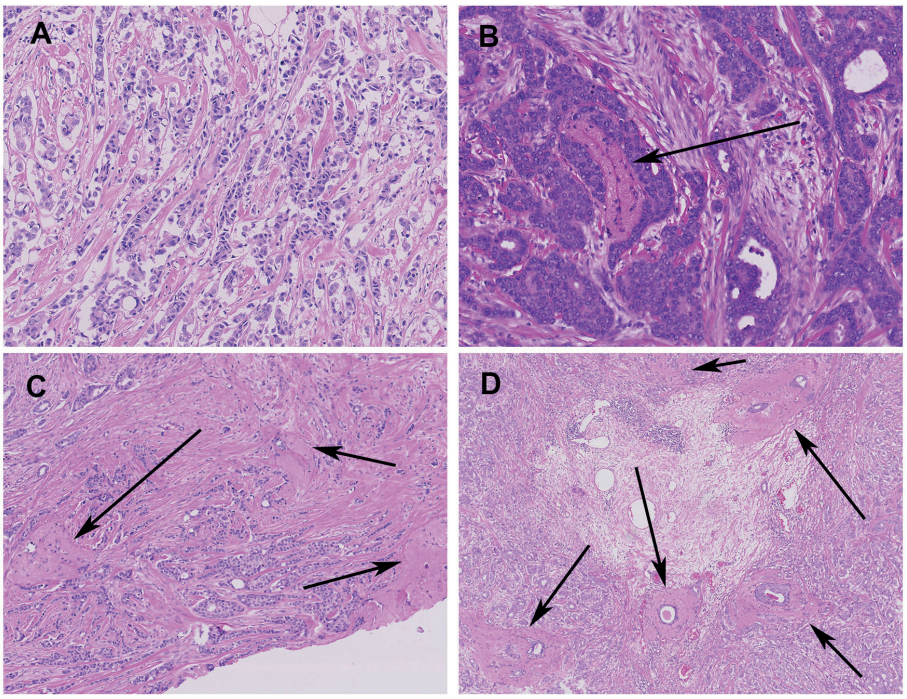

Figure 1 Elastosis in invasive male breast cancer, which can be identified in the H&E staining and classified using a 4-tiered system. Elastosis grade 0: No elastosis can be seen (A), elastosis grade 1: only one small field of elastosis was found in this tumor (B), elastosis grade 2: 5 fields of elastosis were found in this tumor, of which 3 are shown in this image (C) and elastosis grade 3: this tumor demonstrated a high amount of elastosis with a big confluent field of elastosis shown here (D).

Results

Clinicopathological features

All patients, male (n=117) and female (n=135), had invasive BC of no special type (NST) and all tumors were ERα positive. Histopathological features of the male BC cases and female BC cases are summarized in Table 1. The male patients had a median age of 65.4 years (28-98 years), compared to a median age of 58 years (35-79 years) for females ($p<0.001$). Male BC was more frequently graded as a histologic grade 2 (55.6%) compared to female BC (34.1%), which showed a higher percentage of grade 1 and grade 3 cases ($p=0.008$ for grade 1 versus grade 2, $p=0.02$ for grade 2 versus

grade 3, p=0.600 for grade 1 versus grade 3). The mitotic activity index (MAI) was also significantly different with a lower mean MAI in male BC compared to female BC (7.45 versus 10.13, respectively, p=0.011). PgR was positive in significantly more male BC cases compared to female BC cases (p=<0.001).

Table 1 Clinicopathological features of male and female breast cancer patients. Missing data were excluded in the given percentages.

Feature		**Male n=117**	**Female n=135**	**p-value**
Age	Mean	64.4	58.8	<0.001
Grade	I	31 (26.5%)	49 (36.3%)	0.02
	II	65 (55.6%)	46 (34.1%)	
	III	21 (17.9%)	40 (29.6%)	
Mitosis/2mm2	mean	7.45	10.13	0.011
	0-8	85 (72.6%)	70 (51.9%)	0.001
	>8	32 (27.4%)	65 (48.1%)	
PgR	neg	3 (2.6%)	15 (11.1%)	0.012
	pos	113 (97.4%)	120 (88.9%)	
	missing	1	0	
HER2	neg	100 (89.3%)	127 (94.1%)	0.241
	pos	12 (10.7%)	8 (5.9%)	
	missing	5	0	
Elastosis	0	91 (77.8%)	46 (34.1%)	<0.001
	1	20 (17.1%)	32 (23.7%)	
	2	6 (5.1%)	28 (20.7%)	
	3	0	29 (21.5%)	
Elastosis (2 categories)	0+1	111 (94.9%)	78 (57.8%)	<0.001
	2+3	6 (5.1%)	57 (42.2%)	
Neo-adjuvant therapy	no	55 (47.0%)	135 (100%)	
	yes	0	0	
	missing	62 (53.0%)	0	

Discussion

Breast cancer is a well-known and well-studied disease as it is the leading type of cancer in women worldwide, accounting for approximately 30% of the estimated new cases of cancer in the United States in 2019 [8]. In contrast to female BC, male BC is understudied and unfamiliar amongst the public due to its low prevalence. Of all the breast cancers diagnosed in the United States in 2019 only 1% will occur in the male breast [8]. When male BC is diagnosed it is usually treated using treatment algorithms derived from female BC studies. Male BC however, is not as similar to female BC as one might assume, as previous studies have shown differences in the distribution of histologic as well as molecular subtype, age at presentation, as well as differences at the molecular and epigenetic level [9-12 13, 14].

In female BC, it is well shown that the presence of elastosis is correlated to ERα expression [4, 5, 7]. To the best of our knowledge, this correlation had not been studied in male BC before. We therefore studied elastosis in 117 male BC cases and correlated the amount of elastosis to histopathological characteristics and survival. In addition, we compared our results to 135 female BC cases. All cases were ERα positive and all cases were subtyped as invasive carcinoma of no special type, according to the WHO.

In our study, none of the male BC cases showed an abundant amount of elastosis (EG3). Only 6 cases (5.1%) showed a moderate amount of elastosis (EG2). This was significantly lower than the number of female BC cases showing EG2 and 3 (20.7% EG2 and 21.5% EG3). A previous female BC study examining elastosis using a similar grading scheme (grades 0, 1, 2 and 3) found 16.5% of the 272 cases to show a high amount of elastosis (EG3). 33.8% showed no elastosis (EG0), 28.7% a minimal amount (EG1) and 21.0% a moderate amount (EG2). This distribution is similar to our female BC study population and strengthens our finding that the stroma in male and female BC differs, even in multivariate analysis.

In BC, the production of the elastic fibers is thought to originate from both neoplastic epithelial cells as well as from (myo)fibroblasts [2, 3, 20, 21]. A previous study using *in situ* hybridization for elastin mRNA on BC sections and using BC cell lines to examine elastin biosynthesis and regulation in fibroblasts and epithelial cells, showed that the regulatory mechanism of elastin biosynthesis is probably similar to the mechanism in normal elastotic fibroblasts. Cells that showed to produce immunoreactive tropoelastin were epithelial cells, fibroblasts and endothelial cells, with usually more than one cell type involved per studied sample [2]. As the immunoreactive epithelial cells were

located at the periphery and in close proximity to stroma, it is believed that the interaction between the stroma and epithelial cells triggers tropoelastin biosynthesis in the epithelial cells [2]. Other studies have also shown the importance of the stroma/extracellular matrix (ECM) in BC [22, 23]. That elastosis is common in ERα-positive female BC is a well-known fact, but the underlying mechanism of this correlation has not been described to our knowledge. As ERα is known to have influence on gene expression including many genes, perhaps one could speculate that in men, certain genes that play a role in elastic fiber formation are expressed differently compared to women or are more susceptible to ER influence, resulting in lower elastic fiber formation, and as a consequence, a lower amount of elastosis [24]. Why the association between ERα and elastosis is different in male BC remains an unanswered question and further research is needed. This could be investigated by looking at stromal gene signature, that has been done in a previous study revealing different signatures for different stromal elements [25].

In addition to our comparison of elastosis in male and female BC, we correlated the amount of elastosis to different histopathological features, but no significant correlations were found between elastosis and histologic grade, age, MAI and PgR. Also, no significant prognostic value of elastosis was seen, although a limitation of this study is that we could only use overall survival. BC specific survival was not available. In female BC the correlation between survival and elastosis differs, as several studies found an improved survival in cases with a high amount of elastosis but others found no correlation or an inversed one [3-6].

In conclusion, despite high ERα expression, male BC shows significantly less elastosis than female BC with no relevant clinicopathologic correlations or prognostic value. Therefore, elastosis seems to be a less useful ERα tissue biomarker with less clinical significance in male BC than in female BC, again pointing towards important BC sex differences. Although male BC is a rare disease, further research is needed to better understand the underlying pathogenesis of (lack of) elastosis in male BC.

References

1. Wagenseil JE, Mecham RP. New insights into elastic fiber assembly. *Birth defects research. Part C, Embryo today : reviews* 2007;**81**;229-240.
2. Krishnan R, Cleary EG. Elastin gene expression in elastotic human breast cancers and epithelial cell lines. *Cancer research* 1990;**50**;2164-2171.
3. Shivas AA, Douglas JG. The prognostic significance of elastosis in breast carcinoma. *Journal of the Royal College of Surgeons of Edinburgh* 1972;**17**;315-320.
4. Chen Y, Klingen TA, Wik E *et al.* Breast cancer stromal elastosis is associated with mammography screening detection, low ki67 expression and favourable prognosis in a population-based study. *Diagnostic pathology* 2014;**9**;230.
5. Rasmussen BB, Pedersen BV, Thorpe SM, Rose C. Elastosis in relation to prognosis in primary breast carcinoma. *Cancer research* 1985;**45**;1428-1430.
6. Robertson AJ, Brown RA, Cree IA, MacGillivray JB, Slidders W, Beck JS. Prognostic value of measurement of elastosis in breast carcinoma. *Journal of clinical pathology* 1981;**34**;738-743.
7. Rasmussen BB, Rose C, Thorpe SM, Hou-Jensen K, Daehnfeldt JL, Palshof T. Histopathological characteristics and oestrogen receptor content in primary breast carcinoma. *Virchows Archiv. A, Pathological anatomy and histology* 1981;**390**;347-351.
8. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;**69**;7-34.
9. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
10. Ge Y, Sneige N, Eltorky MA *et al.* Immunohistochemical characterization of subtypes of male breast carcinoma. *Breast cancer research : BCR* 2009;**11**;R28.
11. Kornegoor R, Verschuur-Maes AH, Buerger H *et al.* Molecular subtyping of male breast cancer by immunohistochemistry. *Mod Pathol* 2012;**25**;398-404.
12. Anderson WF, Jatoi I, Tse J, Rosenberg PS. Male breast cancer: A population-based comparison with female breast cancer. *J Clin Oncol* 2010;**28**;232-239.
13. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Promoter hypermethylation in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research : BCR* 2012;**14**;R101.
14. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Oncogene amplification in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research and treatment* 2012;**135**;49-58.
15. Cardoso F, Bartlett JMS, Slaets L *et al.* Characterization of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018;**29**;405-417.
16. Vermeulen MA, Slaets L, Cardoso F *et al.* Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England : 1990)* 2017.
17. Lakhani SR, Ellis. I.O., Schnitt, S.J., Tan, P.H., van de Vijver, M.J. *Who classification of tumours of the breast. Fourth edition* 2012.
18. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;**19**;403-410.
19. Wolff AC, Hammond ME, Hicks DG *et al.* Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of american pathologists clinical practice guideline update. *J Clin Oncol* 2013;**31**;3997-4013.

20. Martinez-Hernandez A, Francis DJ, Silverberg SG. Elastosis and other stromal reactions in benign and malignant breast tissue: An ultrastructural study. *Cancer* 1977;**40**;600-606.
21. Parfrey NA, Doyle CT. Elastosis in benign and malignant breast disease. *Human pathology* 1985;**16**;674-676.
22. Soysal SD, Tzankov A, Muenst SE. Role of the tumor microenvironment in breast cancer. *Pathobiology : journal of immunopathology, molecular and cellular biology* 2015;**82**;142-152.
23. Giussani M, Merlino G, Cappelletti V, Tagliabue E, Daidone MG. Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression. *Seminars in cancer biology* 2015;**35**;3-10.
24. Zheng Y, Shao X, Huang Y *et al*. Role of estrogen receptor in breast cancer cell gene expression. *Molecular medicine reports* 2016;**13**;4046-4050.
25. Winslow S, Leandersson K, Edsjo A, Larsson C. Prognostic stromal gene signatures in breast cancer. *Breast cancer research : BCR* 2015;**17**;23.

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Expression of hypoxia-induced proteins in ductal carcinoma *in situ* and invasive cancer of the male breast

Marijn A Vermeulen, Carolien HM van Deurzen, Carolien P Schröder, John WM Martens, Paul J van Diest

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Abstract

Aim: The aim of this study was to determine the role of hypoxia in male breast carcinogenesis by evaluating expression of the hypoxia-related proteins, hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1) in ductal carcinoma *in situ* (DCIS) of the male breast in relation to invasive cancer (IC).

Methods: Tumor tissue blocks of 18 cases of pure DCIS, 58 DCIS cases adjacent to IC (DCIS-AIC) and the 58 IC cases were stained by immunohistochemistry for HIF-1α, CAIX and Glut-1, and expression frequencies and patterns (diffuse and/or perinecrotic) were noted.

Results: HIF-1α overexpression was observed in 61.1% (11/18) of pure DCIS, in 37.9% (22/58) of DCIS adjacent to invasive cancer (DCIS-AIC) and in 36.2% (21/58) of IC cases (not significant (n.s.)). CAIX overexpression was observed in 16.7% (3/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 24.1% (14/58) of IC cases (n.s.). Glut-1 overexpression was observed in 61.1% (11/18) of pure DCIS, in 75.9% (44/58) of DCIS-AIC and in 62.1% (36/58) of IC cases (n.s.). Expression of hypoxia-related proteins was seen around necrosis in a little over one-third of DCIS cases, and often coincided with expression in adjacent IC when present. All these observations indicate that the hypoxia response is already at its maximum in the pre-invasive DCIS stage.

Conclusions: In conclusion, male DCIS frequently shows activated hypoxia response, comparable to male IC. This indicates that the activated hypoxia response previously seen in male IC is not a late bystander but likely a genuine carcinogenetic event.

Introduction

Hypoxia is a condition that occurs when there is a mismatch between oxygen supply and oxygen consumption and this condition has been described in several solid tumors, such as head and neck cancer, cervical cancer as well as breast cancer (BC) [1, 2]. Tumor cells need to adapt to hypoxia in order to survive, and are capable of doing this through several different signaling pathways [3]. The key regulator of the hypoxia response is hypoxia-inducible factor-1 (HIF-1), a heterodimeric protein that consists of the HIF-1α and HIF-1β subunits, the latter being constitutively expressed. Under normoxic circumstances HIF-1α is rapidly degraded but when hypoxia occurs HIF-1α is stabilized, resulting in overexpression [4]. The overexpression of HIF-1 α leads to upregulation of established downstream targets such as carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1) [3]. CAIX is a member of the family of zinc metalloenzymes and its function is to regulate intracellular and extracellular pH [5]. Glut-1 is a membrane bound protein involved in glucose transport [6]. In female BC, expression of hypoxia-related markers have been described in invasive cancer (IC) and expression has been correlated with a decreased overall survival, high risk of metastases and a higher histologic grade in IC [7-9]. This indicates that triggering the hypoxia response contributes to the formation of a more aggressive form of IC.

Male BC is a rare disease, accounting for approximately 1% of all breast carcinomas. In the United States, approximately 2670 men are estimated to develop breast cancer in 2019.[10] Of these men, approximately 5% (range 1%-17%) will be diagnosed with pure ductal carcinoma *in situ* (DCIS), the final precursor stage before invasion takes place [11]. Because male IC has a low prevalence and male DCIS even more so, studies are much less abundant than studies concerning female BC. Although evidence has shown that male BC and female BC differ on several levels, therapy strategies are still largely extrapolated from female BC clinical trials. Differences between male and female BC patients are for example a higher age at diagnosis in male patients, a more advanced disease at time of diagnosis in men, a different distribution in histologic subtypes and differences at molecular level [12-16].

In male BC, only one study has been performed in which HIF-1α, CAIX and Glut-1 have been correlated to clinicopathological features and prognosis in a cohort of 134 IC patients. HIF-1α expression was significantly correlated with a high histologic grade, human epidermal growth factor receptor 2 (*HER2)* amplification and poor survival. Glut-1 expression was correlated with high histologic grade [17]. No data on male DCIS

were yet available. Since triggering of the hypoxia response may already take place in the stage of DCIS, like has been described for female IC, we focused in the present study on the expression of HIF-1α, CAIX and Glut-1 in male DCIS to determine the role of hypoxia in male breast carcinogenesis, and to assess whether changes in hypoxia-related proteins seen earlier in male IC are carcinogenetic events or late bystanders [8, 18] .

Table 1. Overview of the hypoxia-related antibodies and tissue processing methods used

Antibody	Type	Source	Dilution	Antigen retrieval	Antibody incubation time
Glut-1	Polyclonal	DAKO	1:200	EDTA	32 min
CAIX	Polyclonal	Abcam	1:1000	Citrate	60 min
HIF-1α	Monoclonal	BD Biosciences	1:50	EDTA	Overnight

Materials and Methods

Patients material

Patients with DCIS and adjacent IC and patients with pure DCIS were enrolled from a previously selected large male breast cancer cohort (the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program) [19 20]. A (Dutch) subgroup of this initial population was selected based on availability of a representative tumor tissue block for central pathology review, resulting in a total of 18 cases of pure DCIS and 58 cases with DCIS adjacent to IC (DCIS-AIC). Of these 58 cases the IC component was also analysed. Patient and tumor characteristics including age at diagnosis were recorded. Hematoxylin and eosin (H&E) stained slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IC according to the World Health Organization and modified Bloom and Richardson score [21]. DCIS was graded according to the classification by Holland et al [22]. Estrogen receptor alpha (ERα), progesterone receptor (PgR) and HER2 were evaluated using immunohistochemistry and scored according to the Allred-score and ASCO-CAP guidelines [23 24].

The EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program was conducted as global effort to retrospectively assess tumor tissue of men diagnosed with breast cancer between 1989 and 2009. Male patients in the Netherlands were identified through the Dutch Cancer Registry. Paraffin embedded male breast cancer tissue was retrospectively collected by the Dutch Breast Cancer Research Group (BOOG).

Immunohistochemistry

Immunohistochemistry (table 1) was performed on 4-µm thick slides after Silane coating. Slides from one representative tumor tissue block were available. EDTA buffer (pH=9.0 for 20 minutes at 100°C) was used for antigen retrieval for HIF-1α. Slides were incubated with the primary HIF-1α antibody overnight at 4°C and detected by Novolink Polymer (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK). For CAIX, antigen retrieval was carried out in citrate buffer (pH=6.0 for 20 minutes at 100°C). Incubation with the primary CAIX antibody was 60 minutes at 20°C followed by detection using Powervision ready to use (poly-HRP-anti Ms/Rb/RtIgG biotin free; immunologic, ImmunoVision Technologies, Brisbane CA, USA). Antigen retrieval for Glut-1 was performed in EDTA buffer and the slides were incubated with the primary Glut-1 antibody for 32 minutes. Glut-1 staining was performed using a Ventana BenchMark ULTRA automated immunostainer (Ventana Medical Systems, Inc, Tucson, AZ, USA) and staining for HIF-1α and CAIX was carried out manually. All slides were developed with diaminobenzidine. For CAIX and HIF-1α formalin-fixed and paraffin-embedded clear cell renal cell carcinoma was taken along as positive control and for Glut-1 erythrocytes were used as the internal positive control. Appropriate negative control steps were used throughout the procedures. Stainings were scored by consensus of two experienced observers.

Quantification of immunohistochemical staining

For HIF-1α mean nuclear staining percentages were used, regarding ≥5% nuclear staining of all tumor cells in the representative slide positive as before [25]. Any clear membranous staining in Glut-1 and CAIX was scored positive. For all markers, two staining patterns were noted as before: a diffuse pattern throughout the tumor cells and a perinecrotic staining pattern in which membranous staining was restricted to the perinecrotic tumor areas [8, 25]. Figure 1 shows an example of the immunohistochemical stainings.

Statistics

Statistical calculations were performed using SPSS for Windows version 24. To evaluate the correlation of clinicopathological features between the three groups (pure DCIS, DCIS-AIC and IC) and for analysis of the expression of the hypoxia related proteins in the three groups, the Chi-square test, and if appropriate the Fisher-exact test was used. Paired analysis, testing the similarity of protein expression between DCIS-AIC and IC was done using Cohen's Kappa test. P-values below 0.05 were considered significant.

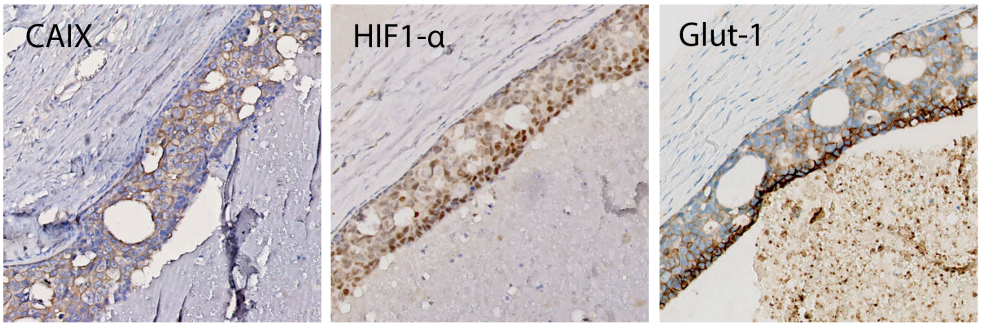

Figure 1. Immunohistochemical stainings for hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1).

Results

The mean age was 62 years (range 38-77 years) for patients with pure DCIS and 64 years (range 38-86 years) for patients with DCIS-AIC/IC (p=0.509). The majority of the IC cases were classified as invasive ductal carcinoma (51/58, 88%). The other cases were classified as mixed (ductal/micropapillary, n=2 and ductal/mucinous, n=1), micropapillary (n=1), mucinous (n=1), encapsulated papillary carcinoma (n=1) or tubular carcinoma (n=1). The clinicopathological characteristics and expression of ERα, PgR and HER2 in pure DCIS, DCIS-AIC and IC are presented in table 2. No significant differences in these clinicopathological features were found between the three groups.

Expression of hypoxia-induced proteins in DCIS and IC

HIF-1α overexpression was observed in 61.1% (11/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 36.2% (21/58) of IC cases (not significant (n.s.)). CAIX overexpression was observed in 16.7% (3/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 24.1%

(14/58) of IC cases (n.s.). Glut-1 overexpression was observed in 61.1% (11/18) of pure DCIS, in 75.9% (44/58) of DCIS-AIC and in 62.1% (36/58) of IC cases (n.s.).

Table 2. Clinicopathological characteristics and expression of ERα, PgR, HER2, HIF-1α, CAIX and Glut-1 in pure ductal carcinoma *in situ* (DCIS), DCIS adjacent to invasive cancer (DCIS-AIC) and invasive cancer (IC) of the male breast (missing data excluded)

		pure DCIS	**DCIS-AIC**	**IC**	**p-value**
	N	**18**	**58**	**58**	
Age	<45	1 (5.6%)	4 (6.9%)	4 (6.9%)	n.s.
	≥45	17 (94.4%)	54 (93.1%)	54 (93.1%)	
Grade♦	1	3 (16.7%)	13 (22.4%)	16 (27.6%)	n.s.
	2	14 (77.8%)	35 (60.3%)	27 (46.6%)	
	3	1 (5.6%)	10 (17.2%)	15 (25.9%)	
Mitoses/2mm²	<7			35 (60.3%)	
	7-12			14 (24.1%)	
	≥13			9 (15.5%)	
ERα	neg	0 (0%)	0 (0%)	0 (0%)	
	pos	18 (100%)	58 (100%)	58 (100%)	
PgR	neg	0 (0%)	3 (5.2%)	4 (6.9%)	n.s.
	pos	18 (100%)	55 (94.8%)	54 (93.1%)	
HER2*	neg	16 (94.1%)	55 (94.8%)	55 (94.8%)	n.s.
	pos	1 (5.9%)	3 (5.2%)	3 (5.2%)	
HIF-1α	neg	7 (38.9%)	36 (62.1%)	37 (63.8%)	n.s.
	pos	11 (61.1%)	22 (37.9%)	21 (36.2%)	
CAIX	neg	15 (83.3%)	36 (62.1%)	44 (75.9%)	n.s.
	pos	3 (16.7%)	22 (37.9%)	14 (24.1%)	
Glut-1**	neg	6 (35.3%)	14 (24.1%)	22 (37.9%)	n.s.
	pos	11 (64.7%)	44 (75.9%)	36 (62.1%)	

♦) Grading for DCIS according to the classification by Holland et al[22] and for IC according to the modified Bloom and Richardson score.

*) 1 case pure DCIS missing HER2 data.

**) 1 case pure DCIS missing Glut-1 data.

In pure DCIS a diffuse staining pattern of HIF-1α was seen in 6/11 positive cases (54.5%). Glut-1 showed a diffuse staining pattern in 7/11 positive cases (63.6%) and CAIX in all of the 3 positive cases (100%). All other positive cases showed a perinecrotic staining pattern. In DCIS (pure DCIS and DCIS-AIC as a group) perinecrotic staining of HIF-1α was significantly correlated with overexpression of CAIX or Glut-1, compared to DCIS cases with a diffuse staining pattern of HIF-1α (p=0.012, table 3).

In DCIS-AIC a diffuse staining pattern was seen in 14/22 (63.6%) of HIF-1α positive cases, in 28/44 (63.6%) of Glut-1 positive cases and in 13/22 (59.0%) of CAIX positive cases. In IC a diffuse staining pattern was seen in 18/21 (85.7%) of HIF-1α positive cases, in 32/36 (88.9%) of Glut-1 positive cases and in 12/14 (85.7%) of CAIX positive cases.

HIF-1α overexpression was more common in tumors with a high mitotic activity index (>7 mitoses/2mm^2) and high grade (grade 3) IC cases (table 4, p=0.013 and p=0.025, respectively). No significant differences were found in DCIS.

Table 5 shows that the expression of HIF-1α, CAIX and Glut-1 in paired DCIS and adjacent IC lesions often coincided (p<0.001 for all three proteins).

Table 3. Correlation between HIF-1α staining pattern and overexpression of CAIX or Glut-1 in male DCIS

HIF-1α positive DCIS (n=33)	**CAIX and Glut-1 negative**	**CAIX and/or Glut-1 positive**	**p-value**
Diffuse HIF-1α positive	8	12	0.012
Perinecrotic HIF-1α positive	0	13	

Table 4. Correlation of HIF-1α overexpression in pure ductal carcinoma *in situ* (DCIS), DCIS adjacent to invasive cancer (DCIS-AIC) and invasive cancer (IC) of the male breast with clinicopathological features and CAIX and Glut-1 expression

		pure DCIS			**DCIS-AIC**			**IC**		
		HIF-1α		**p-value**	**HIF-1α**		**p-value**	**HIF-1α**		**p-value**
		neg	pos		neg	pos		neg	pos	
Age	<45	0	1	n.s.	1	3	n.s.	1	3	n.s.
	≥45	7	10		35	19		36	18	
Grade	1/2	7	10	n.s.	31	17	n.s.	32	11	0.025
	3	0	1		5	5		5	10	
MAI	<7			.			.	27	8	0.013
	≥ 7							10	13	
ERα	neg	0	0	·	0	0	·	0	0	.
	pos	7	11		36	22		37	21	
PgR	neg	0	0	·	2	1	n.s.	2	2	n.s.
	pos	7	11		34	21		35	19	
HER2	neg	6	10	n.s.	35	20	n.s.	36	19	n.s.
	pos	0	1		1	2		1	2	
CAIX	neg	7	8	n.s.	25	11	n.s.	32	12	0.007
	pos	0	3		11	11		5	9	
Glut-1	neg	2	4	n.s.	9	5	n.s.	17	5	n.s.
	pos	4	7		27	17		20	16	

Table 5. Correlation of HIF-1α, CAIX and Glut-1 overexpression in paired ductal carcinoma *in situ* (DCIS) and invasive cancer (IC) of the male breast

		IC								
		HIF-1α			CAIX			Glut-1		
		neg	pos	p-value	neg	pos	p-value	neg	pos	p-value
DCIS	neg	32	4		33	3		11	3	
	pos	5	17	<0.0001	11	11	<0.0001	11	33	<0.0001

Discussion

The aim of this study was to determine the role of hypoxia in male breast carcinogenesis by evaluating expression of the hypoxia-related proteins HIF-1α, CAIX and Glut-1 in DCIS of the male breast in relation to IC. In previous female BC studies HIF-1α expression was similar between DCIS and IC, but no HIF-1α expression was seen in normal breast tissue or benign lesions such as ductal hyperplasia and fibroadenomas, indicating that HIF-1α expression is an early payer in female breast carcinogenesis [8, 18]. In line with these findings in female BC, we found similar levels of HIF-1α expression in male DCIS and IC, paralleled by expression of CAIX and Glut-1. Expression of HIF-1α, CAIX and Glut-1 often coincided in paired DCIS and IC lesions. This indicates that the activated hypoxia response is already at its max in the pre-invasive male DCIS stage and is not a late bystander but likely a genuine carcinogenetic event.

There were no significant correlations between grade and HIF-1α, CAIX or Glut-1 expression in DCIS in contrast to IC that we reported before [17]. In female BC however, it has been described that high grade DCIS shows significantly higher HIF-1α expression. This was shown in 40 DCIS samples in which HIF-1α expression was seen in 85% of 20 poorly differentiated lesions compared to 55% of 20 well differentiated lesions [8]. Our lack of significance could be due to our smaller DCIS sample size with only 11/76 (14.5%) grade 3 lesions, compared to 24/44 (54.5%) grade 3 lesions in the female DCIS study, with only 5 high grade male DCIS samples showing HIF-1α expression. Indeed, expression of hypoxia related proteins in DCIS was generally lower in male DCIS compared to female DCIS: 43.4% vs. 82.1% for HIF-1-α, 32.8% vs. 56.7% for CAIX and 73.3% vs. 25% for Glut-1 [26]. Furthermore, quantification of HIF-1α was performed

differently; >5% nuclear staining was considered positive by us, compared to >1% nuclear staining in the female BC study [26]. Expression of HIF-1α, CAIX and Glut-1 were not previously examined in male DCIS to our knowledge, so our present results cannot be compared with literature.
In our DCIS cases showing HIF-1α expression, the staining was perinecrotic in 39.4%. This staining pattern in DCIS was significantly more correlated to co-expression of CAIX and Glut-1 than a diffuse staining pattern of HIF-1α, like in female BC [27]. This again shows that the hypoxia response is most outspoken around necrosis.

In conclusion, male DCIS frequently shows activated hypoxia response, comparable to male IC. This indicates that the activated hypoxia response previously seen in male IC is not a late bystander but likely a genuine carcinogenetic event.

References

1. Dhani N, Fyles A, Hedley D, Milosevic M. The clinical significance of hypoxia in human cancers. *Semin Nucl Med* 2015;**45**;110-121.
2. Vaupel P, Mayer A, Briest S, Hockel M. Hypoxia in breast cancer: Role of blood flow, oxygen diffusion distances, and anemia in the development of oxygen depletion. *Adv Exp Med Biol* 2005;**566**;333-342.
3. Walsh JC, Lebedev A, Aten E, Madsen K, Marciano L, Kolb HC. The clinical importance of assessing tumor hypoxia: Relationship of tumor hypoxia to prognosis and therapeutic opportunities. *Antioxid Redox Signal* 2014;**21**;1516-1554.
4. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (hif-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997;**272**;22642-22647.
5. Svastova E, Hulikova A, Rafajova M et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase ix to acidify extracellular ph. *FEBS Lett* 2004;**577**;439-445.
6. Younes M, Brown RW, Mody DR, Fernandez L, Laucirica R. Glut1 expression in human breast carcinoma: Correlation with known prognostic markers. *Anticancer Res* 1995;**15**;2895-2898.
7. Chia SK, Wykoff CC, Watson PH et al. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase ix, in invasive breast carcinoma. *J Clin Oncol* 2001;**19**;3660-3668.
8. Bos R, Zhong H, Hanrahan CF et al. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001;**93**;309-314.
9. Dales JP, Garcia S, Meunier-Carpentier S et al. Overexpression of hypoxia-inducible factor hif-1alpha predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. *Int J Cancer* 2005;**116**;734-739.
10. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019;69;7-34.
11. Pappo I, Wasserman I, Halevy A. Ductal carcinoma in situ of the breast in men: A review. *Clinical breast cancer* 2005;**6**;310-314.
12. Anderson WF, Jatoi I, Tse J, Rosenberg PS. Male breast cancer: A population-based comparison with female breast cancer. *J Clin Oncol* 2010;**28**;232-239.
13. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
14. Hill TD, Khamis HJ, Tyczynski JE, Berkel HJ. Comparison of male and female breast cancer incidence trends, tumor characteristics, and survival. *Annals of epidemiology* 2005;**15**;773-780.
15. Kornegoor R, Moelans CB, Verschuur-Maes AH et al. Oncogene amplification in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research and treatment* 2012;**135**;49-58.
16. Vermeulen MA, Doebar SC, van Deurzen CHM, Martens JWM, van Diest PJ, Moelans CB. Copy number profiling of oncogenes in ductal carcinoma in situ of the male breast. *Endocr Relat Cancer* 2018;**25**;173-184.
17. Kornegoor R, Verschuur-Maes AH, Buerger H et al. Fibrotic focus and hypoxia in male breast cancer. **Mod Pathol** 2012;**25**;1397-1404.
18. Okada K, Osaki M, Araki K, Ishiguro K, Ito H, Ohgi S. Expression of hypoxia-inducible factor (hif-1alpha), vegf-c and vegf-d in non-invasive and invasive breast ductal carcinomas. *Anticancer Res* 2005;**25**;3003-3009.

19. Cardoso F, Bartlett JMS, Slaets L et al. Characterization of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018;**29**;405-417.
20. Vermeulen MA, Slaets L, Cardoso F et al. Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England: 1990)* 201.
21. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;**19**;403-410.
22. Holland R, Peterse JL, Millis RR et al. Ductal carcinoma in situ: A proposal for a new classification. *Seminars in diagnostic pathology* 1994;**11**;167-180.
23. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;**11**;155-168.
24. Wolff AC, Hammond ME, Hicks DG *et al*. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of american pathologists clinical practice guideline update. *J Clin Oncol* 2013;**31**;3997-4013.
25. Bos R, van der Groep P, Greijer AE *et al*. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer* 2003;**97**;1573-1581.
26. Chen CL, Chu JS, Su WC, Huang SC, Lee WY. Hypoxia and metabolic phenotypes during breast carcinogenesis: Expression of hif-1alpha, glut1, and caix. Virchows Archiv: *an international journal of pathology* 2010;**457**;53-61.
27. Vleugel MM, Greijer AE, Shvarts A et al. Differential prognostic impact of hypoxia induced and diffuse hif-1alpha expression in invasive breast cancer. *Journal of clinical pathology* 2005;**58**;172-177

5

Copy number profiling of oncogenes in ductal carcinoma *in situ* of the male breast

Marijn A Vermeulen, Shusma C Doebar, Carolien HM van Deurzen, John WM Martens, Paul J van Diest, Cathy B Moelans

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Abstract

Characterizing male breast cancer (BC) and unraveling male breast carcinogenesis is challenging because of the rarity of this disease. We investigated copy number status of 22 BC related genes in 18 cases of pure ductal carcinoma *in situ* (DCIS) and in 49 cases of invasive carcinoma (IC) with adjacent DCIS (DCIS-AIC) in males using multiplex ligation-dependent probe amplification (MLPA). Results were compared to female BC and correlated with survival.

Overall, copy number ratio and aberration frequency including all 22 genes showed no significant difference between the 3 groups. Individual unpaired analysis revealed a significantly higher *MTDH* copy number ratio in IC compared to DCIS-AIC and pure DCIS ($p=0.009$ and $p=0.038$, respectively). *ADAM9* showed a significantly lower copy number aberration frequency in male BC, compared to female BC ($p=0.020$). In DCIS-AIC, *MTDH*, *CPD*, *CDC6* and *TOP2A* showed a lower frequency of copy number increase in males compared to females ($p<0.001$ for all 4 genes). In IC, *CPD* gain and *CCNE1* gain were independent predictors of poor overall survival.

In conclusion, male DCIS and IC showed a similar copy number profile for 21 out of 22 interrogated BC related genes, illustrating their clonal relation and the genetically advanced state of male DCIS. *MTDH* showed a higher copy number ratio in IC compared to adjacent and pure DCIS and may therefore play a role in male breast carcinogenesis. Differences were detected between male and female DCIS for 4 genes pointing to differences in breast carcinogenesis between the sexes.

Introduction

Breast carcinogenesis is a multi-step process involving accumulation of DNA alterations and epigenetic changes. An important event during cancer development is oncogene amplification. Several genes have been described to be frequently amplified in female breast cancer (BC), of which the best-known example is the human epidermal growth factor receptor 2 (*HER2*). *HER2* is amplified in 10-20% of female BC and is correlated to overall survival, time to relapse and response to trastuzumab, a humanized monoclonal anti-*HER2* antibody [1-3]. Other oncogenes that have been described to have clinical implications in female BC include the estrogen receptor (*ESR1*), epidermal growth factor receptor 1 (*EGFR*), *MYC*, topoisomerase IIa (*TOP2A*), fibroblast growth factor receptor 1 (*FGFR1*), cyclin E (*CCNE1*) and cyclin D1 (*CCND1*) [4-10].

Invasive ductal type cancers (IDC) of the breast are thought to arise from ductal carcinoma *in situ* (DCIS) via parallel breast cancer progression pathways in which low grade DCIS progresses to low grade IDC and high grade DCIS to high grade IDC. These parallel pathways have been postulated to have distinct genomic aberrations [11-13]. Progression through grade is a phenomenon that has been rarely observed in BC [14].

The final step in breast carcinogenesis, where the basement membrane of the ducts is breeched and the malignant epithelial cells infiltrate the surrounding stroma, is poorly understood. Several female BC studies have shown similar levels of gene amplification in DCIS and adjacent IC, indicating that these genes play an early role in breast carcinogenesis, but not in the progression from DCIS to invasive carcinoma [12, 15, 16]. Furthermore, not all patients diagnosed with pure DCIS show progression to IC when left untreated. A previous study showed progression from low grade DCIS to IC in 11/28 cases, the remaining cases showing an indolent course [17]. So, unraveling the drivers that control the progression of DCIS to IC has proved to be challenging in female BC, let alone in male BC, where the rarity of the disease hampers thorough investigation. This knowledge is however needed to understand the biological course of male DCIS, to predict patients' outcome and to optimize DCIS treatment strategies. In this study we compare pure DCIS, DCIS adjacent to IC (DCIS-AIC) and IC, as differences at molecular level have been described between these two types of DCIS in females, using RT-PCR [18].

Male BC is a rare disease, accounting for approximately 1% of all BC [19]. Pure DCIS represents approximately 5% (range 1-17%) of all cancers in the male breast [20]. In female BC the diagnosis pure DCIS is made in approximately 20% of all BC, and this difference in DCIS frequency between male and female BC can perhaps be explained by the participation of women in BC screening programs [21].

There are many similarities but also important differences between male and female BC. There are differences in distribution of histologic subtypes as well as molecular subtypes, men tend to be older at the time of diagnosis and have more advanced disease at presentation compared to women [22-25]. Also, there is some evidence suggesting differences in gene amplification frequencies [26]. In a previous male BC study, gain of *CCND1* and *EGFR* was more frequent in male BC compared to female BC, and amplification of *TRAF4* and *EMSY* was more often observed in female BC in comparison to male BC [26].

In the present study we used multiplex ligation-dependent probe amplification (MLPA) to investigate DNA copy number changes of 22 breast cancer related genes in a group of male IC with adjacent DCIS and in a group of male pure DCIS. We correlated these copy number aberrations with clinicopathologic features and 10 year survival data and compared our results to a previous female BC study using a similar MLPA kit [12].

Materials and Methods

Patients

Patients with DCIS and adjacent IC or pure DCIS were enrolled from a previously selected large male BC cohort [27, 28]. A subgroup of this initial population was selected based on availability of a tumor tissue block for central pathology review and sufficient tissue for DNA isolation. This resulted in a total of 51 cases with IC and adjacent DCIS and 20 cases of pure DCIS. Patient and tumor characteristics including age at diagnosis and 10 year overall survival status (defined as death due to any cause) were recorded. Data concerning BRCA1/2 testing was not available. Hematoxylin and eosin (H&E) slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IC according to the World Health Organization and modified Bloom and Richardson score [29]. DCIS was graded according to the classification by Holland et al. [30]. ER, PgR and HER2 were evaluated using immunohistochemistry and scored according to the Allred-score and ASCO-CAP guidelines [31,32]. The areas of interest (pure DCIS, DCIS-AIC and IC) were dissected either manually with a sterile scalpel when big enough or by laser capture microdissection using a Zeiss PALM MD3 laser microdissection system, from 5 sections (4µm) of formalin-fixed-paraffin-embedded (FFPE) tissue blocks. Laser capture microdissection was done in cases with only small areas of DCIS or with abundant inflammatory cells surrounding the area of interest. The DNA was extracted by overnight incubation in proteinase K (10 mg/ml; Roche; Almere; The Netherlands) at 56ºC, followed by boiling for 10 minutes and

centrifugation. Normal male breast tissue was taken along as control. Results from a previous female BC study comparing DCIS and adjacent IC (N=39) using a similar MLPA kit were used to compare copy number status in female and male BC [12]. Clinicopathological data is shown in table 1. Hormone receptor status showed a high concordance (100%) between DCIS and adjacent IC.

Table 1 Clinicopathological data of all male breast cancer cases (invasive carcinoma (IC), male pure ductal carcinoma *in situ* (pure DCIS) and DCIS adjacent to invasive carcinoma).

	Invasive carcinoma	**Adjacent DCIS**	***Pure* DCIS**
Age (years)			
Mean (range)	63.2 (37-85)	63.2 (37-85)	62.3 (37-76)
Histologic subtype IC			
Ductal type carcinoma	46 (90.2 %)		
Mucinous carcinoma	1 (2 %)		
Micropapillary carcinoma	1 (2 %)		
Encapsulated papillary carcinoma	1 (2 %)		
Mixed type			
Ductal/micropapillary	1 (2%)		
Ductal/mucinous	1 (2%)		
Grade			
1	14 (27.5 %)	11 (21.6 %)	3 (15 %)
2	22 (43.1 %)	32 (62.7 %)	16 (80 %)
3	15 (29.4 %)	8 (15.7 %)	1 (5 %)
ER			
Positive	51 (100 %)	51 (100 %)	20 (100 %)
Negative	0 (0 %)	0 (0 %)	0 (0%)
PgR			
Positive	49 (96.1 %)	49 (96.1 %)	20 (100 %)
Negative	2 (3.9 %)	2 (3.9 %)	0 (0%)
HER2			
Positive	2 (3.9 %)	2 (3.9 %)	1 (5.3 %)
Negative	49 (96.1 %)	49 (96.1 %)	18 (94.7 %)
Missing	0	0	1

Multiplex Ligation-dependentProbe Amplification (MLPA)

MLPA analysis was performed on all isolated DNA using the P078-C1 kit (MRC Holland, Amsterdam, The Netherlands), containing 41 probes targeting 22 breast cancer related genes (supplementary table 1). MLPA was performed according to the manufacturer's instructions (MRC Holland), using an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). All tests were done in duplicate and each MLPA run included 7 negative reference samples (3 healthy blood samples, 3 normal male breast FFPE samples and 1 normal female breast FFPE sample). The PCR products were separated by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems). Gene copy numbers were analyzed using GeneScan analysis (Applied Biosystems) and Coffalyser.net software (MRC-Holland). For genes targeted by more than one probe, the mean of all probe-ratios was calculated. Four of the 12 reference probes showed above average copy number variations and were excluded from further analyses (*NRAP* located at 10q25.3, *TGIF1* located at 18p11.31, *CETN3* located at 05q14.3 and *SNCA* located at 04q22.1).

Cut off values were set as described previously with a copy number ratio of <0.7 for gene loss, 1.3 to 2.0 for copy number gain and >2.0 for amplification [12, 26]. Values between 0.7 and 1.3 were considered copy number neutral.

Statistics

Statistical calculations were done using SPSS version 21.0. The Kruskall-Wallis test was used to compare the overall copy number ratio including all 22 genes between the 3 groups, and to compare copy number ratios between the 3 groups for the 22 individual genes. After dichotomization, the Chi-square test was used to compare the frequency of gains, amplifications or losses between groups. Mean copy number aberration frequency, for gains, amplifications and losses, including all genes was analyzed using the Kruskall-Wallis test. Individual genes in pure DCIS and DCIS-AIC were compared using Mann-Whitney test for copy number ratio and Chi-square for dichotomized results.

For paired data (IC and DCIS-AIC) the Wilcoxon signed ranks test was used to compare mean copy number ratio and McNemar's test was used to compare copy number aberration frequency for the 22 individual genes.

The overall copy number ratios between low/intermediate grade and high grade DCIS, as well as between low/intermediate grade and high grade IC were compared by Mann-Whitney test. Dichotomized data per grade category were evaluated by Chi-square. P-values less than 0.05 were considered significant and correction for multiple

comparisons was done using the Holm-Bonferroni method. Survival data were available for all IC and DCIS-AIC cases with a median follow-up of 8.1 years (range 0.86-19.56 years). For univariate survival analysis Kaplan-Meier curves were plotted and analyzed with the log rank test. Multivariate survival analysis was done with Cox regression (backward LR) and included age, mitosis and grade.
Finally, unsupervised hierarchical clustering (Euclidian distance method) of copy number ratios was performed using the statistical program R (www.r-project.org).

Results

Copy number ratio and aberration frequencies in DCIS and invasive carcinoma

One case of invasive carcinoma, one case of DCIS-AIC and two cases of pure DCIS had an insufficient DNA yield and were excluded from further analysis, leaving 49 cases of DCIS with adjacent IC and 18 cases of pure DCIS suitable for copy number analysis. Supplementary table 2 shows raw MLPA copy number data (see http://erc.endocrinology-journals.org/cgi/content/full/ERC-17-0338/DC1).
Table 2 summarizes copy number status for all 22 analyzed genes in each subgroup and Figure 1 illustrates the copy number aberration frequency for each studied gene. The frequencies of losses, gains and amplifications was similar between the three groups (p=0.167, p=0.132 and p=0.361, respectively). Copy number gain/amplification (cut-off >1.3) was most frequently observed for *ZNF703, CCND1* and *MYC* but none of these genes showed a significant difference between the groups.

Overall, the copy number ratio including all 22 genes showed no significant difference between pure DCIS, DCIS-AIC and IC. At the individual gene level, *MTDH* showed a significantly higher copy number ratio in IC as compared to DCIS-AIC and pure DCIS (p=0.009 and p=0.038, respectively). Using a cut-off of >1.3, *MTDH* showed a significantly higher aberration frequency in IC (46.9%) as compared to DCIS-AIC (20.4%) (p=0.005).
The copy number ratio for *PRDM14, C11ORF30* and *FGFR1* was higher in DCIS-AIC compared to pure DCIS (p=0.007, p=0.027 and p=0.042, respectively). However, these genes lost their significance after dichotomization.
No significant differences were found when comparing copy number aberration frequency (gain and amplification) with histologic subtype in IC, although these results should be interpreted with caution due to small sample sizes.

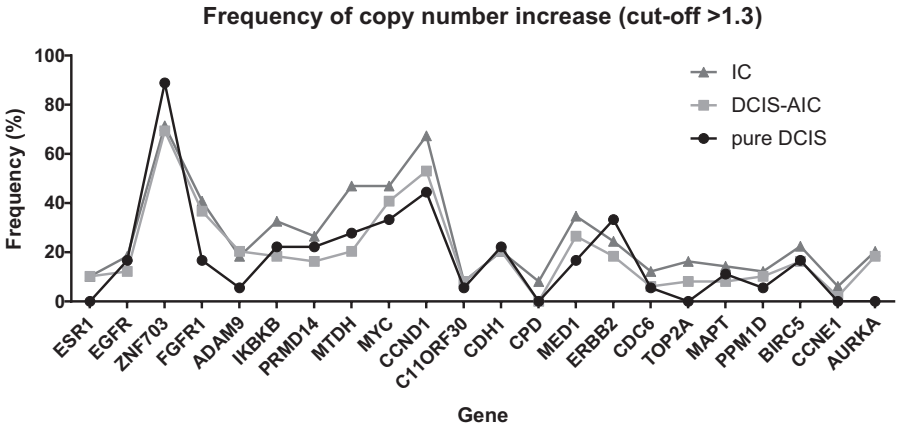

Figure 1. Frequencies of gain/amplification by MLPA for all 22 analyzed genes in male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC).

Paired comparison of DCIS-AIC and adjacent IC showed a high concordance of copy number status for all interrogated genes, with no significant differences present. The highest concordance rates were seen for the genes *CCNE1* (95.9%) and *CDC6* (93.9%). Copy number ratio was significantly higher in IC compared to the paired DCIS-AIC for *MTDH* ($p<0.001$), *MYC* ($p=0.039$), *CPD* ($p=0.015$), *TOP2A* ($p=0.043$) and *PPM1D* ($p=0.036$). Figure 2 shows the median copy number ratio for the 22 analyzed genes and Figure 3 the copy number ratio for *MTDH*, *MYC*, *CPD*, *TOP2A* and *PPM1D* in paired IC and DCIS-AIC.

Correlation between copy number and grade in DCIS-AIC and invasive carcinoma

Copy number ratios and aberration frequencies were compared for DCIS-AIC and IC between low/intermediate grade and high grade lesions. The mean copy number ratio was 1.17 +/− 0.22 versus 1.32 +/− 0.25 for low/intermediate grade versus high grade DCIS-AIC ($p=0.165$), and 1.15 +/− 0.16 versus 1.42 +/− 0.44 for low/intermediate grade versus high grade IC ($p=0.040$). The average number of gains/amplifications in the 22 analyzed genes was 3.7 versus 8.4 for low/intermediate grade versus high grade DCIS-AIC ($p=0.019$), and 4.8 versus 8.3 for low/intermediate grade versus high grade IC ($p=0.037$).

DCIS-AIC showed a significantly higher copy number ratio in high grade lesions for the genes *ESR1* (p=0.047), *PPM1D* (p=0.004), *BIRC5* (p=0.002) and *CCNE1* (p=0.005). After dichotomization (cut-off >1.3), these differences remained significant (p<0.001, p=0.002, p=0.040 and p=0.014, respectively). In addition, *PRDM14* (p=0.040), *CDC6* (p=0.003), *TOP2A* (p=0.018) and *AURKA* (p=0.006) showed a significantly higher copy number aberration frequency in high grade DCIS-AIC lesions. Only *MTDH* showed a significantly higher frequency of amplification in high grade DCIS-AIC (p=0.007).

IC showed a significantly higher copy number ratio in high grade lesions for the genes *EGFR* (p=0.005) and *CCND1* (p=0.005). Dichotomized data (cut-off >1.3) showed a significantly higher aberration frequency for *ESR1* (p=0.007), *EGFR* (p=0.047), *C11ORF30* (p=0.001), *CDC6* (p=0.022) and *PPM1D* (p=0.020) in high grade lesions. *ADAM9* (p=0.029), *MYC* (p=0.031), *CCND1* (p=0.005), *CDH1* (p=0.029), *CDC6* (p=0.013), *TOP2A* (p=0.004) and *PPM1D* (p=0.012) showed significantly more often amplification in high grade lesions.

After correction for multiple comparisons, only *BIRC5* in DCIS-AIC remained significant with regard to copy number ratio difference (1.068 in low/intermediate grade versus 1.353 in high grade). For the dichotomized data, *C11ORF30* in IC (17.1% in low/intermediate grade versus 35.7% in high grade) and *ESR1* in DCIS-AIC (2.4% versus 57.1%) remained significant.

Comparison of DCIS-AIC and IC copy number status between male and female breast cancer

Results from a previous female BC study including 39 patients (IC and adjacent DCIS) were used to compare copy number status between female and male BC [12]. This previous study used a prior version of the MLPA kit used here. Twenty genes were similar in both MLPA kits, with some differences in the probes used for the genes, and were used for analysis.

In IC, *ADAM9* showed a significantly lower copy number aberration frequency (cut-off > 1.3) in male BC (22.5%) compared to female BC (56.4%) (p=0.020). In DCIS, *MTDH*, *CPD*, *CDC6* and *TOP2A* showed a lower frequency of copy number increase in male compared to female BC (p<0.001 for all 4 genes) (Figure 4). The frequencies of amplifications (cut-off >2.0) and losses was similar between female and male BC.

Table 2 Frequencies of losses, gains and amplifications in 22 genes for male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC) including the p-value for gain/amplification (copy number ratio >1.3), amplification (copy number ratio >2.0), and the average copy number aberration frequency for all 22 genes.

		Frequencies (%)										
		pure DCIS (N=18)			**DCIS-AIC (N=49)**			**IC (N=49)**				
Gene	**Chromosome**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**p-value (gain/ amplification, >1,3) Chisquare**	**p-value (amplification, >2,0) Chisquare**
ESR1	6q25.1	0	0	0	1 (2%)	5 (10%)	0	0	5 (10%)	0	0.362	.
EGFR	7p11.2	0	2 (11%)	1 (6%)	0	5 (10%)	1 (2%)	0	8 (16%)	1 (2%)	0.697	0.701
ZNF703	8p11.23	0	11 (61%)	5 (28%)	0	22 (45%)	12 (24%)	1 (2%)	25 (51%)	10 (20%)	0.267	0.395
FGFR1	8p11.22	0	2 (11%)	1 (6%)	1 (2%)	11 (22%)	7 (14%)	1 (2%)	13 (27%)	7 (14%)	0.162	0.443
ADAM9	8p11.22	5 (28%)	0	1 (6%)	8 (16%)	7 (14%)	3 (6%)	9 (18%)	5 (10%)	4 (8%)	0.426	0.901
IKBKB	8p11.21	0	4 (22%)	0	0	7 (14%)	2 (4%)	0	15 (31%)	1 (2%)	0.252	0.682
PRMD14	8p13.3	0	4 (22%)	0	0	7 (14%)	1 (2%)	3 (6%)	12 (24%)	1 (2%)	0.375	0.817
MTDH	8q22.1	0	3 (17%)	2 (11%)	0	9 (18%)	1 (2%)	0	22 (45%)	1 (2%)	0.018	0.237
MYC	8q24.21	0	4 (22%)	2 (11%)	0	17 (35%)	3 (6%)	3 (6%)	14 (29%)	9 (18%)	0.429	0.137
CCND1	11q13.3	0	7 (39%)	1 (6%)	0	18 (37%)	8 (16%)	0	24 (49%)	9 (18%)	0.166	0.241
C11ORF30	11q13.5	0	1 (6%)	0	2 (4%)	3 (6%)	1 (2%)	5 (10%)	3 (6%)	1 (2%)	0.897	0.814
CDH1	16q22.1	0	4 (22%)	0	1 (2%)	9 (18%)	1 (2%)	2 (4%)	8 (16%)	2 (4%)	0.992	0.618
CPD	17q11.2	1 (6%)	0	0	7 (14%)	0	0	5 (10%)	4 (8%)	0	0.061	.
MED1	17q12	0	2 (11%)	1 (6%)	0	11 (22%)	2 (4%)	1 (2%)	10 (20%)	7 (14%)	0.295	0.148
ERBB2	17q12	0	5 (27%)	1 (6%)	0	7 (14%)	2 (4%)	0	9 (18%)	3 (6%)	0.423	0.857
CDC6	17q21.2	0	1 (6%)	0	1 (2%)	2 (4%)	1 (2%)	6 (12%)	4 (8%)	2 (4%)	0.376	0.532
TOP2A	17q21.2	0	0	0	1 (2%)	2 (4%)	2 (4%)	2 (4%)	5 (10%)	3 (6%)	0.109	0.483
MAPT	17q21.31	0	2 (11%)	0	1 (2%)	4 (8%)	0	1 (2%)	6 (12%)	1 (2%)	0.628	0.486
PPM1D	17q23.2	0	1 (6%)	0	1 (2%)	3 (6%)	2 (4%)	1 (2%)	4 (8%)	2 (4%)	0.715	0.672
BIRC5	17q25.3	0	3 (17%)	0	0	8 (16%)	0	1 (2%)	9 (18%)	2 (4%)	0.682	0.231
CCNE1	19q12	0	0	0	1 (2%)	1 (2%)	0	1 (2%)	3 (6%)	0	0.366	.
AURKA	20q13.2	1 (6%)	0	0	2 (4%)	8 (16%)	1 (2%)	3 (6%)	8 (16%)	2 (4%)	0.166	0.566
Total		7	56	15	27	166	50	45	216	68	0.133	0.012

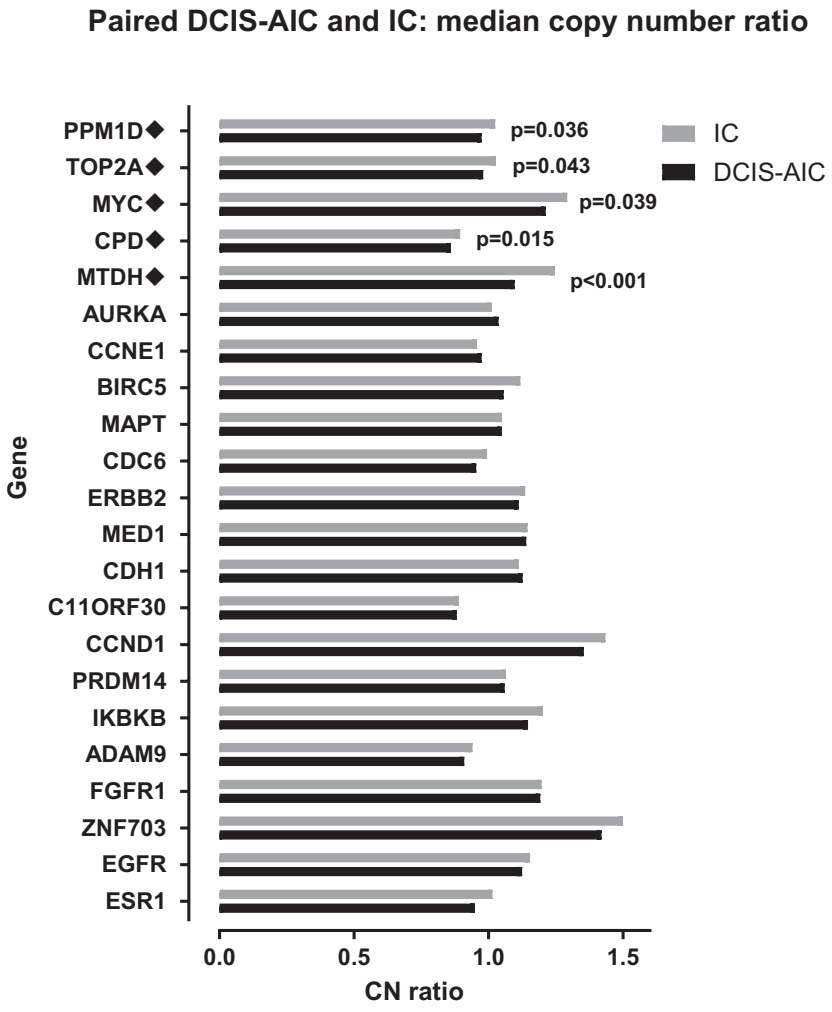

Figure 2. Median copy number ratio for all 22 analyzed genes in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC). Genes with a diamond show a significantly higher copy number ratio in IC.

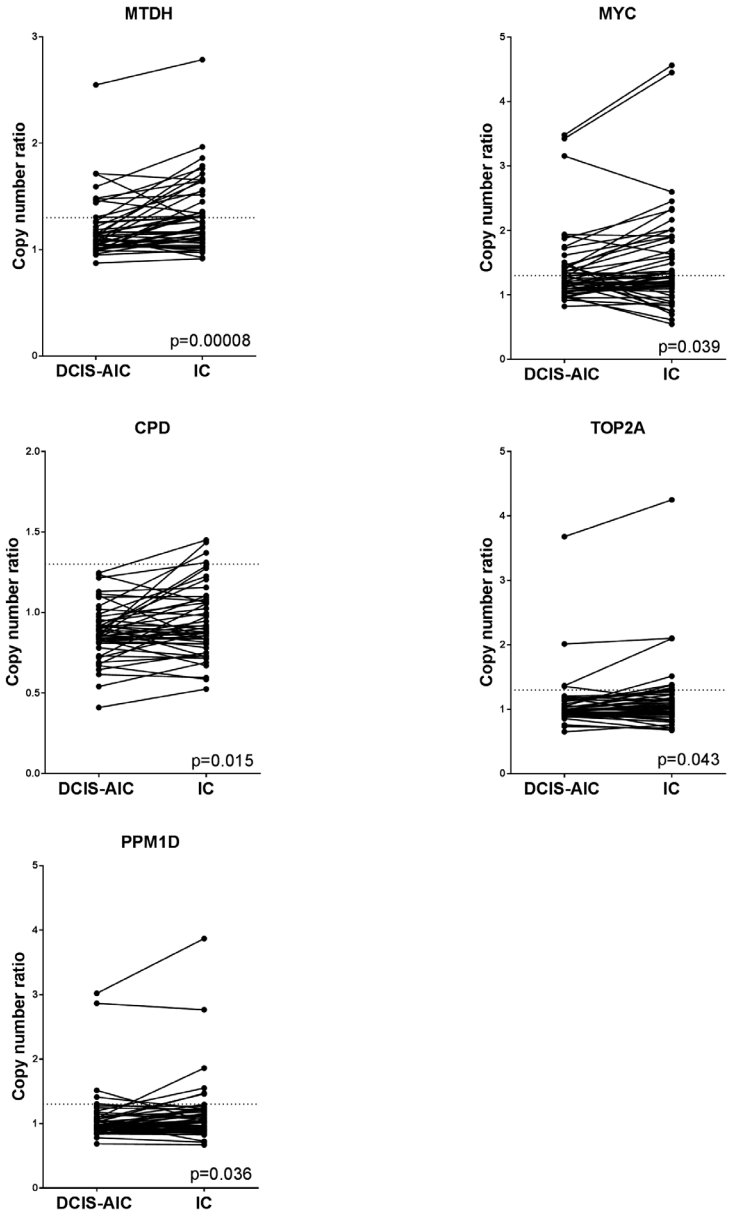

Figure 3. Copy number ratios for *MTDH, MYC, CPD, TOP2A* and *PPM1D* in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC).

In addition, we compared copy number aberration frequencies of 21/22 interrogated genes (*EMSY* data not available) with a large public female breast cancer cohort (METABRIC, www.cbioprtal.org, [33, 34]). Supplementary table 3 shows a high amplification frequency similarity for all genes except for *PRDM14* and *MTDH*, which both showed a difference of at least 10% in amplification frequency, with a higher amplification percentage in the METABRIC population (n=2173).

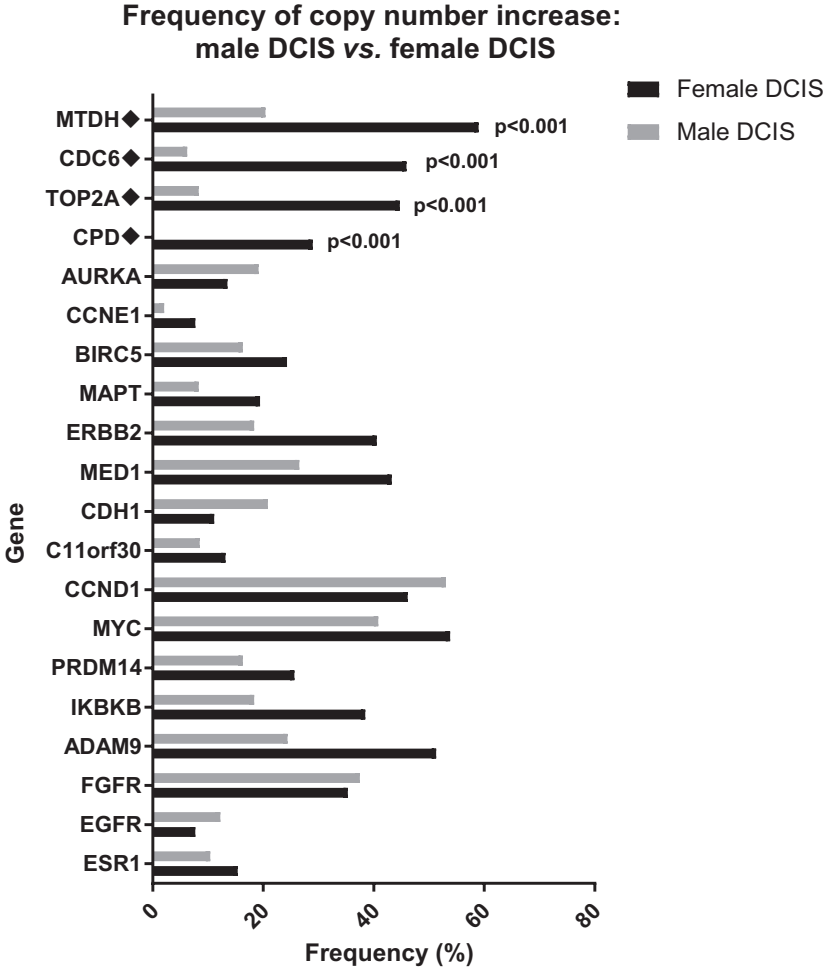

Figure 4. Frequency of copy number increase (cut-off >1.3) in female and male ductal carcinoma *in situ* (DCIS). Genes with a diamond show a significantly higher frequency of copy number gain in female BC.

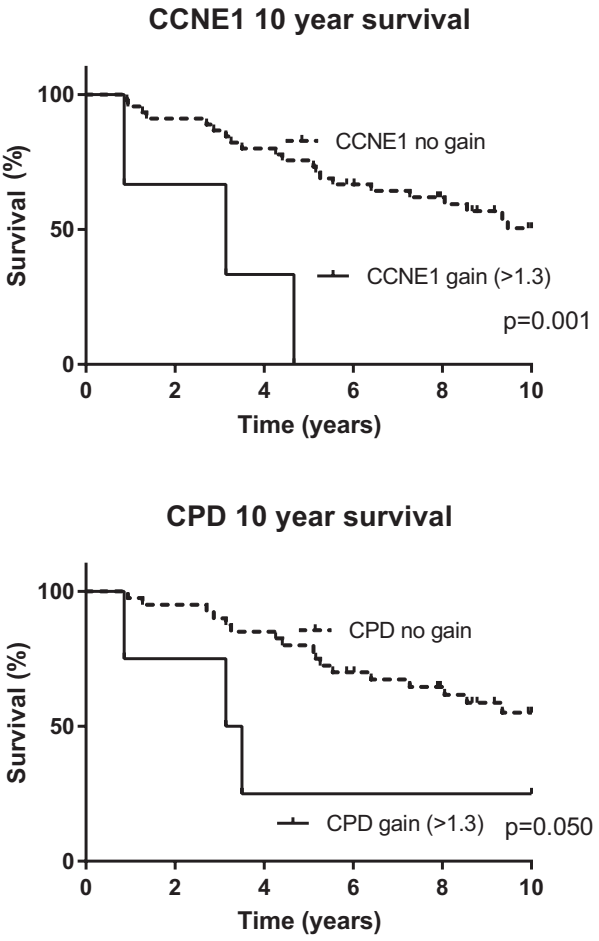

Figure 5. Kaplan-Meier 10-year overall survival plots for *CCNE1* gain and *CPD* gain.

Correlation between copy number alterations and survival

CPD and *CCNE1* gain (no amplifications were observed) in IC were predictors of poor 10 year overall survival (p=0.050 and p=0.001) and remained independent prognosticators when grade, mitoses and age were included in multivariable analysis (p=0.017 (HR 5.1) and p=0.003 (HR 6.9)). Kaplan-Meier curves are presented in Figure 5. None of the other interrogated genes were associated with survival.

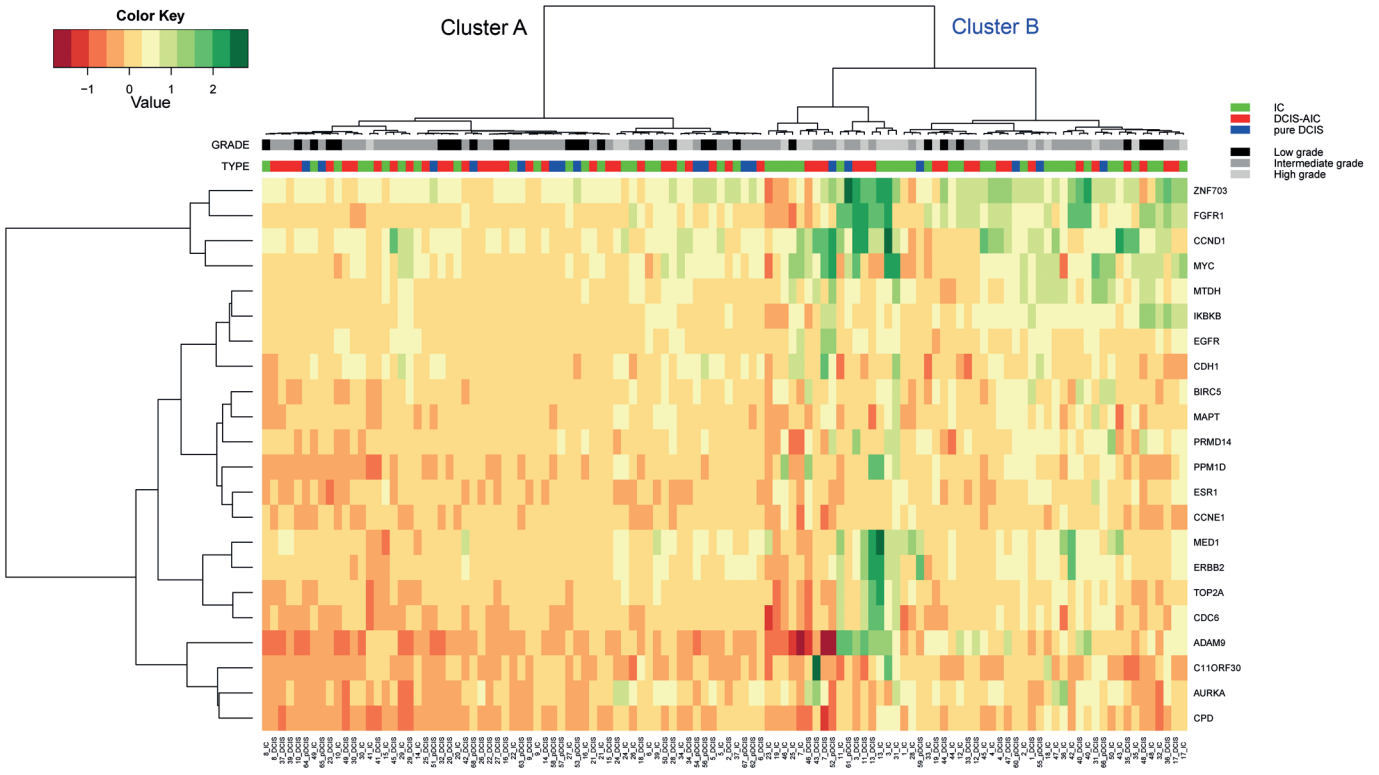

Figure 6. Unsupervised hierarchical cluster analysis of 22 genes in male breast cancer lesions, including pure ductal carcinoma *in situ* (DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC).

Cluster analysis of all male pure DCIS, DCIS-AIC and IC lesions

Unsupervised hierarchical cluster analysis of all pure DCIS, DCIS-AIC and IC showed 2 main clusters that differed significantly according to grade (grade 1/2 vs. grade 3) with more high grade lesions in cluster B (n=29) compared to cluster A (n=20) (p=0.001) (Figure 6). In addition, all genes showed a higher copy number ratio in cluster B. Of the 49 paired DCIS-AIC and IC samples in the cluster analysis, 40 samples (81.6%) were in the same cluster, and of these, 17 pairs (34.7%) clustered closely together indicating that these adjacent *in situ* and invasive components share many genetic alterations.

Discussion

To discover drivers that may control the progression of DCIS to IC, and to establish the precursor role of DCIS in male breast carcinogenesis, we studied copy number status of 22 breast cancer related genes in IC, DCIS-AIC and pure DCIS of the male breast by MLPA. Overall, in line with previous studies on their female counterparts, there were only few copy number differences between male DCIS and IC [12, 15, 16].

Copy number ratios were similar in pure DCIS, DCIS-AIC and IC for most of the studied genes, indicating that copy number gain of the majority of these genes does not seem to play a significant role in the transition from male DCIS to IC. This finding is in line with a previous copy number and gene expression study in female BC [12]. There was however one gene, *MTDH*, that showed a significantly higher copy number ratio and frequency of gain in IC as compared to DCIS-AIC. This implies that gain of *MTDH* could play a role in the progression of DCIS to IC. In a previous MLPA-based male BC study, *MTDH* showed gain/amplification in 46% of the IC samples, similar to our results [26]. *MTDH* is located on chromosome 8 and encodes Metadherin, a transmembrane protein that plays a key role in the activation of several signaling pathways including PI3K/Akt, NFκβ, Wnt/βcatenin and the MAPK pathways [35]. These pathways play a role in cell proliferation, apoptosis, invasion, angiogenesis and metastasis. Metadherin is frequently overexpressed in female BC and overexpression correlates with advanced clinical stage, distant metastasis and an aggressive phenotype [36]. Moelans et. al. compared *MTDH* copy number in 39 paired cases of female DCIS-AIC and IC but found no significant differences in copy number ratio, suggesting that this event may be specific for male breast carcinogenesis [12].

Interestingly, almost all of the analyzed genes showed copy number changes in DCIS, indicating that copy number gain is a relatively early event in male breast carcinogenesis.

Paired analysis of IC and DCIS-AIC samples showed a high concordance of gain/amplification status between individual patients, supported by cluster analysis. This confirms the clonal relation between male DCIS and IC, as has also been accepted in female breast carcinogenesis [12]. CCND1, a cell cycle regulatory protein, showed a high copy number aberration frequency in all three groups with 49% *CCND1* gain and 18% *CCND1* amplification in IC. *CCND1* amplification is more frequent in ER positive and PgR tumors, so these high frequencies can be explained by the high rate of ER positivity (all cases being ER positive) and PR positivity (96% of DCIS-AIC/IC cases and 100% of pure DCIS cases being positive) in our male BC cohort [37].

Several genes showed a higher aberration frequency in high grade lesions compared to low grade lesions (*ESR1*, *PPM1D*, *BIRC5*, *CCNE1*, *PRDM14*, *CDC6*, *TOP2A* and *AURKA* for DCIS-AIC and *ESR1*, *EGFR*, *C11ORF30*, *CDC6* and *PPM1D* for IC). Also, the average copy number ratio was higher in high grade IC compared to low/intermediate grade IC. After correction for multiple comparisons, *BIRC5* copy number ratio and *ESR1* gain in DCIS-AIC and *C11ORF30* gain in IC were significantly higher/more frequent in high grade lesions. Although the sample sizes of high grade DCIS-AIC and high grade IC were small (n=7 and n=14, respectively), this does suggest that tumors with a higher copy number gain have a tendency to have higher histological grade, as previously demonstrated in male BC [26]. *BIRC5* codes for the protein Survivin, a regulatory protein involved in cell proliferation and apoptosis. It has been extensively studied in female BC where an increased expression of Survivin was correlated with a higher risk of recurrence and with a decreased overall survival rate [38, 39]. *ESR1* codes for estrogen receptor alpha, a transcription factor located on chromosome 6q25 and an important therapeutic target in female BC with tamoxifen being the standard endocrine therapy for ER-positive breast cancers [4]. In a previous study using MLPA, *ESR1* amplification and gain were shown in 2% and 6% of 135 female breast tumors, respectively [40]. *C11ORF30* (also known as *EMSY)* is a transcription regulatory protein that can compromise BRCA2 function in sporadic breast cancer and ovarian cancer [41]. In female BC it has been associated with a reduced overall survival in ER-positive patients [42].

Upon comparison of our findings with female BC, a high concordance was evident, especially for IC. For DCIS, 4 genes (*MTDH*, *CPD*, *CDC6* and *TOP2A*) showed a higher frequency of gain in female BC, although no differences in amplification frequency were observed. Copy number aberration frequencies for 21 genes were also compared with a large female breast cancer cohort (METABRIC, www.cbioprtal.org [33, 34]), showing a high amplification frequency similarity.

Two of the 22 studied genes showed a correlation with overall survival. *CCNE1* and *CPD* gain were both indicative of a decreased 10 year overall survival, however, the number

of cases showing gain of these genes (n=3 and n=4, respectively) were small and none of the cases showed amplification. Also, treatment regimens and lymph node status were not known so could not be included in the survival analysis. Therefore, results should be interpreted with caution. High levels of Cyclin E have been described to have prognostic value in female breast cancer, especially as a predictor of endocrine therapy failure [43, 44].

CPD has been investigated in breast cancer cell lines (MCF-7 cells), where prolactin/17β-estradiol induced cell surface *CPD* increased intracellular NO production which increased the survival and inhibited apoptosis [45].

Although a limitation of this study is the relatively small study population, it should be noted that male BC is rare, male DCIS is even rarer, and our DCIS samples have been extracted from a large cohort study, and were enriched for tumor cells by scalpel or laser microdissection. We used MLPA for copy number analysis, a multiplex PCR-based method that simultaneously assesses relative copy numbers of a variety of genes in a quantitative way. The major advantage of this technique is that it requires only minimal amounts of small DNA fragments which makes it very suitable to study small lesions in paraffin-embedded tissue, such as DCIS [1]. The MLPA kit used was pre-designed by the manufacturer, and contains 22 cancer related genes that often show copy number aberrations in female BC [12, 46]. Although there are some genetic differences between male and female BC, we expected the bigger part of these genes to play a role in male breast carcinogenesis as well [46]. We did not include *PIK3CA*, *TP53* and *GATA3*, possible important genomic drivers in female BC and described to be frequently mutated in female BC [47]. In this study we only focused on copy number variations and not on specific mutations.

In conclusion, this MLPA-based study showed a similar copy number status for 21 out of 22 studied breast cancer related genes in male DCIS and IC, illustrating the clonal relation between male DCIS and adjacent IC, and the genetically advanced state of male DCIS. *MTDH* showed a higher copy number ratio and aberration frequency in IC compared to DCIS and could therefore play a role in the transition of male DCIS to IC.

5

References

1. Moelans CB, de Weger RA, van Blokland MT *et al.* Her-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and in situ hybridization. *Cellular oncology : the official journal of the International Society for Cellular Oncology* 2009;**31**;1-10.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the her-2/neu oncogene. *Science (New York, N.Y.)* 1987;**235**;177-182.
3. Hudis CA. Trastuzumab--mechanism of action and use in clinical practice. *The New England journal of medicine* 2007;**357**;39-51.
4. Holst F, Stahl PR, Ruiz C *et al.* Estrogen receptor alpha (esr1) gene amplification is frequent in breast cancer. *Nature genetics* 2007;**39**;655-660.
5. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. *Breast cancer research and treatment* 2012;**136**;331-345.
6. Rodriguez-Pinilla SM, Jones RL, Lambros MB *et al.* Myc amplification in breast cancer: A chromogenic in situ hybridisation study. *Journal of clinical pathology* 2007;**60**;1017-1023.
7. Almeida D, Gerhard R, Leitao D, Davilla C, Damasceno M, Schmitt F. Topoisomerase ii-alfa gene as a predictive marker of response to anthracyclines in breast cancer. *Pathology, research and practice* 2014;**210**;675-679.
8. Turner N, Pearson A, Sharpe R *et al.* Fgfr1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer research* 2010;**70**;2085-2094.
9. Lundgren C, Ahlin C, Holmberg L, Amini RM, Fjallskog ML, Blomqvist C. Cyclin e1 is a strong prognostic marker for death from lymph node negative breast cancer. A population-based case-control study. *Acta oncologica (Stockholm, Sweden)* 2015;**54**;538-544.
10. Holm K, Staaf J, Jonsson G *et al.* Characterisation of amplification patterns and target genes at chromosome 11q13 in ccnd1-amplified sporadic and familial breast tumours. *Breast cancer research and treatment* 2012;**133**;583-594.
11. Hwang ES, DeVries S, Chew KL *et al.* Patterns of chromosomal alterations in breast ductal carcinoma in situ. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004;**10**;5160-5167.
12. Moelans CB, de Weger RA, Monsuur HN, Maes AH, van Diest PJ. Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: A multiplex ligation-dependent probe amplification study. *Analytical cellular pathology (Amsterdam)* 2010;**33**;165-173.
13. Burger H, de Boer M, van Diest PJ, Korsching E. Chromosome 16q loss--a genetic key to the understanding of breast carcinogenesis. *Histology and histopathology* 2013;**28**;311-320.
14. Schymik B, Buerger H, Kramer A *et al.* Is there 'progression through grade' in ductal invasive breast cancer? *Breast cancer research and treatment* 2012;**135**;693-703.
15. Burkhardt L, Grob TJ, Hermann I *et al.* Gene amplification in ductal carcinoma in situ of the breast. *Breast cancer research and treatment* 2010;**123**;757-765.
16. Aubele M, Mattis A, Zitzelsberger H *et al.* Extensive ductal carcinoma in situ with small foci of invasive ductal carcinoma: Evidence of genetic resemblance by cgh. *Int J Cancer* 2000;**85**;82-86.

17. Sanders ME, Schuyler PA, Dupont WD, Page DL. The natural history of low-grade ductal carcinoma in situ of the breast in women treated by biopsy only revealed over 30 years of long-term follow-up. *Cancer* 2005;**103**;2481-2484.
18. Doebar SC, Sieuwerts AM, de Weerd V, Stoop H, Martens JWM, van Deurzen CHM. Gene expression differences between ductal carcinoma in situ with and without progression to invasive breast cancer. *The American journal of pathology* 2017;**187**;1648-1655.
19. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015;**65**;5-29.
20. Pappo I, Wasserman I, Halevy A. Ductal carcinoma in situ of the breast in men: A review. *Clinical breast cancer* 2005;**6**;310-314.
21. Leonard GD, Swain SM. Ductal carcinoma in situ, complexities and challenges. *J Natl Cancer Inst* 2004;**96**;906-920.
22. Ge Y, Sneige N, Eltorky MA *et al.* Immunohistochemical characterization of subtypes of male breast carcinoma. *Breast cancer research : BCR* 2009;**11**;R28.
23. Anderson WF, Jatoi I, Tse J, Rosenberg PS. Male breast cancer: A population-based comparison with female breast cancer. *J Clin Oncol* 2010;**28**;232-239.
24. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
25. Kornegoor R, Verschuur-Maes AH, Buerger H *et al.* Molecular subtyping of male breast cancer by immunohistochemistry. *Mod Pathol* 2012;**25**;398-404.
26. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Promoter hypermethylation in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research : BCR* 2012;**14**;R101.
27. Cardoso F, Bartlett J, Slaets L *et al.* Characterization of male breast cancer: First results of the eortc10085/tbcrc/big/nabcg international male bc program. *Cancer Research 75(9 Supplement):S6-05-S6-05*, 2015.
28. Vermeulen MA, Slaets L, Cardoso F *et al.* Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England : 1990)* 2017.
29. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;**19**;403-410.
30. Holland R, Peterse JL, Millis RR *et al.* Ductal carcinoma in situ: A proposal for a new classification. *Seminars in diagnostic pathology* 1994;**11**;167-180.
31. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;**11**;155-168.
32. Wolff AC, Hammond ME, Hicks DG *et al.* Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of american pathologists clinical practice guideline update. *J Clin Oncol* 2013;**31**;3997-4013.
33. Pereira B, Chin SF, Rueda OM *et al.* The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. *Nature communications* 2016;**7**;11479.
34. Curtis C, Shah SP, Chin SF *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012;**486**;346-352.
35. Shi X, Wang X. The role of mtdh/aeg-1 in the progression of cancer. *International journal of clinical and experimental medicine* 2015;**8**;4795-4807.
36. Tokunaga E, Nakashima Y, Yamashita N *et al.* Overexpression of metadherin/mtdh is associated with an aggressive phenotype and a poor prognosis in invasive breast cancer. *Breast cancer (Tokyo, Japan)* 2014;**21**;341-349.

37. Reis-Filho JS, Savage K, Lambros MB *et al*. Cyclin d1 protein overexpression and ccnd1 amplification in breast carcinomas: An immunohistochemical and chromogenic in situ hybridisation analysis. *Mod Pathol* 2006;**19**;999-1009.
38. Li Y, Ma X, Wu X, Liu X, Liu L. Prognostic significance of survivin in breast cancer: Meta-analysis. *The breast journal* 2014;**20**;514-524.
39. Davis LM, Harris C, Tang L *et al*. Amplification patterns of three genomic regions predict distant recurrence in breast carcinoma. *The Journal of molecular diagnostics : JMD* 2007;**9**;327-336.
40. Moelans CB, de Wegers RA, Monsuurs HN, Maess AH, van Diest PJ. Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: A multiplex ligation-dependent probe amplification study. *Cellular oncology (Dordrecht)* 2011;**34**;475-482.
41. Hughes-Davies L, Huntsman D, Ruas M *et al*. Emsy links the brca2 pathway to sporadic breast and ovarian cancer. *Cell* 2003;**115**;523-535.
42. Kirkegaard T, Nielsen KV, Jensen LB *et al*. Genetic alterations of ccnd1 and emsy in breast cancers. *Histopathology* 2008;**52**;698-705.
43. Span PN, Tjan-Heijnen VC, Manders P, Beex LV, Sweep CG. Cyclin-e is a strong predictor of endocrine therapy failure in human breast cancer. *Oncogene* 2003;**22**;4898-4904.
44. Keyomarsi K, Tucker SL, Buchholz TA *et al*. Cyclin e and survival in patients with breast cancer. *The New England journal of medicine* 2002;**347**;1566-1575.
45. Abdelmagid SA, Too CK. Prolactin and estrogen up-regulate carboxypeptidase-d to promote nitric oxide production and survival of mcf-7 breast cancer cells. *Endocrinology* 2008;**149**;4821-4828.
46. Moelans CB, de Weger RA, Monsuur HN, Vijzelaar R, van Diest PJ. Molecular profiling of invasive breast cancer by multiplex ligation-dependent probe amplification-based copy number analysis of tumor suppressor and oncogenes. *Mod Pathol* 2010;**23**;1029-1039.
47. Comprehensive molecular portraits of human breast tumours. *Nature* 2012;**490**;61-70.

Supplementary Table 1 Contents of the P078-C1 MLPA kit. The gene (n=22), chromosomal position, mapview position, number of probes, transcript description and references are given.

Gene	Chromosome	Mapview position	No. of probes	Transcript description	Ref
ESR1	6q25.1	06-152.423833 06-152.457203	2	Nuclear transcription factor	(Holst et al. 2007; Ooi, et al. 2012)
EGFR	7p11.2	07-055.233952 07-055.191054	2	Receptor tyrosine kinase involved in signal transduction	(Masuda et al. 2012; Park, et al. 2007)
ZNF703	8p11.23	08-037.672630 08-037.675173	2	Transcription factor	(Sircoulomb, et al. 2011; Spellman and Gray 201
FGFR1	8p11.22	08-038.434093 08-038.391534	2	Receptor tyrosine kinase involved in signal transduction	(Turner et al. 2010)
ADAM9	8p11.22	08-038.993938	1	Metalloproteinase	(Mazzocca, et al. 2005)
IKBKB	8p11.21	08-042.292897 08-042.302668	2	Serine threonine kinase associated with signal transduction	(Chin, et al. 2006)
PRMD14	8p13.3	08-071.130073	1	Transcriptional regulatory protein	(Nishikawa, et al. 2007)
MTDH	8q22.1	08-098.742494 08-098.788083	2	Transcription co-activator	(Shi and Wang 2015)
MYC	8q24.21	08-128.822147 08-128.817868	2	Transcription factor involved in cell proliferation and apoptosis	(Rodriguez-Pinilla et al. 2007)
CCND1	11q13.3	11-069.175090 11-069.167778	2	Cell cycle regulatory protein involved in signal transduction	(Holm et al. 2012)
C11ORF30	11q13.5	11-075.902087 11-075.926543	2	Transcription regulatory protein	(Hou, et al. 2014; Kirkegaard et al. 2008)
CDH1	16q22.1	16-067.404849 16-067.328707	2	Cellular adhesion molecule	(Cleton-Jansen 2002)
CPD	17q11.2	17-025.795018	1	Carboxypeptidase involved in protein metabolism	(Abdelmagid and Too 2008)
MED1	17q12	17-034.840858	1	Transcription regulatory protein	(Cui, et al. 2012; Nagalingam, et al. 2012)
ERBB2	17q12	17-035.118096 17-035.122165 17-035.136342 17-035.127182	4	Receptor tyrosine kinase involved in signal transduction	(Hudis 2007)
CDC6	17q21.2	17-035.699283	1	Cell cycle control protein	(Gonzalez, et al. 2006; Petrakis, et al. 2012)
TOP2A	17q21.2	17-035.818297 17-035.812698 17-035.816651	3	DNA topoisomerase protein	(Almeida et al. 2014)
MAPT	17q21.31	17-041.423082	1	Structural protein involved in microtubule assembly and stabilization	(Baquero, et al. 2011)
PPM1D	17q23.2	17-056.055700	1	Serine threonine phosphatase	(Lambros, et al. 2010)
BIRC5	17q25.3	17-073.722036 17-073.724340 17-073.722396	3	Regulatory protein involved in cell proliferation and apoptosis	(Davis et al. 2007)
CCNE1	19q12	19-035.005212 19-035.000150	2	Cell cycle regulatory protein involved in signal transduction	(Lundgren et al. 2015)
AURKA	20q13.2	20-054.391595 20-054.381824	2	Serine threonine kinase involved in regulation of cell cycle progression	(Staff, et al. 2010)

Supplementary Table 3 Comparison of copy number aberration frequencies between invasive male breast cancer and a female breast cancer cohort (N=2173, METABRIC).

				METABRIC	MALE BC	METABRIC	MALE BC
Gene	**N**	**Missing**	**Total**	**gain (%)**	**gain (%)**	**amplification (%)**	**amplification (%)**
ESR1	2509	336	2173	6,44	10,0	2,30	0,0
EGFR	2509	336	2173	14,13	16,0	2,39	2,0
ZNF703	2509	336	2173	12,10	51,0	14,27	20,0
FGFR1	2509	336	2173	12,47	27,0	13,12	14,0
ADAM9	2509	336	2173	12,33	10,0	11,23	8,0
IKBKB	2509	336	2173	15,14	31,0	10,26	2,0
PRDM14	2509	336	2173	21,58	24,0	15,74	2,0
MTDH	2509	336	2173	22,46	45,0	19,24	2,0
MYC	2509	336	2173	22,09	29,0	25,49	18,0
CCND1	2509	336	2173	11,18	49,0	16,29	18,0
EMSY	2509	2509	0		6,0		2,0
CDH1	2509	336	2173	2,95	16,0	0,18	4,0
CPD	2509	336	2173	8,10	8,0	2,76	0,0
MED1	2509	336	2173	8,51	20,0	11,92	14,0
ERBB2	2509	336	2173	8,42	18,0	15,74	6,0
CDC6	2509	336	2173	8,65	8,0	6,53	4,0
TOP2A	2509	336	2173	8,74	10,0	4,92	6,0
MAPT	2509	336	2173	8,70	12,0	1,10	2,0
PPM1D	2509	336	2173	14,17	8,0	10,08	4,0
BIRC5	2509	336	2173	16,57	18,0	5,71	4,0
CCNE1	2509	336	2173	7,87	6,0	2,81	0,0
AURKA	2509	336	2173	20,66	16,0	6,35	4,0

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6

Promoter hypermethylation in ductal carcinoma *in situ* of the male breast

Marijn A Vermeulen, Carolien HM van Deurzen, Shusma C Doebar, WWJ de Leng, John WM Martens, Paul J van Diest, Cathy B Moelans

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Abstract

Ductal carcinoma *in situ* (DCIS) of the male breast is very rare and has hardly been studied molecularly. In males, we compared methylation status of 25 breast cancer-related genes in pure DCIS (n = 18) and invasive breast carcinoma (IBC) with adjacent DCIS (DCIS-AIC) (n = 44) using methylation-specific multiplex ligation-dependent probe amplification. Results were compared to female breast cancer (BC). There were no significant differences in methylation features between male pure DCIS, DCIS-AIC and IBC after correction for multiple comparisons. In paired analysis of IBC and adjacent DCIS, *CADM1* showed a significantly higher absolute methylation percentage in DCIS (p = 0.002). In cluster analysis, two clusters stood out with respectively infrequent and frequent methylation (*GATA5*, *KLLN*, *PAX6*, *PAX5*, *CDH13*, *MSH6* and *WT1* were frequently methylated). Compared to female DCIS, methylation was in general much less common in male DCIS, especially for *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11*. In contrast, *THBS1* and *GATA5* were more frequently methylated in male DCIS. In conclusion, there is frequent methylation of *GATA5*, *KLLN*, *PAX6*, *PAX5*, *CDH13*, *MSH6* and *WT1* in male DCIS. Since there was little change in the methylation status for the studied genes from pure male DCIS to DCIS-AIC and IBC, methylation of these seven genes is more likely to occur early in male breast carcinogenesis. Based on the current markers male DCIS seems to be an epigenetically more advanced precursor of male BC, although in comparison to its female counterpart it appears that fewer loci harbor methylation, pointing to differences between male and female breast carcinogenesis with regard to the studied loci.

Introduction

Male breast cancer (BC) is a rare disease accounting for approximately 1% of all breast cancers [1]. According to the American Cancer Society an estimated 2670 men will develop breast cancer in the United States in 2019, and of these men approximately 5% will be diagnosed with pure DCIS making this entity even more rare [1, 2]. Because of the low prevalence of this disease, it has been studied to a far lesser extent than its female counterpart. Therapy is still mostly extrapolated from female BC clinical trials, although there is evidence that male BC differs from female BC on several levels. For example, male BC is diagnosed at older age, men have more advanced disease at the time of diagnosis and there are differences in the distribution of histologic subtypes and molecular characteristics, for instance regarding gene amplification and epigenetic alterations [3-8].

Epigenetic changes, including DNA methylation, play an essential role in carcinogenesis besides established players such as mutations, copy number changes and genetic rearrangements. DNA methylation is a process in which DNA methyltransferase enzymes (DNMTs) add a methyl group to the 5'-carbon of cytosine in the context of cytosine guanine dinucleotides (CpG sites), a process which is associated with gene silencing, thereby causing inactivation of tumor suppressor genes [9, 10]. This epigenetic event is of great interest because it is a mark which can be reversed by epigenetic modifiers such as DNMT inhibitors and therefore, targeting epigenetically silenced tumor suppressor genes can be considered as a therapeutic option in cancer [10]. In female BC, using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), frequent promoter hypermethylation of *PAX6*, *BRCA2*, *PAX5*, *WT1*, *CDH13* and *MSH* has been described in both DCIS and invasive breast carcinoma (IBC) [11]. Using the same technique, prognostic value of methylation status conversion of *SFRP4-1* and *HLTF-2* and promoter hypermethylation of *ID4-2*, *SFRP4-1* and *DAPK1* in female BC metastases has been suggested [12]. In male BC, promoter hypermethylation was frequent for *MSH6*, *WT1*, *PAX5*, *CDH13*, *GATA5* and *PAX6* and a high overall methylation status of 25 interrogated genes was correlated with poor survival in a group of 108 male BC patients [7]. Because not every DCIS lesion progresses to invasive breast carcinoma (IBC) and accurately predicting DCIS behavior is still not possible, it is important to identify drivers that control progression to IBC so treatment strategies can be optimized [13].

The aim of this study was to investigate male breast carcinogenesis by, for the first time, assessing the methylation status of 25 breast cancer-related genes in male DCIS, and comparing methylation in DCIS adjacent to IBC (DCIS-AIC) in 44 patients to that

in male pure DCIS in 18 patients using MS-MLPA. We also correlated methylation in paired and unpaired DCIS and IBC samples, and compared our results to previous MS-MLPA data on female DCIS, using a similar kit [11].

Materials and Methods

Patients

Unstained slides of formalin fixed paraffin embedded (FFPE) tissue blocks containing DCIS with adjacent IBC (n=51) or containing pure DCIS (n=20) were collected from a previously selected large male BC cohort [14, 15]. This subgroup was selected based on availability of a tumor tissue block for central pathology review and sufficient tissue for DNA isolation using the unstained slides available to us. Unfortunately, no tissue block or unstained slides containing only normal breast tissue from the same patient was available. Patient and tumor characteristics including age at diagnosis and hormone receptor status (estrogen receptor alpha (ERα), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) were recorded. Data concerning *BRCA1/2* testing was not available. According to the Dutch legislation, use of anonymous left-over material for scientific purposes is allowed without informed consent if patients have been informed and given the opportunity to opt out, which is part of the standard treatment contract with patients in our hospital. Since we used anonymized archival pathology material which does not interfere with patient care and does not imply the physical involvement of the patient, our study is exempt from ethical approval and informed consent [16].

Hematoxylin and eosin (H&E) slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IBC according to the World Health Organization and the modified Bloom and Richardson score [17]. ERα, PgR and HER2 were evaluated using immunohistochemistry and scored according to the Allred-score and the ASCO-CAP guidelines [18, 19]. The areas of interest (pure DCIS, DCIS-AIC and IBC as well as normal tissue in present) were dissected either manually with a sterile scalpel or by laser capture microdissection, using a Zeiss PALM MD3 laser microdissection system, from 5 consecutive sections (4μm) of FFPE tissue blocks. Laser capture microdissection was done in cases with only small areas of DCIS or with abundant inflammatory cells surrounding the area of interest (n=7). DNA was extracted by overnight incubation in lysis buffer with proteinase K (10 mg/ml; Roche; Almere; The Netherlands) at 56ºC, followed by boiling for 10 minutes and centrifugation.

Results from a previous female BC study evaluating methylation status of DCIS and adjacent IBC (N=33) using a similar MS-MLPA kit were used to compare 23 genes between female and male DCIS [11].

Clinicopathological data of all analyzed male breast cancer cases (IBC, DCIS-AIC and pure DCIS) are shown in table 1. Hormone receptor status showed perfect concordance (100%) between DCIS and adjacent IBC.

Table 1. Baseline characteristics of investigated male invasive breast carcinomas, DCIS adjacent to invasive breast carcinoma (IBC) and pure ductal carcinoma *in situ*.

	IBC	**Adjacent DCIS**	***Pure* DCIS**
	n=44	n=44	n=18
Age (years)			
Mean (range)	63.3 (37-85)	63.2 (37-85)	62.6 (37-76)
Histologic subtype IC			
Ductal type carcinoma	41 (93.2 %)		
Mucinous carcinoma	1 (2.3 %)		
Micropapillary carcinoma	1 (2.3 %)		
Mixed type			
Ductal/micropapillary	1 (2.3%)		
Grade			
1	14 (31.8 %)	11 (25.0 %)	3 (17 %)
2	19 (43.2 %)	27 (61.4 %)	14 (78 %)
3	11 (25.0 %)	6 (13.6 %)	1 (6.0 %)
ER			
Positive	44 (100 %)	44 (100 %)	18 (100 %)
Negative	0 (0 %)	0 (0 %)	0 (0%)
PR			
Positive	42 (95.5 %)	42 (95.5 %)	18 (100 %)
Negative	2 (4.5 %)	2 (4.5 %)	0 (0%)
HER2			
Positive	1 (2.3 %)	1 (2.3 %)	1 (5.6 %)
Negative	43 (97.7 %)	43 (97.7 %)	17 (94.4 %)

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

MS-MLPA was performed according to the manufacturer's instructions on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA) using the ME002-C1 kit (MRC Holland, Amsterdam, The Netherlands), containing 25 tumor suppressor genes (supplementary table 1). The ME002-C1 kit was chosen based on in-house experience and literature that shows an association between these genes and breast cancer, concerning frequent methylation and prognosis/survival (e.g. *PAX6* [11]; *BRCA1* [20]; *ATM* [21]; *TP73* [22]; *WT1* [11, 23]; *GSTP1* [20, 24]; *CADM1* [25]; *RARB* [20, 22, 24]). The principle of MS-MLPA has been described elsewhere [26]. In summary, MS-MLPA uses probes that are designed for quantifying methylation. These probes contain a restriction site that is recognized by the methylation sensitive HhaI enzyme. After incubation of two tubes (containing the same DNA sample) with the probemix, only one of the tubes is incubated with HhaI. This tube will only generate a probe amplification product after PCR if the CpG site is methylated. Using the ratio between the signal, incubated with and without HhaI, the level of methylation can be calculated. Appropriate negative and positive controls were taken along each MS-MLPA run.

The PCR products were separated by capillary electrophoresis using a 3730 DNA analyzer (Applied Biosystems). Methylation analysis was performed using GeneScan analysis (Applied Biosystems) and Coffalyser Software (MRC-Holland, version 13.1) after the data of each sample was normalized by dividing the signal of each probe by the signal of all reference probes (not degraded by HhaI). Then, the ratio of the relative probe peaks of the undigested sample (not containing HhaI) and the corresponding digested sample (containing HhaI) was calculated for each probe to obtain the final methylation status. A promoter methylation ratio above 0.15 (corresponding to >15% methylation) was considered as promoter hypermethylation, based on previous studies [7, 11]. The mean methylation index (MMI) was defined as the number of methylated genes divided by the number of genes tested.

Statistics

Statistical calculations were done using SPSS for Windows version 21.0. MMI in pure DCIS and DCIS-AIC was compared using the Mann-Whitney test (unpaired data) and in DCIS-AIC and IBC using the Wilcoxon signed ranks test (paired data). Individual gene comparison was done using the Mann-Whitney test (absolute methylation percentages) and Chi-square test (dichotomized data) in pure DCIS and DCIS-AIC, and using the Wilcoxon signed ranks test (absolute methylation percentages) and McNemar test (dichotomized data) in paired DCIS-AIC and IBC.

The differences between female and male DCIS and differences in histologic grades in DCIS-AIC and IBC were compared using Mann-Whitney and Chi-square tests for absolute methylation percentages and dichotomized data, respectively. P-values below 0.05 were considered significant and correction for multiple comparisons was done using the Holm-Bonferroni method. Finally, unsupervised hierarchical clustering (Euclidian distance method) of absolute methylation percentages was performed using the statistical program R (www.r-project.org) for male cases (female cases previously described [11]).

Results

Methylation status of male DCIS and IBC

18 cases of pure DCIS and 44 cases of DCIS-AIC and matched IBC had sufficient DNA-yield for further analysis. Using absolute methylation percentages, *CADM1* showed a significantly higher methylation percentage in DCIS-AIC (mean 12.3%) compared to IBC (mean 9.0%) (paired analysis, p=0.002) and significance remained after correction for multiple comparisons. Absolute methylation percentages of *PAX5* and *CDH13* were significantly higher in DCIS-AIC (mean 28.1% and 29.0%, respectively) compared to pure DCIS (20.0% and 18.0%, respectively) (p=0.018 for both). These findings however, lost their significance after correction for multiple comparisons.

Using the 15% threshold, no significant differences were found for individual genes between pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC in dichotomized unpaired analysis. In paired analysis of IBC and DCIS-AIC (n=44) using this 15% threshold, *MGMT* showed a significantly higher percentage of methylated cases in IBC (25% of cases) compared to DCIS-AIC (9.1% of cases) (p=0.039), but significance was lost after correction for multiple comparisons. In this analysis *CADM1* showed no significant difference in number of cases with >15% methylation between DCIS-AIC and IDC (p=0.219).

Table 2 shows methylation frequencies for all 25 interrogated genes. Figure 1 illustrates the methylation frequencies in male DCIS and IBC. There was no significant difference in the number of methylated genes between pure DCIS and DCIS-AIC (p=0.402) and between DCIS-AIC and IBC (p=0.205), all having an average of 7 methylated genes. The number of methylated genes varied between 2-10 for pure DCIS, 1-11 for DCIS-AIC and 3-13 for IBC. The MMI was 0.26 for pure DCIS, 0.28 for DCIS-AIC and 0.30 for IBC (p=0.240 and p=0.205 for pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC, respectively).

Table 2. Comparison of percentage of methylated cases (cut-off value 15%) of the 25 analyzed genes in pure ductal carcinoma *in situ* (DCIS), DCIS with adjacent invasive carcinoma (DCIS-AIC) and the adjacent invasive breast carcinoma (IBC) in males.

Gene	Hypermethylation (%)			Chromosome
	pure DCIS	DCIS-AIC	IBC	
MSH6	100	98	100	2p16.3
KLLN	89	91	89	10q23.31
GATA5	83	75	89	20q13.33
WT1	78	75	82	11p13
PAX5	44	68	75	9p13.2
THBS1	72	57	64	15q14
CDH13	39	70	64	16q23.3
PAX6	22	36	43	11p13
GSTP1	11	32	41	11q13.2
MGMT	22	9	25	10q26.3
TP53	22	27	18	17p13.1
TP73	11	16	14	1p36.32
ESR1	6	5	9	6q25.1
CD44	0	7	9	11p13
CADM1	11	16	7	11q23.3
RARB	11	2	5	3p24.2
BRCA2	11	5	2	13q13.1
PYCARD	6	2	2	16p11.2
VHL	0	0	0	3p25.3
CDKN2A	6	0	0	9p21.3
ATM	0	0	0	11q22.3
CHFR	0	0	0	12q24.33
RB1	0	0	0	13q14.2
BRCA1	0	0	0	17q21.31
STK11	6	0	0	19p13.3

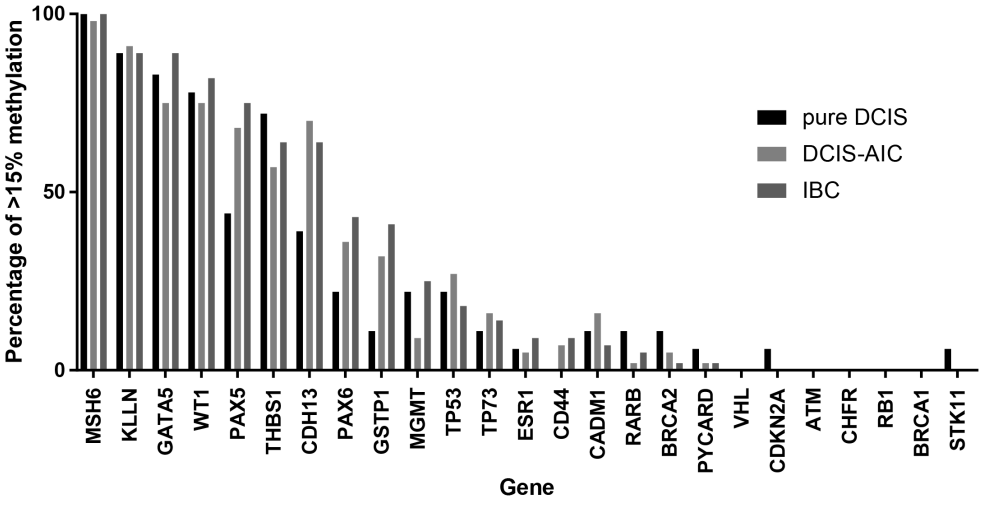


Figure 1. Percentage of cases showing >15% methylation concerning 25 breast cancer related genes in pure ductal carcinoma *in situ* (DCIS), DCIS with adjacent invasive carcinoma (DCIS-AIC) and the adjacent invasive breast carcinoma (IBC) in males.

Correlation of methylation with tumor grade

The percentage of methylated cases and absolute methylation percentages were compared between low/intermediate grade and high grade DCIS-AIC and between low/intermediate grade and high grade IBC. *PAX6* and *CDH13* showed higher absolute methylation percentages in high grade DCIS-AIC, but there was no significance after correction for multiple comparisons (p=0.025 and p=0.033, respectively). No significant differences in percentage of methylated cases (binary) were found for these genes between low/intermediate grade and high grade DCIS-AIC.

In IBC the genes *PAX5*, *PAX6*, *GSTP1*, *ATM* and *GATA5* showed a similar pattern but were also not significantly different after correction for multiple comparisons (p=0.004, p=0.005, p=0.047, p=0.036, p=0.038, respectively). Of these genes, only *PAX6* showed a significant difference in percentage of methylated cases between low/intermediate grade and high grade IBC (p=0.04), which was also lost after correction for multiple comparisons.

The average number of methylated genes was not significantly different between grades for DCIS-AIC or IBC after correction for multiple comparisons.

Cluster analysis

Figure 2 shows the results of unsupervised hierarchical cluster analysis of 18 interrogated genes in all pure DCIS, DCIS-AIC and IBC cases. The 18 genes that showed the highest spread in absolute methylation percentage were included in the cluster analysis. This was done to prevent that less variable genes may blur and influence the results. There were 3 recognizable gene clusters. One cluster (cluster A) consisted of *GATA5, KLLN*, *PAX5*, *CDH13*, *MSH6, PAX6* and *WT1*, all frequently methylated (>43% hypermethylated cases in IBC). A second cluster (cluster B) was formed by genes that were less frequently methylated, varying between 2-25% methylated cases in IBC and consisted of *MGMT, TP73*, *TP53*, *BRCA2*, *ESR1, CADM1, THBS1, CD44,* and *RARB*. A small cluster (cluster C) only consisting of *GSTP1* and *PYCARD* showed variable levels of methylation, with averages of 41% and 2% hypermethylated IBC cases, respectively. For male BC cases two large clusters (cluster I and cluster II) could be identified with 20 IBC cases in cluster I and 24 IBC cases in cluster II. 32 DCIS-AIC with corresponding IBC cases clustered together in cluster I and cluster II. Age, Mitotic Activity Index (MAI) and grade were not significantly different between IBC in these two clusters.

Comparison of methylation status between male and female DCIS

Methylation was much less common in male DCIS-AIC for a variety of genes. After correction for multiple comparisons, the genes *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11* showed a significantly lower absolute methylation percentage in male DCIS-AIC (all p-values equal or smaller than 0.002). *THBS1, MSH6* and *GATA5* showed significantly higher absolute methylation percentages ($p<0.001$, $p<0.001$ and $p=0.001$, respectively).

Of the genes showing significance in absolute methylation percentage analysis, *ESR1*, *BRCA2* and *STK11* also showed a significant lower percentage of methylated cases in male DCIS-AIC compared to female DCIS-AIC ($p=0.002$, $p<0.001$ and $p<0.001$, respectively) and *THBS1* and *GATA5* a higher percentage of methylated cases in male DCIS-AIC compared to female DCIS-AIC ($p=0.002$ and $p=0.002$, respectively). Figure 3 illustrates the percentage of methylated cases of the same 23 interrogated genes (identical CpG sites and technique) in male and female DCIS-AIC.

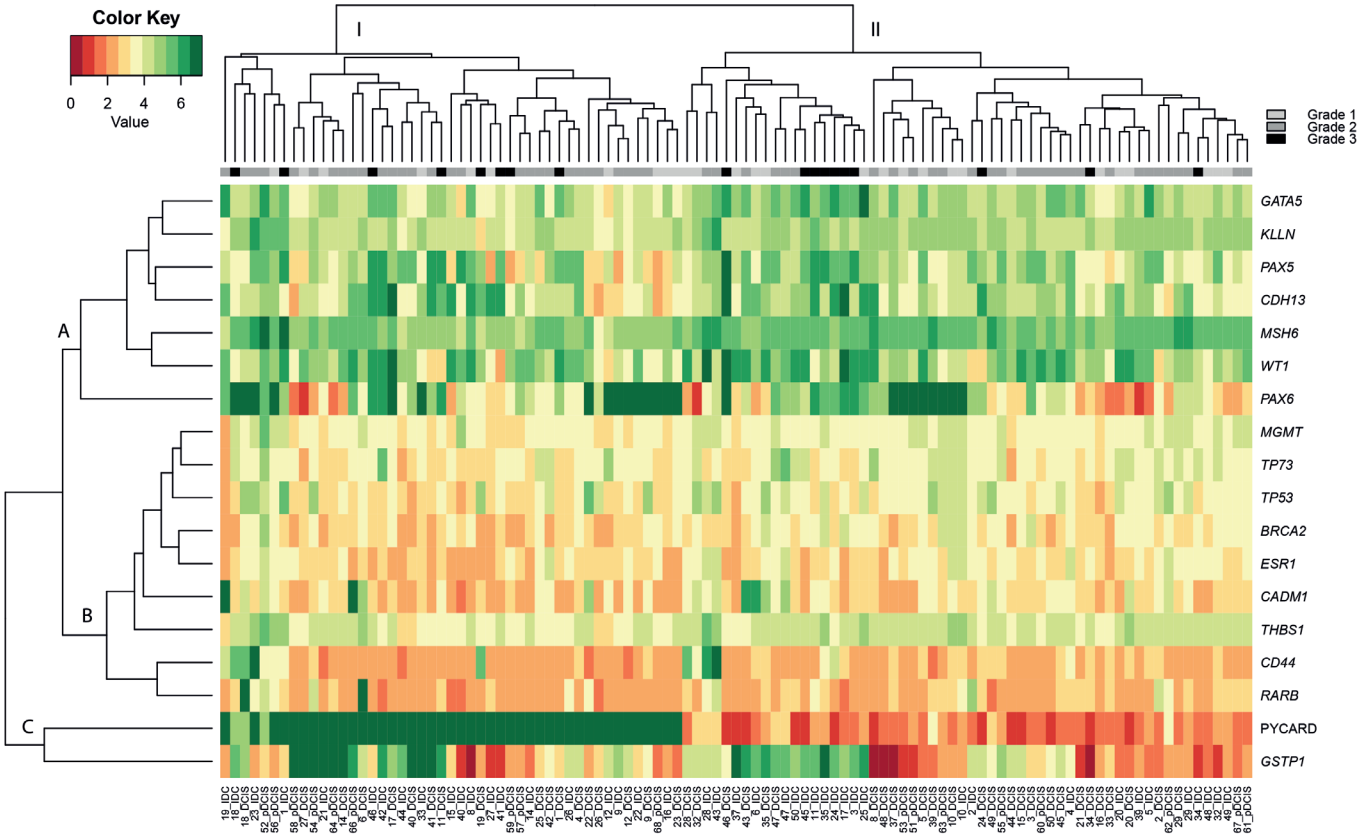


Figure 2. Hierarchical cluster analysis showing 3 gene clusters and 2 clusters of cases in pure ductal carcinoma *in situ*, ductal carcinoma *in situ* adjacent to invasive carcinoma and invasive carcinoma of the male breast and 2 clusters of BC cases.

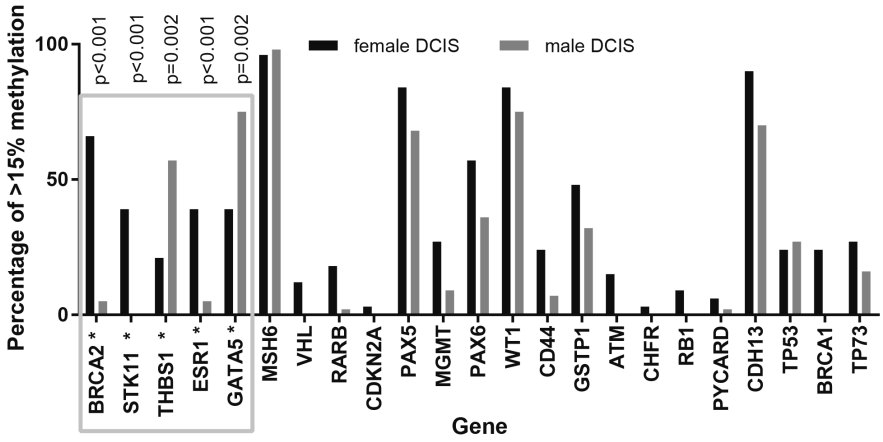


Figure 3. Percentage of cases showing >15% methylation concerning 22 breast cancer related genes in male and female ductal carcinoma *in situ* (DCIS). Genes with an asterisk were significantly different for both the absolute methylation percentage and percentage of methylated cases, between male and female DCIS after correction for multiple comparisons. P-values for methylation status are shown.

Discussion

To investigate the role of promoter hypermethylation in male BC during tumor progression, we compared the methylation status of 25 breast cancer related tumor suppressor genes between 18 cases of pure DCIS and 44 cases of DCIS-AIC, and between these DCIS-AIC cases and paired IBC using MS-MLPA. In addition to the absolute methylation percentages, we used dichotomized data with a cut-off of 15% methylation as established in previous studies.

For methylation detection, multiple methods are available such as Methylation-Specific PCR (MSP) or Quantitative Multiplex MSP (QM-MSP). We chose MS-MLPA as this is a robust, inexpensive and easy-to-perform technique. In comparison to MSP it uses methylation-sensitive HhaI enzyme instead of sodium bisulfite conversion of unmethylated cytosine residues which is known to be a difficult part in the process to standardize and can lead to DNA degradation [27, 28]. In addition, MS-MLPA has shown good correlation with QM-MSP in a study comparing different techniques in breast cancer [29-31].

Most IBCs were ductal carcinoma NOS and most tumors were grade 2, consistent with literature [7, 14]. Using absolute methylation percentages, promoter methylation in pure DCIS vs DCIS-AIC and DCIS-IBC vs IBC was overall not significantly different after correction for multiple comparisons. Using the 15% threshold there was no significant difference between pure DCIS vs DCIS-AIC and DCIS-AIC vs IBC in the number of methylated tumor suppressor genes. Previous female BC studies showed similar methylation levels in DCIS and IBC, in line with our findings [11, 32, 33]. In the current study *CADM1* had a significantly higher absolute methylation percentage in DCIS-AIC compared to the adjacent IBC, although the percentage of methylated cases using the 15% threshold was not significantly different. *CADM1* (cell adhesion molecule 1) is a tumor suppressor gene located at chromosome 11q23.2 and encodes a membrane glycoprotein that plays a role in cell adhesion. It has been described to be frequently inactivated in various tumors such as non-small cell lung carcinoma [34]. In breast cancer, *CADM1* promoter methylation was found in 23/50 (46%) primary breast cancers using pyrosequencing [35]. Lower methylation levels in more advanced lesions, such as IBC compared to DCIS, may be explained by the reversibility of hypermethylation and tumor heterogeneity, and has previously been described in female BC, where *CADM1* showed a significantly lower methylation frequency using a 15% cut-off in breast cancer metastases compared to the primary tumor [12].

In at least 60% of both DCIS-AIC and IBC lesions methylation was high for *KLLN*, *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5*, genes that also clustered together in our cluster analysis, together with *PAX6*. Of these genes, *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5* were shown to be frequently methylated (>50%) in a previous male BC study and *WT1*, *CDH13*, *MSH6* and *PAX5* in a previous female BC study [7, 11]. *KLLN* was not included in the MS-MLPA kit used in these previous studies. This indicates that methylation of these genes, especially *WT1*, *CDH13*, *MSH6*, *GATA5* and *PAX5*, could play an important role in BC, and specifically in male breast carcinogenesis. Methylation of these genes seems to be an early event in breast carcinogenesis as we saw no significant differences between DCIS and IBC.

A limitation of this study is that we were unable to analyze the normal breast tissue in the samples. Only 6 samples had enough surrounding normal tissue left, which was mostly fat and in close proximity to the abnormal tissue. DNA concentration of these samples was not sufficient and MS-MLPA results were therefore not reliable. MS-MLPA analysis on normal male breast tissue was done in the previous male BC study by Kornegoor et. al., where normal male breast tissue (N=10) derived from autopsies

showed low methylation levels in 25 genes. Only *MSH6*, *ESR1*, *PAX5* and *CDH13* showed methylation (40%, 20%, 10% and 10%, respectively) [7]. This finding supports the assumption that methylation of several genes is an early event and that these genes are not likely to be silenced in normal male breast cancer tissue. Genes that were rarely methylated in our study were *PYCARD*, *CHFR* and *CDKN2A*, of which the latter two also showed low methylation (<2%) in the male BC study by Kornegoor et al. [7]. We did not compare protein expression by immunohistochemistry to MS-MLPA results since previous studies have shown only weak concordance between immunohistochemistry and methylation [28, 30, 31, 36].

In this study we observed several differences between low/intermediate and high grade IBC, and between low/intermediate and high grade DCIS-AIC. In high grade IBC, genes *PAX5*, *PAX6*, *GSTP1*, *ATM* and *GATA5* showed a higher absolute methylation frequency and a higher percentage of cases showing *PAX6* methylation was seen in high grade IBC. In DCIS-AIC, *PAX6* and *CDH13* showed a higher absolute methylation percentage in high grade lesions. These findings could indicate that methylation of these genes may be important in the development of these high grade lesions.
Compared to females, methylation levels for *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11* were significantly lower in male DCIS-AIC after correction for multiple comparisons. In addition, *ESR1*, *BRCA2* and *STK11* also showed a significantly lower methylation frequency in male versus female DCIS-AIC.
Estrogen receptor α (ERα) is a transcription factor encoded by *ESR1*. Activation of ERα plays a role in cell proliferation and differentiation in various cells [37]. In breast cancer, ERα-negativity is associated with a poor response to endocrine therapy and a poor prognosis [38].

In female BC similar methylation levels have been described between DCIS and IBC [11]. Our finding of lower methylation levels of ESR1 in male DCIS compared to female DCIS is in line with previous findings in male IBC [7]. This finding points to possible differences in male and female breast carcinogenesis concerning promoter methylation.
BRCA2 encodes for the protein BRCA2 and is involved in DNA repair [39]. The lower *BRCA2* methylation levels in male versus female DCIS may be explained by the higher rate of *BRCA2* mutation carriers in the male BC population. *BRCA2* mutations occur in 4-40% of male BC and in approximately 3.7% of female BC [40, 41]. *BRCA1/2* methylation as a "second hit" has been described to be an infrequent event in *BRCA1/2* mutation carriers, although a recent systematic review by Vos et al. could not support this assumption [42-44]. Unfortunately, no data regarding *BRCA2* mutation

status were available. Germline mutations in *STK11*, encoding LKB1, are associated with Peutz-Jeghers syndrome, a syndrome associated with a 24-54% lifetime risk of developing breast cancer [45]. In sporadic breast cancer *STK11* mutations are rare but hypermethylation of this tumor suppressor gene has been described in papillary breast carcinomas [46].

THBS1, *GATA5* and *MSH6* showed higher methylation levels in male DCIS-AIC compared to females, of which the former two (*THBS1* and *GATA5*) also showed a higher methylation status in men. *THBS1* encodes TSP-1 (thrombospondin 1), a glycoprotein that plays a role in angiogenesis inhibition, leading to suppression of tumor growth [47]. In the male BC study by Kornegoor et.al., no significant differences were found in *THBS1* methylation between male and female BC, so these results should be interpreted with caution and should be confirmed in a larger male BC cohort [7].
GATA factors are a family of transcription regulatory proteins and *GATA4*, *GATA5* and *GATA6* regulate development of endoderm derived organs. Loss of *GATA5* expression due to promoter hypermethylation has been reported in a variety of cancers, such as colorectal, gastric, lung and ovarian cancer [48-51]. In colorectal cancer *GATA5* has been described as an important tumor suppressor gene that is hypermethylated early during carcinogenesis [48].
In conclusion, there is relatively frequently methylation of *GATA5*, *KLLN*, *PAX5*, *CDH13*, *MSH6* and *WT1* in male DCIS. Since there was little difference in methylation status for common tumor suppressor genes from pure male DCIS to DCIS-AIC and IBC, methylation of these 6 genes could play an important early role in male breast carcinogenesis. Male DCIS seems to be a genetically advanced precursor of male breast cancer, although it seems to harbor less methylation than its female counterpart, pointing to differences in male and female breast carcinogenesis.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;**69**;7-34.
2. Pappo I, Wasserman I, Halevy A. Ductal carcinoma in situ of the breast in men: A review. *Clinical breast cancer* 2005;**6**;310-314.
3. Hill TD, Khamis HJ, Tyczynski JE, Berkel HJ. Comparison of male and female breast cancer incidence trends, tumor characteristics, and survival. *Annals of epidemiology* 2005;**15**;773-780.
4. Anderson WF, Jatoi I, Tse J, Rosenberg PS. Male breast cancer: A population-based comparison with female breast cancer. *J Clin Oncol* 2010;**28**;232-239.
5. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
6. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Oncogene amplification in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research and treatment* 2012;**135**;49-58.
7. Kornegoor R, Moelans CB, Verschuur-Maes AH *et al.* Promoter hypermethylation in male breast cancer: Analysis by multiplex ligation-dependent probe amplification. *Breast cancer research : BCR* 2012;**14**;R101.
8. Lacle MM, Kornegoor R, Moelans CB *et al.* Analysis of copy number changes on chromosome 16q in male breast cancer by multiplex ligation-dependent probe amplification. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2013;**26**;1461-1467.
9. Plass C. Cancer epigenomics. *Human molecular genetics* 2002;**11**;2479-2488.
10. Connolly R, Stearns V. Epigenetics as a therapeutic target in breast cancer. *Journal of mammary gland biology and neoplasia* 2012;**17**;191-204.
11. Moelans CB, Verschuur-Maes AH, van Diest PJ. Frequent promoter hypermethylation of brca2, cdh13, msh6, pax5, pax6 and wt1 in ductal carcinoma in situ and invasive breast cancer. *The Journal of pathology* 2011;**225**;222-231.
12. Schrijver WA, Jiwa LS, van Diest PJ, Moelans CB. Promoter hypermethylation profiling of distant breast cancer metastases. *Breast cancer research and treatment* 2015;**151**;41-55.
13. Sanders ME, Schuyler PA, Dupont WD, Page DL. The natural history of low-grade ductal carcinoma in situ of the breast in women treated by biopsy only revealed over 30 years of long-term follow-up. *Cancer* 2005;**103**;2481-2484.
14. Vermeulen MA, Slaets L, Cardoso F *et al.* Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England : 1990)* 2017.
15. Cardoso F, Bartlett JMS, Slaets L *et al.* Characterization of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018;**29**;405-417.
16. Wendler D. One-time general consent for research on biological samples. *BMJ (Clinical research ed.)* 2006;**332**;544-547.
17. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;**19**;403-410.
18. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;**11**;155-168.

19. Wolff AC, Hammond ME, Hicks DG *et al.* Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of american pathologists clinical practice guideline update. *J Clin Oncol* 2013;**31**;3997-4013.
20. Sharma G, Mirza S, Yang YH *et al.* Prognostic relevance of promoter hypermethylation of multiple genes in breast cancer patients. *Cellular oncology : the official journal of the International Society for Cellular Oncology* 2009;**31**;487-500.
21. Ye C, Cai Q, Dai Q *et al.* Expression patterns of the atm gene in mammary tissues and their associations with breast cancer survival. *Cancer* 2007;**109**;1729-1735.
22. Marzese DM, Hoon DS, Chong KK *et al.* DNA methylation index and methylation profile of invasive ductal breast tumors. *The Journal of molecular diagnostics : JMD* 2012;**14**;613-622.
23. Gupta S, Joshi K, Wig JD, Arora SK. High frequency of loss of allelic integrity at wilms' tumor suppressor gene-1 locus in advanced breast tumors associated with aggressiveness of the tumor. *Indian journal of cancer* 2009;**46**;303-310.
24. Cho YH, Shen J, Gammon MD *et al.* Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. *Breast cancer research and treatment* 2012;**131**;197-205.
25. Heller G, Geradts J, Ziegler B *et al.* Downregulation of tslc1 and dal-1 expression occurs frequently in breast cancer. *Breast cancer research and treatment* 2007;**103**;283-291.
26. Nygren AO, Ameziane N, Duarte HM *et al.* Methylation-specific mlpa (ms-mlpa): Simultaneous detection of cpg methylation and copy number changes of up to 40 sequences. *Nucleic acids research* 2005;**33**;e128.
27. Moelans CB, Atanesyan L, Savola SP, van Diest PJ. Methylation-specific multiplex ligation-dependent probe amplification (ms-mlpa). *Methods Mol Biol* 2018;**1708**;537-549.
28. Jeuken JW, Cornelissen SJ, Vriezen M *et al.* Ms-mlpa: An attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of mgmt promoter hypermethylation in gliomas. *Lab Invest* 2007;**87**;1055-1065.
29. Suijkerbuijk KP, Pan X, van der Wall E, van Diest PJ, Vooijs M. Comparison of different promoter methylation assays in breast cancer. *Anal Cell Pathol (Amst)* 2010;**33**;133-141.
30. Lopez F, Sampedro T, Llorente JL *et al.* Utility of ms-mlpa in DNA methylation profiling in primary laryngeal squamous cell carcinoma. *Oral Oncol* 2014;**50**;291-297.
31. Furlan D, Sahnane N, Mazzoni M *et al.* Diagnostic utility of ms-mlpa in DNA methylation profiling of adenocarcinomas and neuroendocrine carcinomas of the colon-rectum. *Virchows Archiv : an international journal of pathology* 2013;**462**;47-56.
32. Verschuur-Maes AH, de Bruin PC, van Diest PJ. Epigenetic progression of columnar cell lesions of the breast to invasive breast cancer. *Breast cancer research and treatment* 2012;**136**;705-715.
33. Park SY, Kwon HJ, Lee HE *et al.* Promoter cpg island hypermethylation during breast cancer progression. *Virchows Archiv : an international journal of pathology* 2011;**458**;73-84.
34. Fukami T, Fukuhara H, Kuramochi M *et al.* Promoter methylation of the tslc1 gene in advanced lung tumors and various cancer cell lines. *International journal of cancer* 2003;**107**;53-59.
35. Takahashi Y, Iwai M, Kawai T *et al.* Aberrant expression of tumor suppressors cadm1 and 4.1b in invasive lesions of primary breast cancer. *Breast cancer (Tokyo, Japan)* 2012;**19**;242-252.
36. Mao X, Qiao Z, Fan C, Guo A, Yu X, Jin F. Expression pattern and methylation of estrogen receptor alpha in breast intraductal proliferative lesions. *Oncology reports* 2016;**36**;1868-1874.
37. Lin Z, Reierstad S, Huang CC, Bulun SE. Novel estrogen receptor-alpha binding sites and estradiol target genes identified by chromatin immunoprecipitation cloning in breast cancer. *Cancer research* 2007;**67**;5017-5024.

38. Duffy MJ, Harbeck N, Nap M *et al.* Clinical use of biomarkers in breast cancer: Updated guidelines from the european group on tumor markers (egtm). *European journal of cancer (Oxford, England : 1990)* 2017;**75**;284-298.
39. Xia B, Sheng Q, Nakanishi K *et al.* Control of brca2 cellular and clinical functions by a nuclear partner, palb2. *Mol Cell* 2006;**22**;719-729.
40. Ottini L, Palli D, Rizzo S, Federico M, Bazan V, Russo A. Male breast cancer. *Critical reviews in oncology/hematology* 2010;**73**;141-155.
41. King MC, Marks JH, Mandell JB. Breast and ovarian cancer risks due to inherited mutations in brca1 and brca2. *Science (New York, N.Y.)* 2003;**302**;643-646.
42. Dworkin AM, Spearman AD, Tseng SY, Sweet K, Toland AE. Methylation not a frequent "second hit" in tumors with germline brca mutations. *Familial cancer* 2009;**8**;339-346.
43. Vos S, van Diest PJ, Moelans CB. A systematic review on the frequency of brca promoter methylation in breast and ovarian carcinomas of brca germline mutation carriers: Mutually exclusive, or not? *Critical reviews in oncology/hematology* 2018;**127**;29-41.
44. Vos S, Moelans CB, van Diest PJ. Brca promoter methylation in sporadic versus brca germline mutation-related breast cancers. *Breast cancer research : BCR* 2017;**19**;64.
45. Beggs AD, Latchford AR, Vasen HF *et al.* Peutz-jeghers syndrome: A systematic review and recommendations for management. *Gut* 2010;**59**;975-986.
46. Esteller M, Avizienyte E, Corn PG *et al.* Epigenetic inactivation of lkb1 in primary tumors associated with the peutz-jeghers syndrome. *Oncogene* 2000;**19**;164-168.
47. Ren B, Yee KO, Lawler J, Khosravi-Far R. Regulation of tumor angiogenesis by thrombospondin-1. *Biochim Biophys Acta* 2006;**1765**;178-188.
48. Hellebrekers DM, Lentjes MH, van den Bosch SM *et al.* Gata4 and gata5 are potential tumor suppressors and biomarkers in colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2009;**15**;3990-3997.
49. Akiyama Y, Watkins N, Suzuki H *et al.* Gata-4 and gata-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Molecular and cellular biology* 2003;**23**;8429-8439.
50. Guo M, Akiyama Y, House MG *et al.* Hypermethylation of the gata genes in lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004;**10**;7917-7924.
51. Peters I, Gebauer K, Dubrowinskaja N *et al.* Gata5 cpg island hypermethylation is an independent predictor for poor clinical outcome in renal cell carcinoma. *Oncology reports* 2014;**31**;1523-1530.

Supplementary table 1. Contents of the the ME002-C1 kit (MRC Holland, Amsterdam, The Netherlands), containing 25 tumor suppressor genes.

Gene	Chromosome	Mapview position	Length (nt)	Gene name
BRCA1	17q21.31	17-038.530922	140	Breast Cancer 1, Early Onset
BRCA2	13q13.1	13-031.787801	148	Breast Cancer 2, Early Onset
*ATMb**	11q22.4	11-107.598881	161	ATM Serine/Threonine Kinase
TP53	17p13.1	17-007.531511	167	Tumour protein P53
KLLN	10q23.31	10-089.613066	183	Killin
*MGMTa**	10q26.2	10-131.155095	191	O-6-Methylguanine-DNA Methyltransferase
PAX5	9p13.2	09-037.024651	211	Paired box 5
CDH13	16q23.3	16-081.218154	219	Cadherin 13, H-cadherin
TP73	1p36.32	01-003.559219	240	Tumour protein P73
WT1	11p13	11-032.413841	247	Wilms Tumor 1
VHL1	3p25.3	03-010.158544	265	Von Hippel-Lindau
GSTP1	11q13.2	11-067.108109	274	Glutathione S-Transferase Pi 1
CHFR	12q24.33	12-131.974372	293	Checkpoint With Forkhead And Ring Finger Domains
ESR1	6q25.1	06-152.170607	301	Estrogen receptor 1
*RB1b**	13q14.2	13-047.775508	319	Retinoblastoma 1
MSH6	2p16.3	02-047.863865	328	MutS homologue 6
*MGMTb**	10q26.3	10-131.155600	346	O-6-Methylguanine-DNA Methyltransferase
THBS1	15q14	15-037.660496	355	Thrombospondin 1
CADM1	11q23.3	11-114.880363	364	Cell adhesion molecule 1
STK11	19p13.3	19-001.157467	382	Serine/Threonine Kinase 11
PYCARD	16p11.2	16-031.121292	398	PYD and CARD Domain Containing
PAX6	11p13	11-031.789463	409	Paired box 6
*ATMa**	11q22.3	11-107.660311	418	ATM Serine/Threonine Kinase
CDKN2A	9p21.3	09-021.984268	425	Cyclin-dependent kinase inhibitor 2A
GATA5	20q13.33	20-060.484610	434	GATA Binding Protein 5
RARB	3p24.2	03-025.444383	453	Retinoic acid receptor beta
CD44	11p13	11-035.116991	462	CD44 Molecule (Indian blood group)
*RB1a**	13q14.2	13-047.775703	472	Retinoblastoma 1

7

Summarizing discussion

Summarizing discussion

Male breast cancer is a rare disease, that accounts for approximately 1% of all breast cancers [1]. Approximately 5% of these cases are diagnosed as ductal carcinoma *in situ* (DCIS) and 95% as invasive breast cancer (BC), making DCIS of the male breast very uncommon [2]. Due to its low prevalence, dedicated male breast cancer research is challenging, and only few studies have been able to include a large cohort. Studies on male breast cancer that have been performed have shown similarities with female breast cancer but also differences on several levels but, as a consequence of the limited number of studies, male breast cancer management is largely extrapolated from female breast cancer studies. Therefore, there is an urgent need for more research in this field in order to improve risk stratification and optimize patient management.

For the present thesis we included patients from the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program, a large male BC cohort [3, 4]. One study included all enrolled and analyzed male patients and the other studies included a (Dutch) subgroup of this initial population. The male patients in The Netherlands were identified through the Dutch Cancer Registry, and paraffin embedded male BC tissue was retrospectively collected by the Dutch Breast Cancer Research Group (BOOG). Results of the different studies from this thesis are summarized in this chapter, along with a general discussion.

Female BC has been extendedly studied and various histopathological characteristics with prognostic value have been described, such as histological grade, the presence of a fibrotic focus (a hypoxia related phenomenon) and density of tumor infiltrating lymphocytes (TILs) [5-12]. The prognostic significance of these features in male BC remained unclear [3, 13-15]. In **chapter 2** we therefore studied these features in 1483 male BC cases and correlated these with clinical outcome. The median age was 68.4 years and the most frequent histological subtype was invasive carcinoma of No Special Type (86.6%), formerly known as invasive ductal carcinoma not otherwise specified. Only 1.4% of the cases were invasive lobular carcinomas. Carcinomas were graded as grade 1 (21.8%), grade 2 (50.1%) or grade 3 (28.1%). Although overall histological grade was not significantly correlated with relapse free survival (RFS, p=0.099) or overall survival (OS, p=0.129), a high mitotic count (≥8 mitoses/2mm^2) was associated with unfavorable outcome. A fibrotic focus, present in 32% of cases, was associated with reduced RFS and OS. The majority of patients had either a minimal or mild density of TILs (25.4% and 60.3%, respectively). Remaining cases were scored as moderate (12.5%) or severe (1.8%). We found an improved outcome in patients with a higher density of TILs. The presence of lymphovascular invasion was not significantly correlated to prognosis.

When scoring the hematoxylin & eosin stained slides of part of the male BC cases described in chapter 2, the lack of elastosis in these cases caught our eye. This was a remarkable finding, as the majority of male breast cancer is estrogen receptor alpha (ERα) positive, and elastosis is a very common phenomenon in female breast cancer and related to ERα expression. Therefore, we decided to quantify the amount of elastosis in a similar 4-tiered scoring system (EG0-EG3) as described previously [16]. In **chapter 3** we scored 117 male BC ERα-positive cases that were available to us and correlated the findings to 135 ERα-positive female breast cancer cases. The latter were collected at the department of pathology of the University Medical Center Utrecht between 2017 and 2018. Despite all tumors being ERα-positive, male BC showed any degree of elastosis in only 26/117 (22.2%) cases with no cases showing EG3 (the highest elastosis grade), while female BC cases showed any elastosis in 89/135 cases (65.9%) with 21.5% showing EG3 ($p < 0.001$). This difference retained significance in multivariate logistic regression correcting for confounders. In male BC cases no significant correlations were found between the amount of elastosis and age, grade, mitotic activity index, and progesterone receptor (PgR). In addition, in univariate and multivariate survival analysis no significant prognostic value of elastosis was seen.
As elastosis and ERα expression are correlated in female BC, presence of elastosis can be used as surrogate tissue biomarker for ERα expression [16-20]. As this correlation does not seem to be present in male BC, elastosis seems a less useful surrogate tissue biomarker than in female BC.

Hypoxia is an important condition that occurs when there is a mismatch between oxygen supply and oxygen consumption [21, 22], awarded with the 2019 Nobel Prize for Medicine. In order to survive, tumor cells can adapt to hypoxia through several signaling pathways, with the key regulator being HIF-1α that shows overexpression when hypoxia occurs [23, 24]. This overexpression leads to up-regulation of CAIX and Glut-1 [23].
In female BC, expression of these markers has been described and correlated with decreased OS, high risk of metastases and higher histological grade, indicating that the hypoxia response contributes to the formation of a more aggressive form of BC [25-27]. Although the hypoxia response had been previously investigated in male invasive BC, no data on male DCIS were yet available [15]. In female BC, the hypoxia response seems to already be triggered in the stage of DCIS but if this is the same for men was still unanswered [26, 28]. So, in **chapter 4** we focused on the immunohistochemical expression of HIF-1α, CAIX and Glut-1 as hypoxia markers in male DCIS (n=76) and male invasive carcinoma (n=58) to determine the role of hypoxia in male breast carcinogenesis.

HIF-1α, CAIX and Glut-1 overexpression were not significantly different between pure DCIS, DCIS adjacent to invasive carcinoma and invasive carcinoma. Expression of hypoxia related proteins was seen around necrosis in a little over 1/3 of DCIS cases, and often coincided with expression in adjacent invasive carcinoma when present. The findings indicate that the hypoxia response is likely a genuine carcinogenetic event in the pre-invasive DCIS stage and not a late bystander. This potentially offers new therapeutic options for prevention of invasive male BC.

Breast development and progression is a multi-step process involving accumulation of DNA alterations and epigenetic changes. Oncogene amplification is one of the important events in this process with a well-known example being amplification of the human epidermal growth factor receptor 2 (*HER2*) in female BC [29]. In female BC, similar levels of gene amplification in DCIS and adjacent invasive carcinoma have been found, indicating that these genes play an early role in breast carcinogenesis, but not in the progression from DCIS to invasive carcinoma [30-32]. In male BC this had not previously been studied to our knowledge. In **chapter 5** we used multiplex ligation-dependent probe amplification (MLPA) to investigate DNA copy number changes of 22 BC related genes in a group of male invasive carcinoma with adjacent DCIS (n=49) and in a group of male pure DCIS (n=18). We correlated copy number aberrations with clinicopathological features and survival and compared our results to a previous female BC study [32]. Overall, copy number ratio and aberration frequency including all 22 genes showed no significant difference between pure DCIS, DCIS adjacent to invasive carcinoma and invasive carcinoma, illustrating their clonal relation and the genetically advanced state of male DCIS. *MTDH* showed a higher copy number ratio in invasive carcinoma compared to adjacent DCIS and pure DCIS (p=0.009 and p=0.038, respectively) and may therefore play a role in male breast carcinogenesis. Differences were detected between male and female DCIS for 4 genes (*MTDH*, *CPD*, *CDC6* and *TOP2A)*, pointing to differences in breast carcinogenesis between the sexes.

Another relevant event in the multi-step process of breast carcinogenesis is promotor hypermethylation. This is a process in which DNA methyltransferase enzymes (DNMTs) add a methyl group to the 5'-carbon of cytosine in the context of cytosine guanine dinucleotides (CpG sites), associated with gene silencing and thereby causing inactivation of tumor suppressor genes [33, 34]. Targeting these epigenetically silenced tumor suppressor genes is considered a therapeutic option in cancer [34]. In female BC frequent promoter hypermethylation of *PAX6*, *BRCA2*, *PAX5*, *WT1*, *CDH13* and *MSH* has been described in both DCIS and invasive carcinoma [35]. Using methylation-specific

MLPA (MS-MLPA) in **chapter 6**, we compared methylation status of 25 BC-related genes in male pure DCIS (n=18) and male invasive carcinoma with adjacent DCIS (n=44) and compared results to female BC. *GATA5, KLLN*, *PAX6, PAX5*, *CDH13*, *MSH6* and *WT1* were frequently methylated and there was little change in the methylation status for these 7 genes from pure male DCIS to DCIS-AIC and invasive BC, indicating that methylation of these genes is likely to occur early in male breast carcinogenesis. Compared to female DCIS, methylation was in general much less common in male DCIS, especially for *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* and *STK11*, pointing to differences between male and female breast carcinogenesis with regard to the studied loci.

Overall conclusion

In this thesis, we describe for the first time the improved outcome of male BC patients with a higher density of TILs, we describe paucity of elastosis in ERα-positive BC and the potentially important role of hypoxia in male breast carcinogenesis. In addition, we have identified several oncogenes and tumor suppressor genes that may play a role the progression of male DCIS to invasive BC. Thereby, this thesis has contributed to understanding of male breast carcinogenesis and progression, and has identified potential new therapeutic options.

Future perspectives

As male BC is rare, research dedicated to this disease is best carried out in an international network. One of these networks was launched in 2006, called the 'International Male Breast Cancer Program'. This is a collaboration of the European Organization for Research and Treatment of Cancer (EORTC) in collaboration with the Translational Breast Cancer Research Consortium (TBCRC), the North American Breast Cancer Group (NABCG) and the Breast International Group (BIG). This program consists of three parts: a retrospective collection of male BC treated in participating centers over 20 years, for whom centralized clinical information and tumor samples were collected (part I); a prospective registry of newly diagnosed cases during a period of approximately 30 months, with clinical data and tumor samples (part II); and prospective clinical studies to optimize the management of these patients (part III) [3]. Part I has been completed and published, together with a study describing precursor lesions in this cohort and our study, described in chapter 1 [3, 36]. The results of the second and third part of this program will probably be a leap forward in optimizing treatment strategies for male BC patients.

Although quite a number of studies have pointed to the differences hidden behind the similarities in male breast cancer, treatment regimens are still mostly extrapolated from female breast cancer studies. Our thesis is in line with previous studies showing differences between male and female breast cancer on several levels. Although our thesis shows that DCIS is as genetically advanced as the invasive carcinoma counterpart in comparison to female BC regarding hypoxia response, copy number aberrations and promotor hypermethylation, male BC (including DCIS) should probably be treated differently than female BC. Future studies, in line with ours, could investigate protein expression of several important genes, as we did not have resources to do this.

The differences between male and female BC will probably have therapeutic consequences in the future and also for pathologists, likely there will be changes in diagnosing and grading the tumors. One aspect is the finding in chapter 2 of the absence of prognostic value for histological grade. This is an important finding, as male BC is still graded according to the modified Bloom and Richardson grading system that is also used for female BC [12]. The absence of prognostic value could potentially be explained by using OS instead of breast cancer-specific survival or by the adjuvant treatment, which was not standardized in our study. Nevertheless, a modified grading system for male BC could be developed if our results were to be confirmed by others, perhaps more focusing on proliferation related features, hypoxia related proteins and/or TILs.

The differential importance of elastosis in male BC is biologically interesting and deserves to be further studied by stromal gene signatures and RNA sequencing.

Further molecular clues to understand male breast carcinogenesis may be derived from studies on earlier precursors than DCIS, but these are rare and difficult to identify [37]. At the other end of the spectrum, there have been no studies on distant male BC metastases yet which would be interesting.

7

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;**69**;7-34.
2. Pappo I, Wasserman I, Halevy A. Ductal carcinoma in situ of the breast in men: A review. *Clinical breast cancer* 2005;**6**;310-314.
3. Cardoso F, Bartlett JMS, Slaets L *et al.* Characterization of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Annals of oncology : official journal of the European Society for Medical Oncology* 2018;**29**;405-417.
4. Vermeulen MA, Slaets L, Cardoso F *et al.* Pathological characterisation of male breast cancer: Results of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *European journal of cancer (Oxford, England : 1990)* 2017.
5. Thomas JS, Kerr GR, Jack WJ *et al.* Histological grading of invasive breast carcinoma--a simplification of existing methods in a large conservation series with long-term follow-up. *Histopathology* 2009;**55**;724-731.
6. Schwartz AM, Henson DE, Chen D, Rajamarthandan S. Histologic grade remains a prognostic factor for breast cancer regardless of the number of positive lymph nodes and tumor size: A study of 161 708 cases of breast cancer from the seer program. *Archives of pathology & laboratory medicine* 2014;**138**;1048-1052.
7. Cutuli B, Le-Nir CC, Serin D *et al.* Male breast cancer. Evolution of treatment and prognostic factors. Analysis of 489 cases. *Critical reviews in oncology/hematology* 2010;**73**;246-254.
8. Salgado R, Denkert C, Demaria S *et al.* The evaluation of tumor-infiltrating lymphocytes (tils) in breast cancer: Recommendations by an international tils working group 2014. *Annals of oncology : official journal of the European Society for Medical Oncology* 2015;**26**;259-271.
9. Adams S, Gray RJ, Demaria S *et al.* Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase iii randomized adjuvant breast cancer trials: Ecog 2197 and ecog 1199. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2014;**32**;2959-2966.
10. Avisar E, McParland E, Dicostanzo D, Axelrod D. Prognostic factors in node-negative male breast cancer. *Clinical breast cancer* 2006;**7**;331-335.
11. Baak JP, Colpaert CG, van Diest PJ *et al.* Multivariate prognostic evaluation of the mitotic activity index and fibrotic focus in node-negative invasive breast cancers. *European journal of cancer (Oxford, England : 1990)* 2005;**41**;2093-2101.
12. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 1991;**19**;403-410.
13. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN. Breast carcinoma in men: A population-based study. *Cancer* 2004;**101**;51-57.
14. Mujtaba SS, Ni YB, Tsang JY *et al.* Fibrotic focus in breast carcinomas: Relationship with prognostic parameters and biomarkers. *Annals of surgical oncology* 2013;**20**;2842-2849.
15. Kornegoor R, Verschuur-Maes AH, Buerger H *et al.* Fibrotic focus and hypoxia in male breast cancer. *Mod Pathol* 2012;**25**;1397-1404.
16. Shivas AA, Douglas JG. The prognostic significance of elastosis in breast carcinoma. *Journal of the Royal College of Surgeons of Edinburgh* 1972;**17**;315-320.
17. Chen Y, Klingen TA, Wik E *et al.* Breast cancer stromal elastosis is associated with mammography screening detection, low ki67 expression and favourable prognosis in a population-based study. *Diagnostic pathology* 2014;**9**;230.

18. Rasmussen BB, Pedersen BV, Thorpe SM, Rose C. Elastosis in relation to prognosis in primary breast carcinoma. *Cancer research* 1985;**45**;1428-1430.
19. Rasmussen BB, Rose C, Thorpe SM, Hou-Jensen K, Daehnfeldt JL, Palshof T. Histopathological characteristics and oestrogen receptor content in primary breast carcinoma. *Virchows Archiv. A, Pathological anatomy and histology* 1981;**390**;347-351.
20. Robertson AJ, Brown RA, Cree IA, MacGillivray JB, Slidders W, Beck JS. Prognostic value of measurement of elastosis in breast carcinoma. *Journal of clinical pathology* 1981;**34**;738-743.
21. Dhani N, Fyles A, Hedley D, Milosevic M. The clinical significance of hypoxia in human cancers. *Semin Nucl Med* 2015;**45**;110-121.
22. Vaupel P, Mayer A, Briest S, Hockel M. Hypoxia in breast cancer: Role of blood flow, oxygen diffusion distances, and anemia in the development of oxygen depletion. *Adv Exp Med Biol* 2005;**566**;333-342.
23. Walsh JC, Lebedev A, Aten E, Madsen K, Marciano L, Kolb HC. The clinical importance of assessing tumor hypoxia: Relationship of tumor hypoxia to prognosis and therapeutic opportunities. *Antioxid Redox Signal* 2014;**21**;1516-1554.
24. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (hif-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997;**272**;22642-22647.
25. Chia SK, Wykoff CC, Watson PH *et al.* Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase ix, in invasive breast carcinoma. *J Clin Oncol* 2001;**19**;3660-3668.
26. Bos R, Zhong H, Hanrahan CF *et al.* Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001;**93**;309-314.
27. Dales JP, Garcia S, Meunier-Carpentier S *et al.* Overexpression of hypoxia-inducible factor hif-1alpha predicts early relapse in breast cancer: Retrospective study in a series of 745 patients. *Int J Cancer* 2005;**116**;734-739.
28. Okada K, Osaki M, Araki K, Ishiguro K, Ito H, Ohgi S. Expression of hypoxia-inducible factor (hif-1alpha), vegf-c and vegf-d in non-invasive and invasive breast ductal carcinomas. *Anticancer Res* 2005;**25**;3003-3009.
29. Moelans CB, de Weger RA, van Blokland MT *et al.* Her-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and in situ hybridization. *Cellular oncology : the official journal of the International Society for Cellular Oncology* 2009;**31**;1-10.
30. Burkhardt L, Grob TJ, Hermann I *et al.* Gene amplification in ductal carcinoma in situ of the breast. *Breast cancer research and treatment* 2010;**123**;757-765.
31. Aubele M, Mattis A, Zitzelsberger H *et al.* Extensive ductal carcinoma in situ with small foci of invasive ductal carcinoma: Evidence of genetic resemblance by cgh. *Int J Cancer* 2000;**85**;82-86.
32. Moelans CB, de Wegers RA, Monsuurs HN, Maess AH, van Diest PJ. Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: A multiplex ligation-dependent probe amplification study. *Cellular oncology (Dordrecht)* 2011;**34**;475-482.
33. Plass C. Cancer epigenomics. *Human molecular genetics* 2002;**11**;2479-2488.
34. Connolly R, Stearns V. Epigenetics as a therapeutic target in breast cancer. *Journal of mammary gland biology and neoplasia* 2012;**17**;191-204.
35. Moelans CB, Verschuur-Maes AH, van Diest PJ. Frequent promoter hypermethylation of brca2, cdh13, msh6, pax5, pax6 and wt1 in ductal carcinoma in situ and invasive breast cancer. *The Journal of pathology* 2011;**225**;222-231.

36. Doebar SC, Slaets L, Cardoso F *et al*. Male breast cancer precursor lesions: Analysis of the eortc 10085/tbcrc/big/nabcg international male breast cancer program. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2017;**30**;509-518.
37. Verschuur-Maes AH, Kornegoor R, de Bruin PC, Oudejans JJ, van Diest PJ. Do columnar cell lesions exist in the male breast? *Histopathology* 2014;**64**;818-825.

7

Nederlandse samenvatting

Nederlandse samenvatting

Borstkanker is een ziekte die meestal bij vrouwen voorkomt, maar ook mannen kunnen borstkanker krijgen. In Nederland kregen 131 mannen in 2018 de diagnose borstkanker, wat circa 1% is van alle mensen met borstkanker. De resterende 99% betreft dus borstkanker bij vrouwen. Daarnaast is het voorstadium of niet-invasieve stadium van borstkanker, ductaal carcinoma *in situ* (DCIS) genoemd, bij mannen zeer zeldzaam. Van alle gevallen van borstkanker bij mannen is slechts 5% DCIS (in 2018 in Nederland 12 gevallen). De relatieve zeldzaamheid van borstkanker bij mannen, en in het bijzonder DCIS, zorgt ervoor dat er weinig onderzoek is gedaan met (voldoende) grote patiëntengroepen. Het is echter wel belangrijk dat er naar deze ziekte bij mannen voldoende onderzoek wordt gedaan omdat er bekend is dat er naast overeenkomsten, ook meerdere verschillen zijn vergeleken met borstkanker bij vrouwen, onder andere op eiwitniveau en moleculair niveau.

Mannen zijn ten tijde van de diagnose borstkanker ouder dan vrouwen waarbij de gemiddelde leeftijd waarop de diagnose wordt gesteld 67 jaar is, in vergelijking met 62 jaar bij vrouwen. Vaak is de ziekte uitgebreider dan bij vrouwen wat zeer waarschijnlijk komt doordat mannen meestal pas na meer dan 6 maanden naar een arts gaan vanaf het moment dat ze klachten krijgen. Deze klachten zijn meestal een pijnloze zwelling, tepelvloed of een zweer.

De mannelijke borst is anders dan een vrouwelijke borst waarbij het verschil voornamelijk zit in het ontbreken van klierweefsel (lobjes). De mannelijke borst bestaat daarom hoofdzakelijk uit vet, steunweefsel en spaarzame rudimentaire klierbuizen.
Borstkanker bij mannen is voornamelijk gedreven door endocriene (hormonale) stimulatie, waarbij de oestrogeen receptor alpha (ERα) maar ook de androgeen receptor (AR) vrijwel altijd tot expressie komt in deze tumoren. De oestrogenen in de circulatie zijn bij vrouwen voor de overgang voornamelijk afkomstig uit het ovarium, maar bij mannen en vrouwen na de overgang voornamelijk van androgenen (zoals testosteron) die worden geproduceerd in de bijnier en testis en vervolgens elders in het lichaam (vooral in het vetweefsel) worden omgezet in oestrogeen. Borstkanker bij mannen is, door deze hoge ERα expressie, dus ook vaker hormoongevoelig dan bij vrouwen.

Er zijn verschillende risicofactoren voor het ontwikkelen van borstkanker bij mannen. 15-20% van de mannen heeft één of meerdere familieleden met borstkanker of

eierstokkanker. De belangrijkste genetische risicofactor is een mutatie in het *BRCA2* gen. *BRCA1* mutaties spelen ook een rol, maar een veel minder grote rol dan bij vrouwen. Andere risico's zijn onder andere Klinefelter syndroom (een chromosoomafwijking met teveel X-chromosomen), obesitas, levercirrose en bestraling.

Wanneer borstkanker ontstaat, is er vrijwel altijd eerst een voorstadium, waarbij neoplastische (afwijkende, tumorvormende) cellen groeien in de klierbuizen, maar nog niet binnendringen in het weefsel daaromheen. Als dit wel gebeurt, groeien de tumorcellen in het steunweefsel van de borst. Dit heet invasieve borstkanker.

Omdat borstkanker bij mannen en vrouwen zeker niet identiek is, moet deze ziekte als uniek worden beschouwd. Er is dus onderzoek nodig naar deze ziekte om zo meer helderheid te verschaffen over bijvoorbeeld genetische aspecten, pathologische aspecten en ontstaanswijze. Om dit onderzoek zo betrouwbaar mogelijk te doen zijn (voldoende) grote patiëntengroepen nodig. Het materiaal dat voor het onderzoek in dit proefschrift gebruikt is, is afkomstig van een groot internationaal mannen borstkanker cohort, de "*EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program*". Dit initiatief is opgezet om retrospectief borstkankerweefsel van mannen te kunnen onderzoeken die gediagnosticeerd zijn tussen 1989 en 2009. In een van de studies hebben wij het hele cohort onderzocht en in de andere studies is een Nederlandse subpopulatie van dit cohort gebruikt die retrospectief verzameld werd door de Borstkanker Onderzoek groep (BOOG). Van deze laatste groep werd weefsel afkomstig van een weefselblokje beschikbaar gesteld om zowel immunohistochemisch (eiwit) als moleculair onderzoek te verrichten.

Het doel van dit proefschrift is om borstkanker bij mannen verder te karakteriseren en om beter te proberen begrijpen hoe borstkanker bij mannen precies ontstaat door DCIS te vergelijken met invasieve borstkanker.

In **hoofdstuk 2** beschrijven we verschillende histologische aspecten van 1483 borstkanker casus bij mannen, en correleren deze aspecten met prognose (zowel de algemene overleving als de duur tot aan een eventueel recidief). De gemiddelde leeftijd van de patiëntenpopulatie was 68.4 jaar en het tumortype (histologisch subtype) was meestal invasief carcinoom, geen speciaal type (no special type). Lobulair carcinoom was erg zeldzaam (1.4%). Borstkanker wordt normaliter gegradeerd in 3 groepen (graad 1, 2 en 3), gebaseerd op atypie van de cellen, aantal celdelingen en de mate van buisvorming. In onze patiëntenpopulatie was 21.8% van de tumoren graad 1, 50.1%

graad 2 en 28.1% graad 3. Bij vrouwen borstkanker is bekend dat hoe hoger de graad, hoe agressiever de tumor is. Dit heeft prognostische consequenties. In onze groep met mannenborstkanker had de graad geen prognostische waarde, echter bleek het aantal celdelingen (≥8 celdelingen per $2mm^2$) wel geassocieerd met een ongunstige uitkomst. De aanwezigheid van een fibrotisch focus (een soort littekengebied in de tumor) was in onze groep ook geassocieerd met een slechtere prognose. Ook de hoeveelheid ontstekingsinfiltraat, dus de hoeveelheid lymfocyten (witte bloedcellen) die te midden van de tumor liggen, had invloed op de prognose in onze studie. Als er veel infiltraat te zien was, was de uitkomst gunstiger. De aanwezigheid of afwezigheid van bloed- of lymfvat invasie met tumorcellen had geen prognostische waarde.

In **hoofdstuk 3** hebben we gekeken naar elastose. Elastose is de aanwezigheid van grote aggregaten elastische vezels in borstkanker. Dit fenomeen is bekend bij vrouwen borstkanker, waar er een duidelijke associatie is met ERα-positiviteit van de tumor. Je kunt de aanwezigheid van elastose, wat zichtbaar is in een HE gekleurde coupe, dus gemakkelijk gebruiken als een surrogaat weefsel biomarker voor ERα expressie. Wij gebruikten een scoringsysteem om elastose te kwantificeren, waarbij score 0 overeenkwam met afwezigheid van elastose en score 3 met veel elastose. Wij hebben in dit proefschrift een groep van 117 ERα-positieve borstkanker casus bij mannen vergeleken met 135 ERα-positieve borstkanker casus bij vrouwen. Deze laatste groep vrouwen zijn gediagnosticeerd in het Universitair Medisch Centrum Utrecht tussen 2017 en 2018. Ondanks dat alle tumoren ERα-positief waren, toonde slechts 22.2% van de borsttumoren in mannen enige vorm van elastose waarbij er geen enkel geval veel elastose (graad 3) toonde, terwijl 65.9% van de borsttumoren bij vrouwen enige vorm (graad 1, 2 of 3) van elastose toonde waarvan 21.5% graad 3 elastose was. Dit verschil was significant ($p<0.001$), ook na multivariate logistische regressie. Er waren geen significante correlaties tussen elastose en leeftijd, graad, aantal celdelingen en progesteron receptor in borstkanker bij mannen. Ook had elastose geen prognostische waarde. Aangezien er geen correlatie werd gevonden tussen ERα expressie en aanwezigheid van elastose, lijkt elastose een minder waardevolle surrogaat biomarker met minder klinische waarde dan bij vrouwen.

In **hoofdstuk 4** werd activatie van de hypoxie (zuurstoftekort) respons bestudeerd. Hypoxie is een staat van disbalans tussen zuurstoftoevoer/aanbod en zuurstofverbruik, waarbij er dus meer verbruik is dan aanbod. Tumorcellen kunnen in een staat van hypoxie overleven, door zich hieraan aan te passen. Een belangrijke speler is hierbij HIF-1α (een transcriptiefactor, die expressie van andere genen reguleert), die tot

overexpressie komt bij hypoxie. Deze overexpressie leidt tot opregulatie van de eiwitten CAIX en Glut-1. Met immuunhistochemie kan je eventuele overexpressie van deze markers beoordelen. Bij vrouwen borstkanker is overexpressie van hypoxie-gerelateerde markers gecorreleerd met een slechtere overleving, hoger risico op metastasering (uitzaaiingen) en een hogere tumorgraad, wat suggereert dat de hypoxie respons bijdraagt aan een meer agressieve vorm van borstkanker. Daarnaast lijkt de hypoxie respons al aanwezig te zijn in DCIS. In ons onderzoek hebben we gekeken naar expressie van HIF-1α, CAIX en Glut-1 in 76 casus van DCIS en 58 invasieve tumoren om te kijken of de hypoxie respons al is opgetreden in het voorstadium van borstkanker, zoals ook bij vrouwen bekend is, of nog niet. Wij vonden geen significant verschil in overexpressie van de markers tussen DCIS en invasieve tumoren, er op wijzend dat de hypoxie al in het pre-invasieve DCIS stadium aanwezig is en waarschijnlijk een oprechte carcinogene gebeurtenis is, en niet een bijstander in een later stadium van de borstkankerontwikkeling bij mannen.

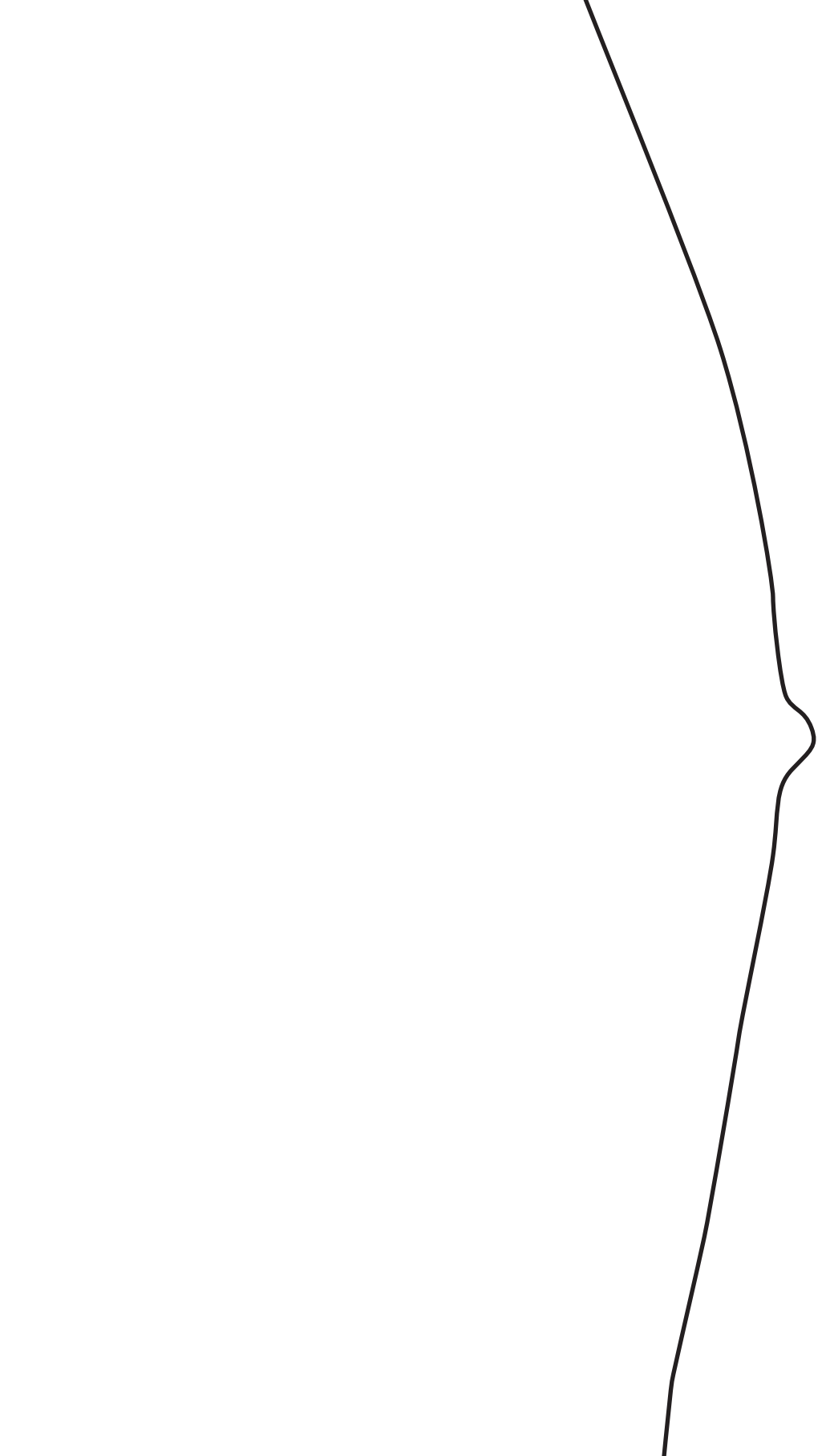
Ontwikkeling van borstkanker gaat via meerdere stappen waarbij een opeenstapeling van DNA veranderingen en epigenetische veranderingen (veranderingen in gen-functie zonder dat de moleculaire DNA-structuur veranderd) betrokken zijn. In **hoofdstuk 5** hebben we met behulp van MLPA (multiplex ligation-dependent probe amplification), een moleculaire techniek om meerdere genen tegelijk te analyseren, gekeken naar het aantal kopieën van 22 borstkanker-gerelateerde (proto)oncogenen. Een proto-oncogen is een gen dat in normale omstandigheden een rol speelt bij o.a. celdeling, remming van celdood of verminderde celdifferentiatie. Als een proto-oncogen een toegenomen activiteit krijgt, door bijvoorbeeld een mutatie of door toename van chromosomale kopieën (amplificatie), wordt dit proto-oncogen een oncogen genoemd en kan dit bijdragen aan de ontwikkeling van kanker. Een bekend voorbeeld hiervan is amplificatie (gencopie toename) van de humane epidermale groeifactor receptor 2 (*HER2)* in borstkanker. In deze studie hebben wij het aantal kopieën van 22 (proto)oncogenen onderzocht in invasieve borstkanker (n=49), DCIS aangrenzend aan de invasieve borstkanker (n=49) en puur DCIS (n=18) bij mannen, en de resultaten vergeleken met resultaten van een eerdere vrouwen borstkanker studie. Wij vonden geen verschil in het gemiddelde aantal kopieën van alle 22 genen tussen DCIS en invasieve borstkanker. Ook de frequentie van een afwijkend aantal kopieën was tussen deze 3 groepen niet verschillend. Bij analyse per individueel gen vonden wij een significant hoger aantal kopieën van *MTDH* in invasieve kanker vergeleken met DCIS. Deze bevindingen illustreren de clonale relatie tussen DCIS en invasieve borstkanker en de genetisch vergevorderde status van DCIS bij mannen. *MTDH* zou

mogelijk een rol kunnen spelen in de carcinogenese. De genen *MTDH*, *CPD*, *CDC6* en *TOP2A* toonden verschillen tussen mannen- en vrouwen borstkanker, wijzend op verschillen in borstkanker carcinogenese tussen man en vrouw.

In **hoofdstuk 6** beschrijven we promotor hypermethylatie, welke wij door middel van een methylatie specifieke MLPA (MS-MLPA) hebben onderzocht. Promotor hypermethylatie is een epigenetische verandering wat kan resulteren in het stilleggen van het gen waardoor de functie van dit gen verloren gaat. Als het een tumor suppressor gen betreft kan dit leiden tot het ontstaan van kanker, aangezien deze genen normaal gesproken een rol hebben in het remmen van de groei en ontwikkeling van tumorcellen. De rol van promotor hypermethylatie in het ontstaan van borstkanker bij mannen hebben wij onderzocht door 25 borstkanker gerelateerde tumor suppressor genen te vergelijken in invasieve kanker (n=44), DCIS aangrenzend aan de invasieve kanker (n=44) en puur DCIS (n=18). De resultaten werden vergeleken met resultaten van een eerder gepubliceerd vrouwen borstkanker onderzoek. *GATA5, KLLN*, *PAX6, PAX5*, *CDH13*, *MSH6* en *WT1* toonde frequent methylatie waarbij weinig verschil in methylatie status te zien was tussen DCIS en invasieve kanker. Dit wijst op een vroege rol van hypermethylatie van deze 7 genen in borstkanker carcinogenese bij mannen. Vergeleken met vrouwen DCIS werd er veel minder methylatie gevonden, in het bijzonder voor *VHL*, *ESR1*, *CDKN2A*, *CD44*, *CHFR*, *BRCA2*, *RB1* en *STK11*, alweer wijzend op verschillen tussen de ontwikkeling van borstkanker bij mannen en vrouwen.

Samenvattend benadrukt dit promotieonderzoek dat borstkanker bij mannen een unieke ziekte is, die niet één-op-één vergeleken kan worden met borstkanker bij vrouwen. Deze verschillen zijn op meerdere niveaus aanwezig. Ook beschrijven wij een betere overleving bij mannen borstkanker patiënten die een hogere densiteit hebben van lymfocyten in de tumor. Daarnaast zien wij dat bepaalde genetische veranderingen en epigenetische veranderingen al vroeg aanwezig zijn tijdens het ontstaan van borstkanker bij mannen, namelijk al in de DCIS fase. Het gen *MTDH* zou mogelijk een rol kunnen spelen in de ontwikkeling van borstkanker bij mannen.

8



Dankwoord

Dankwoord

Mijn promotie is voortgekomen uit mijn wetenschappelijke stage tijdens mijn opleiding tot patholoog. Promoveren was niet direct het doel. Het doel was om een nuttige en leerzame tijd te hebben en om een artikel te schrijven. Echter bleek gaandeweg het onderzoek in breedste zin veel leuker dan vooraf bedacht en hebben mensen om mij heen mij zodanig geënthousiasmeerd en geholpen dat dit proefschrift tot stand is gekomen. Deze mensen wil ik in dit hoofdstuk bedanken.

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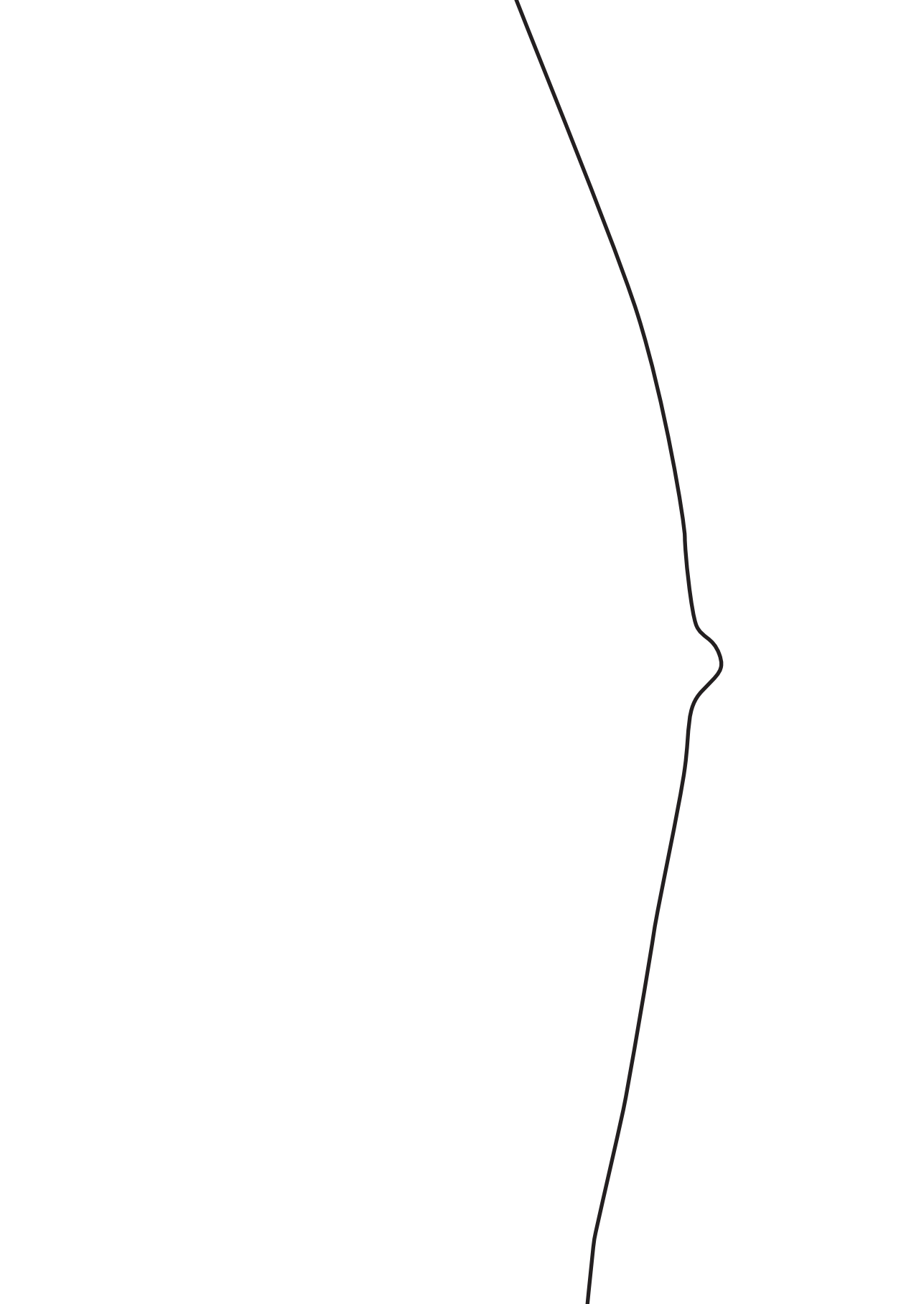

About the author

About the author

Marijn Annette Scheijde-Vermeulen werd geboren op 23 maart 1982 in Rotterdam. Ze groeide op in Nigeria, Tunesië, Syrië en Thailand en verhuisde op haar 12e naar Nederland waar ze tot haar 16e op Internaat 't Veerhuis in Oegstgeest woonde. Nadat ze in 2000 haar eindexamen op Het Rijnlands Lyceum in Oegstgeest behaald had, heeft ze haar propedeuse Biologie aan de Universiteit Leiden behaald.

Vervolgens begon zij aan haar studie Geneeskunde, eveneens in Leiden. Tijdens haar studie heeft zij gedurende 5 maanden in het Bottom Hospital in Lilongwe, Malawi haar wetenschapsstage gedaan.

Na het behalen van haar artsexamen in 2007 heeft zij gedurende een jaar op de spoedeisende hulp gewerkt in het VUMC. Vervolgens is zij begonnen aan de huisarts-opleiding in Utrecht.

Na een jaar bleek haar hart niet te liggen bij de huisartsgeneeskunde en is zij in januari 2011 begonnen aan de opleiding pathologie in het Universitair Medisch Centrum Utrecht (opleiders prof. dr. J. van den Tweel, drs. R. Leguit en prof. dr. M. van Dijk). Voor het perifere deel van de opleiding was zij werkzaam in het Diakonessenhuis te Utrecht en in het Gelre Ziekenhuizen te Apeldoorn). Na haar opleiding is zij in september 2017 gestart als patholoog in het Prinses Máxima Centrum in Utrecht met als aandachtsgebied pediatrische hemato-oncologie en solide tumoren bij kinderen.

Marijn is getrouwd met Martijn Scheijde en ze wonen samen met Nout (7 jaar) en Anne (5 jaar) in Utrecht.