INTEGRATING CLINICOPATHOLOGICAL AND MOLECULAR DATA IN THE BREAST CANCER PATIENT

Towards precision medicine

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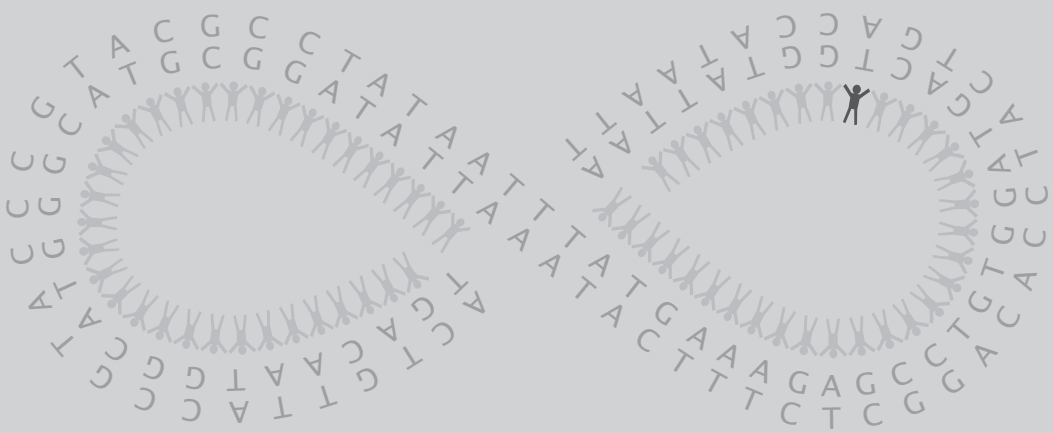

Chapter 1

General introduction

INCIDENCE AND ETIOLOGY

With an estimated 1.67 million new cases diagnosed in 2012, breast cancer is the most common malignancy and the leading cause of cancer related death in women of the western world [1;2]. Currently it is estimated that one in eight women will develop breast cancer at some point in life. However, with a growing aged population, and an increased adoption of cancer-causing behaviors, it is expected that the global burden of (breast) cancer will further increase in the coming decades [3;4].

In 2000, Hanahan and Weinberg proposed that carcinogenesis is embodied in defects of regulatory circuits governing cell proliferation and homeostasis. It was suggested that the comprehensive cancer cell genotypes are a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth [5]. These six biological alterations, induced by genomic instability which a tumor acquires during a multistep development pathway, are also known as 'the hallmarks of cancer' and consist of: 1. sustaining proliferative signaling, 2. evading growth suppression, 3. activating tissue invasion and metastasis, 4. enabling replicative immortality, 5. inducing angiogenesis and 6. resisting cell death. In 2011, after recognition of the importance of tumor microenvironment, Hanahan and Weinberg added two additional hallmarks, namely, reprogramming of energy metabolism and evasion of immune recognition [6].

TREATMENT AND PROGNOSIS

In general, treatment of breast cancer employs a multidisciplinary approach involving surgery, radiation, and systemic treatment. Today, treatment choices are mainly influenced by the tumor, node and metastasis (TNM) classification. The main aim of the TNM classification is to provide an estimation of the prognosis in order to guide therapy choice and create treatment uniformity in oncologic disease [7;8].

Generally, patients with early stage breast cancer undergo primary surgical resection (lumpectomy or mastectomy) of the tumor and regional lymph nodes, with or without radiation therapy. Subsequently, adjuvant systemic treatment may be offered based on patient and tumor characteristics such as tumor size, tumor grade, number of affected lymph nodes, age at diagnosis, co-morbidities, hormone receptor and human epidermal growth factor-2 (HER-2) status as well as patient preference.

Breast cancer mortality rates have been steadily declining since the early 1990's [9]. Survival of breast cancer patients largely depends on disease stage at diagnosis, in which a great inter-stage difference is seen. Currently, a five-year survival rate of 95% is seen in stage I breast cancer, which, regardless of current onco-pathology knowledge and treatment modalities, drastically drops to 18% in stage IV breast cancer patients [10;11].

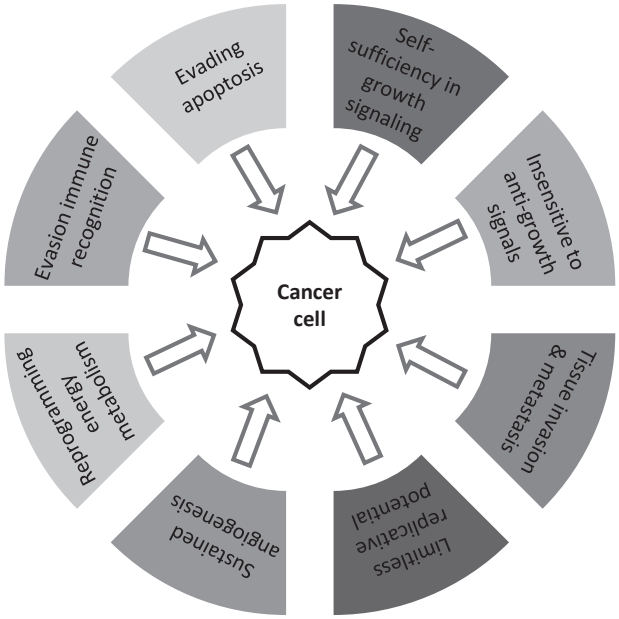

Figure 1: Global overview of the hallmarks of cancer as proposed by Hanahan and Weinberg.

BREAST CANCER IN THE ELDERLY

For most women, increasing age is the primary risk factor for breast cancer. Currently, almost half of the annually diagnosed breast cancer cases arise in women above the age of 65 years [4;9;12]. With the continuously increasing life expectancy and the decreased birth rates of the last decades, a larger proportion of the general population will be categorized as older. Consequently, the number of older women diagnosed with breast cancer will likely rise in the coming years, increasing the burden on society and on already overtaxed health care systems.

Elderly breast cancer patients differ from their younger counterparts in several aspects. For instance, with regard to tumor biology, it has been shown that breast tumors of older patients have lower proliferation rates, which result in slower tumor growth. Furthermore, they are genetically more stable and are more likely to be hormone-sensitive [13]. On the other hand, older patients tend to be diagnosed with larger tumors and increased nodal involvement, which may partly be the result of delayed diagnosis [14;15]. In addition to tumor biology differences, age-related physiological changes might affect metabolism, which may drive oncogenesis and also alter drug functionality and tolerability (Figure 2) [16].

With higher age, women with breast cancer not only have a higher risk of dying from other causes than breast cancer, known as competing mortality, but, compared to younger counterparts, also have an increased risk of breast cancer mortality [17].

Consequently, absolute benefits of anti-cancer therapy may be less clear in this specific subset of breast cancer patients. Furthermore, in contrast to the younger breast cancer patients, breast cancer survival in the older population has not improved in recent years, further increasing the survival gap between young and old breast cancer patients [18].

If the functional status of the older breast cancer patients is not sufficiently taken into account, the result may be both undertreatment (not treated with adjuvant therapy or treated with drugs of insufficient additive value) and over-treatment (cured with solely local therapy or limited adjuvant treatment) of this specific breast cancer population. This could explain the lack of survival gain for older patients, emphasizing the importance of individualized treatment strategies to improve breast cancer care in the older breast cancer population.

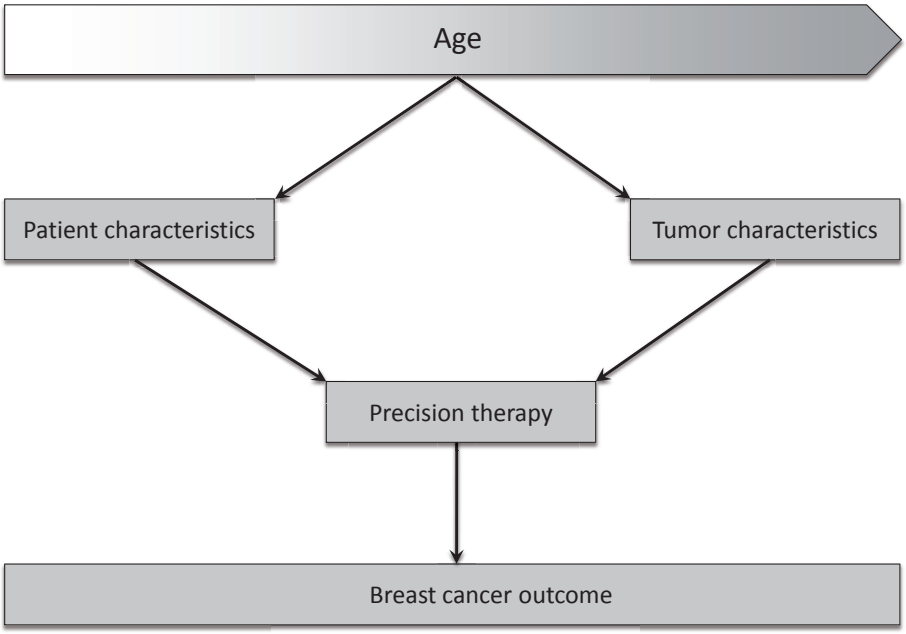

Figure 2: Global overview of the effect of age on patient and tumor characteristics, consequently leading to different treatment modalities with a focus on personalized care, aiming for the best clinical outcome for each patient.

NON-EVIDENCE-BASED MEDICINE IN THE ELDERLY

Despite the high cancer incidence and cancer-related mortality in the elderly [19], our knowledge about aging and its role in oncogenesis, and about optimal treatment for older patients is still far from adequate. The international society of geriatric oncology (SIOG) has established guidelines for breast cancer treatment in the elderly, but confirms that in many areas, solid evidence is lacking [12]. This is mainly due to underrepresentation of older breast cancer patients in clinical trials, due in large part to eligibility criteria

that have excluded the elderly for different reasons [20]. Therefore, current breast cancer treatment guidelines are largely based on studies performed in younger breast cancer patients [12]. However, given the aforementioned differences between older and younger breast cancer patients, guidelines for younger patients are not automatically applicable to elderly breast cancer patients. It is for this reason that the use of currently available online decision making tools, such as Adjuvant! Online, which are mainly based on research-data from studies performed in younger breast cancer patients, which estimate clinical outcome and assist in making treatment choices, should be interpreted with caution for this specific subset of breast cancer patients.

As a result, there is a lack of evidence-based guidelines to inform the most appropriate treatment of breast cancer disease in the older breast cancer population.

One of the major characteristics of the older cancer population treated in everyday clinical practice is the heterogeneity observed among patients of the same calendar age. Consequently, older breast cancer patients often receive less standard therapy compared to their younger counterparts [21-23];older patients presenting with breast cancer have less surgical resection, less frequently receive adjuvant radiation therapy following breast conserving surgical intervention and have an overall higher rate of primary endocrine therapy [21]. These differences in treatment among older and younger patients are largely due to co-morbidities or the declining general health status of the older women which is also associated with an increased risk of treatment-related complications and death [24]. It is for this reason that oncogeriatric breast cancer research is increasingly focusing on individualized, tailored treatment for the older breast cancer patient. The ultimate aim is to find the most appropriate care for each individual in the heterogeneous elderly breast cancer population by predicting who will die *with* (those harboring a low risk of recurrence and a high risk of competing mortality) and who will die *from* (those with a high risk of recurrent disease) breast cancer.

Currently, usage of a comprehensive geriatric assessment (CGA) is widely accepted to guide therapeutic decision making in the elderly breast cancer patient [25]. However, a systematic review published in 2012 showed that frailty screening by the clinician was not sufficient to qualify patients for a CGA [26]. Furthermore, the performance of a CGA is laborious, with high observer bias risk. Therefore, prognostic markers distinguishing between and taking into account the functional status of a patient would be of great value in clinical decision making with regard to breast cancer treatment in the elderly population.

PROGNOSTIC AND PREDICTIVE MARKERS

By definition, a *prognostic* factor is capable of providing information on clinical outcome at the time of diagnosis independent of therapy. Usually these markers are indicators of growth, invasion and metastatic potential. A *predictive* factor is capable of providing information on the likelihood of response to a given therapeutic modality [27;28]. Although often separated, in breast cancer several factors are both prognostic and predictive. As explained above, it is highly desired to have reliable *prognostic* markers that could help select those patients most at risk of recurrence or cancer-related death. In addition, clinically applicable predictive markers would aide in the tailoring of adjuvant therapy by identifying of which treatment a patient would most optimally benefit, thus saving them from unnecessary exposure to potentially toxic and expensive therapies.

To date, tumor stage has had the greatest influence on treatment decisions. However, new insights and advances in the molecular biology of breast cancer have started to influence prognostication and treatment decisions. The cellular and molecular heterogeneity of breast cancer, as well as the large number of genes involved in controlling cell growth, death, and differentiation emphasize the importance of studying multiple genetic and epigenetic alterations in concert. Over the last decades gene expression profiling studies have identified several molecular breast cancer subtypes, also called the intrinsic breast cancer subtypes, with greatly differing prognosis. In short, this subtype shows that estrogen receptor (ER)-positive and ER-negative tumors are fundamentally distinct molecular diseases [29]. There are two predominantly ER-positive intrinsic molecular subtypes (luminal A and luminal B, which carry the best prognosis) and two predominantly ER-negative intrinsic subtypes (HER-2-enriched and basal-like). The intrinsic molecular subtypes are largely distinguished by the expression of genes involved in luminal epithelial differentiation (ER and progesterone receptor (PR) genes), proliferation (Ki67 gene), human epidermal growth factor receptor-2 pathway (HER-2 gene), and basal differentiation [29]. Other promising molecular prognostic assays are the 21-gene Recurrence Score (RS) (Onco*type* DX Breast Cancer Assay (Genomic Health, Redwood City, CA, USA)), the Amsterdam 70-gene profile (Mammaprint (Agendia, Amsterdam, the Netherlands)), and the PAM50 Risk of Recurrence score assay (Prosigna, Nanostring Technologies, Inc., Seattle, USA)) [30-32]. In all breast cancer patients, but especially in the increasingly frail elder patient, predicting the clinical behavior of a tumor through a combination of clinical, pathological and biological characteristics is of great value as it may lead to tailored, optimally beneficial treatment.

AIM OF THIS THESIS

The work presented in this thesis is part of the collaborative FOCUS project (**F**emale breast cancer in the elderly; **O**ptimizing **C**linical guidelines **US**ing clinico-pathological & molecular data), seeking insight into breast cancer disease in the elderly population in order to improve care in this often affected but frequently neglected patient group. As it cannot be expected that clinical trials focusing on older patients with breast cancer will abate the current knowledge-gap in tumor-biology and treatment in the near future, the aim of this thesis is to define normal tissue, breast cancer, and therapeutic sensitivity differences in observational, population-based cohorts consisting of elderly breast cancer patients. The ultimate goal is to improve risk stratification and consequently treatment benefit for the individual patient, paving the way for the clinical introduction of precision medicine, especially in the older breast cancer population.

The FOCUS project consists of four domains; analysis of a large observational cohort of elderly patients; age- specific analyses of clinical trial data; a prospective study investigating patient preferences; and a pathology study aiming to elucidate and unravel the differences and/or similarities in tumor biology of elderly breast cancer patients compared to younger counterparts. The studies presented in this thesis consist of analyses of pathology studies combined with the observational cohort data and clinical trial data.

USED PATIENT COHORTS

JANE cohort

Data from the JANE cohort was used in chapters 2, 3, 4, and 9. The JANE cohort is a population-based cohort consisting of 822 breast cancer patients. JANE is comprised of heterogeneous, non-metastasized, primarily surgically treated breast cancer patients, without a history of previous malignancy, who were treated at the Leiden University Medical Center (LUMC) between 1985 and 1996. Breast tissue was collected from the department of pathology in the LUMC, after which all samples were histologically confirmed malignant according to current pathological standard. All samples were handled in a coded fashion, according to national ethical guidelines: "Code for Proper Secondary Use of Human Tissue" of the Dutch Federation of Medical Scientific Societies. Information on patient and tumor characteristics, treatment, follow-up and outcome were recorded for all patients by medical record review. The main advantage of this cohort is that we were able to collect detailed information of a large number of unselected patients, reflecting the large heterogeneity among the general breast cancer population.

TEAM trial

Data from the Dutch Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial were used in chapters 5 and 7. Originally, the TEAM trial was a randomized, phase 3, multinational, open-label study conducted between January 2001 and January 2006 in postmenopausal women with hormone-receptor positive breast cancer. In short, postmenopausal patients with histologically confirmed breast carcinoma who completed local therapy with curative intent (i.e., without evidence of metastatic disease) and no history of previous malignancy (with a disease-free interval of less than 5 years), were eligible. Overall, 9.766 patients were randomized to receive either exemestane, 25 mg once daily for 5 years, or tamoxifen, 20 mg once daily for 2.5 to 3 years, followed by exemestane, 25 mg once daily for 2 to 2.5 years, for a total of 5 years within 10 weeks of completion of surgery and, if indicated, chemotherapy. Appropriate approvals from the ethical committees and written informed consent from all patients were obtained. Patients were assessed every 3 months during the first year of treatment and at least once a year thereafter. Clinical outcome data was retrieved, and vital status was established by medical record review or through linkage with the municipal population registries. For the studies performed in this thesis, only tumor material from the patients enrolled in the TEAM trial in the Netherlands was available for experimental purposes. A large advantage of using data and material from the TEAM trial, was the structured follow up on recurrence and cause of death, which provided a unique opportunity to study associations between age, tumor characteristics and breast cancer outcomes.

FOCUS cohort

Data of the FOCUS cohort was used in chapters 6, 8, and 10. The FOCUS cohort is a population-based cohort of breast cancer patients aged 65 years or older, who were diagnosed in the geographically defined Comprehensive Cancer Center Region West in the Netherlands, between 1997 and 2004. Overall, 3.672 patients were included. Information on patient characteristics, tumor characteristics, treatment, follow-up and outcome were recorded for all patients. Co-morbidity was defined as presence of co-morbidity at time of diagnosis, and categorized by the 10th edition of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). Vital status was established either directly from the patient's medical record or through linkage with the municipal population registries. The main advantage of this cohort is that we were able to collect detailed information and tumor and normal tissue samples of a large number of unselected older patients, reflecting the large heterogeneity among elderly breast cancer patients in the general population.

OUTLINE OF THIS THESIS

Four major topics will be discussed in this thesis; for overview purposes this thesis is therefore subdivided into overarching parts.

Molecular differentiation, immune evasion, and sustaining proliferative signaling and resisting cell death are important mechanisms that cancer cells acquire during tumor development [5;6] and are therefore studied in ***part I*** of this thesis. ***Part II*** discusses the predictive value of the biomarkers HER-2 and the insulin growth factor-1 receptor (IGF1R) in relation with treatment. ***Part III*** investigates the effect of aging on tumor development, and the functional status of the patient. Ultimately in ***part IV***, the use of predictive and prognostic biomarkers in clinical practice, its utility and the road to precision medicine are discussed.

Deregulation of the proliferative and apoptotic signaling pathways are two important hallmarks of tissue homeostasis disturbance, ultimately leading to tumor development [5]. Previous studies have shown contradicting results with respect to the relation of apoptosis or proliferation in tumor specimens and patient outcome in breast cancer [33;34]. As tumor growth is characterized by a fine balance between cellular multiplication and cell death, we hypothesize in **chapter 2**, that the level of imbalance between these two signaling pathways might indicate tumor aggressiveness more accurately than single marker studies.

Over the last two decades, it was shown that the immune system has a substantial effect on tumor development and spread [35]. It is believed that under certain conditions, tumors possess the ability to edit themselves, in order to improve their survival through a selection process, leading to a poorly immunogenic tumor variant which is able to evade immune recognition, consequently leading to tumor progression [36-39]. Research aimed at unraveling the tumor cell mechanisms leading to immune evasion showed multiple potential target points in order to obtain the diminished immune susceptible phenotype; First, down-regulation of classical human leukocyte antigen (HLA) class I expression, which minimizes the level of tumor-associated antigen (TAA) expression on the tumor cell surface, leads to less immune recognition and subsequently less destruction by cytotoxic T-cells (CTL) [40]. Second, expression of non-classical HLA class I molecules, HLA-E and HLA-G, on the tumor cell surface: under normal circumstances HLA-E is found in most tissues that express classical HLA-I and is thought to provide an important 'self-recognition-signal' to the immune system [41]. In contrast, HLA-G is rarely expressed in healthy tissue but is shown to be frequently up-regulated in extravillous trophoblastic cells, where it mediates immunotolerance during pregnancy, and in tumor tissue [42]. Simultaneous expression of both non-classical HLA class I subtypes, HLA-E and HLA-G, has been associated with evasion of natural killer (NK) cell recognition, resulting

in further escape from immune attack [42;43]. A third mechanism is the attraction of immunosuppressive regulatory T-cells (Tregs) into the tumor microenvironment, leading to suppression of CTL activity [44].

Overall, a complex association was seen between these known immune markers, highlighting the need for combined marker analyses [45-47]. Therefore, in **chapter 3** we evaluated the association of these immune markers, separately and combined, with the clinical outcome of the breast cancer patients. In **chapter 4**, we performed the same analysis in breast cancer patients stratified for tumor histology, to investigate whether there is a difference in tumor immune escape between invasive ductal carcinoma and invasive lobular carcinoma. This was of particular interest due to the fact that these two histologically different breast tumors tend to present with different clinical properties. Finally, in **chapter 5** we studied the tumor immune characteristics in relation to clinical outcome in a large, clinical trial controlled hormone receptor-positive (HR+ve) breast cancer cohort, in which the effect of endocrine therapy was investigated, as previous research hinted at a possible immuno-modulatory effect of endocrine therapy [48].

Identification of breast cancer molecular subtypes has proven that breast cancer is a heterogeneous disease, requiring different adjuvant treatment [49-51]. In the older breast cancer population, where a large part of the tumors are HR+ve, have lower proliferation rates and patients have an increased risk of dying of other causes than breast cancer, we investigated the prognostic value of the molecular subtypes in this specific subgroup of breast cancer patients (**chapter 6**).

In ***part II,*** **Chapter 7** of this thesis, the benefit of aromatase inhibiting treatment in high IGF-1R expressing HR+ve breast tumors compared to estrogen receptor-blocking therapy was noted. This effect was committed to the activating capacity of IGF-1R by estrogen and insulin growth factor [52]. This beneficial effect was further enhanced when metformin, a well-known reducer of hepatic glucose production and insulin, due to improvement of the peripheral insulin sensitivity, was added to the breast cancer-related endocrine treatment.

With the dreaded side effects of anti-HER-2 treatment, its use in the already frail elderly population is reluctant. Currently, no literature can be found to support this clinical decision. Furthermore, recent studies show that HER-2-positive breast carcinomas with a PIK3CA mutation are less likely to respond to anthracycline-taxane-based chemotherapy plus HER-2 treatment [53]. Therefore, in **chapter 8** the clinical consequence of HER-2 overexpression on the breast tumor surface of elderly (≥65 years) patients, with or without PIK3CA mutations, and the effect of chemotherapy, was investigated. The aim of this study was to define whether we could identify a subgroup of elderly breast cancer patients who could potentially still benefit from anti-HER-2 treatment, despite the risk of the dreaded side effects.

Still a matter of ongoing debate, and an important question to address, is 'Why does cancer risk increase as we age?' The current attribution that cancer risk increases due to the so-called multi-hit hypothesis, stating that time is necessary for cells to accumulate sufficient genetic mutations to push them over a certain mutagenic threshold and into full-blown carcinogenesis [54;55], fails to explain why cancer risk is greatly reduced by calorie restriction and physical exercise, even in situations where chemical carcinogens would normally evoke a 100% cancer penetrance, and why a high-fat diet and a sedentary lifestyle has the opposite effect [56]. Recent work proposed that it is not simply the time necessary to accumulate sufficient hits that account for the increased rate of cancer with age, but the decline in metabolic homeostasis and gene regulation that occurs normally as we age [55;57]. A hallmark of cancer is a shift away from oxidative phosphorylation (OXPHOS) toward anaerobic glycolysis, to provide cells with sufficient substrates for biomass [57]. This reprogramming, also known as the Warburg-effect [58], is driven by several pathways, of which hypoxia-inducible factor-1 (HIF1α) is an important component [59]. Recent evidence has emerged, from studies performed in *C. Elegans* and mammals [57;60], for an important role of HIF1α in aging, supporting the proposition of a decline in metabolic homeostasis as a driver of aging, which also primes for a carcinogenic environment. ***Part III*** of this thesis will focus on the difference in young and old breast cancer patients with regard to HIF1α targets in the tumor (**chapter 9**) and in normal breast tissue (**chapter 10**), in relation with the functional status of the patient and clinical outcome parameters.

Over the last decades the public health sector witnessed a vast and rapid development of genomic profiling techniques, with the promise of precision medicine as a strong driving force. Prediction of pathway deregulation coupled to molecular target identification using genome-wide approaches may provide an opportunity to guide treatment [61]. ***Part IV***, **Chapter 11** discusses the impact of current clinically approved multi-gene assays such as the Onco*type* DX Breast Cancer Assay (Genomic Health, Redwood City, CA, USA) and the Mammaprint (Agendia, Amsterdam, the Netherlands) on surgery.

Finally, an overall summary and discussion on the content of this thesis are presented in **chapter 12**.

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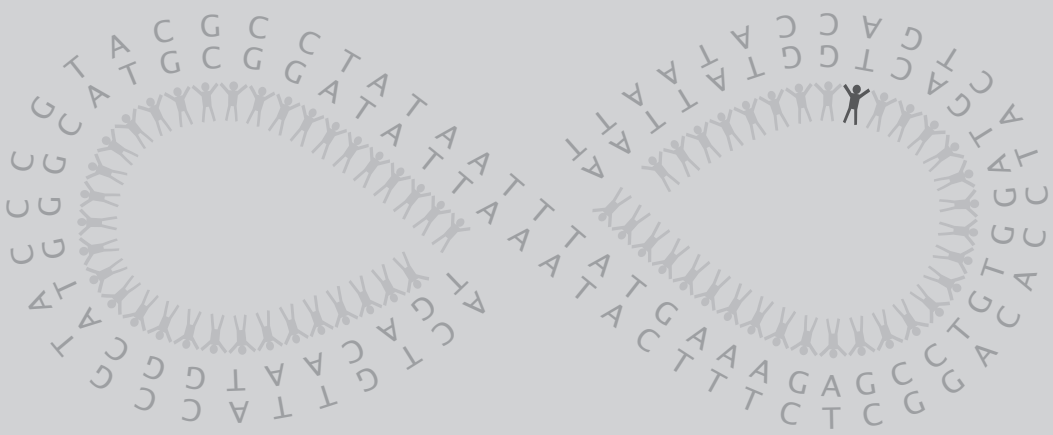
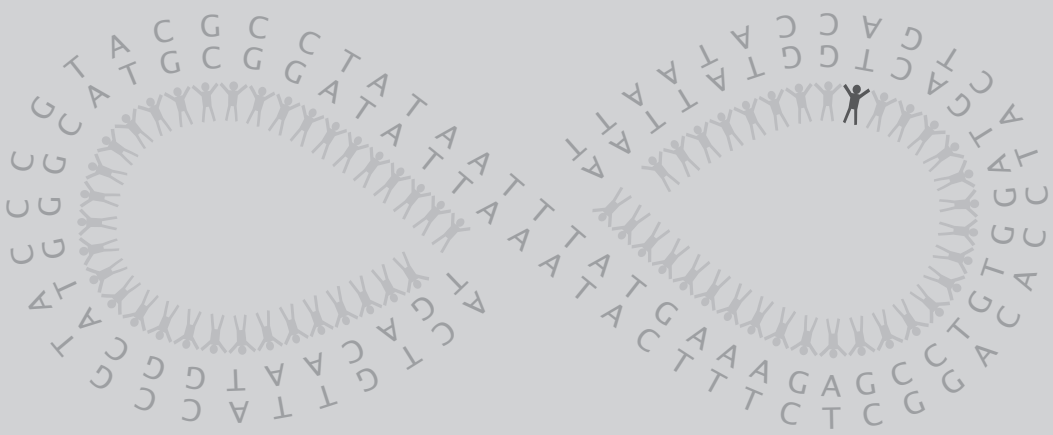


Part I

Prognostic biomarkers in breast cancer



Chapter 2

The prognostic value of apoptotic and proliferative markers in breast cancer

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ABSTRACT

Introduction

Increasing ability of early breast cancer diagnosis leading to more early stage detection, better survival and low relapse marks one of the milestones achieved over the decades. Foregoing poses a challenge for clinicians regarding optimal treatment, in which over- and under-treatment should be avoided. Classical prognostic and predictive factors fall short for individualized adjuvant therapy selection in this patient group. The key to better characterization may be found in the biology underlying individual tumors. We hypothesized that markers related to cellular proliferation and apoptosis and the balance between these two processes in tumor development will be predictive for clinical outcome.

Material and Method

Our study population (n=822) consisted of all early stage breast cancer patients primarily treated with surgery in our center between 1985-1996. Sections of available tumor tissue (87%, 714/822) were immunohistochemically stained for expression of p53, active-caspase-3 and Ki67. In 43% (304/714) and 18% (126/714) of this cohort respectively a biochemical C2P® risk prediction and caspase-3 assay were performed.

Results

Expression data of the mentioned markers, single or combined, were analyzed. Results showed that both single and combined markers, whether of apoptotic or proliferative origin had associations with clinical outcome. An additive effect was seen for the hazard ratios when data on p53, active caspase-3 and Ki67 status were combined. The assembled prognostic apoptotic-proliferative subtype showed significant association for both the OS (p=0.024) and RFP (p=0.001) in the multivariate analyses of grade I breast tumors.

Conclusion

Combined markers of tumor cell apoptosis and proliferation represents tumor aggressiveness. The apoptotic-proliferative subtypes that we present in this study represent a clinical prognostic profile with solid underlying biological rationale and poses a promising method for accurate identification of grade I breast cancer patients in need of an aggressive therapeutic approach, thus contributing to precision medicine in breast cancer disease.

INTRODUCTION

The introduction of population-based screening for breast cancer (BC) with the aid of mammography led to a shift towards early-stage (<2cm) node-negative BC detection with better prognosis [1]. This development contributed to a continuous decline in BC-related deaths despite the increasing incidence of BC in developed countries over the past decades. Nevertheless BC still remains one of the leading causes of cancer death in women in the western world [1].

Early diagnosis poses a challenge for clinicians regarding optimal treatment. With a relatively low relapse rate in patients detected with early BC, individual estimation of the therapeutic benefit for these patients is of crucial importance, in which over- and under-treatment has to be avoided. Defining individual tumor-specific characteristics could lend a helping hand in this consideration.

Classical prognostic and predictive factors like tumor size, histology, tumor grade, lymph node and hormone receptor status are routinely assessed for every BC patient. Nonetheless, characterizing the tumor by identification of new or additional (bio)markers may lead to a better insight into the tumor biology and thus to its clinical behavior.

It is widely accepted that the presence of certain local factors determine tumor development, such as angiogenesis and the level of tumor cell proliferation and apoptosis. The inability to undergo apoptosis is thought to contribute to tumorigenesis and tumor progression [2]. Recent work showed that identification of the proliferation marker Ki67 proved to be of fixed prognostic value, even in an independent fashion [3;4]. Bearing in mind that healthy tissue signifies a fine proliferative-apoptotic balance, we propose that tumor growth may be more accurately determined by the outcome of the balance between tumor cell proliferation on one side and apoptosis on the other. It is for this reason that we in this study aimed to identify clinically relevant biomarkers quantifying apoptosis and proliferation in breast tumors, which could be of major prognostic and predictive value. To achieve this we assessed the presence of p53, active caspase-3 and the proliferative markers Ki67 and C2P® (Sysmex, Kobe,Japan) in post-operative tumor material of early stage BC patients. Lastly, we constructed an apoptotic-proliferative subtype risk model based on the combination and rate of expressed markers. Reporting was done according to the REMARK criteria [5].

PATIENTS AND METHODS

Patients and tumors

Our retrospectively analyzed patient population comprised of all non-metastasized BC patients primarily treated with surgery, with or without adjuvant systemic therapy in

the Leiden University Medical Center between 1985 and 1996 (n=822). Exclusion criteria were bilateral tumors or a prior history of cancer (other than basal cell carcinoma or cervical carcinoma *in situ*). The following data were known: age at diagnosis, tumor grade, histological tumor type, TNM stage, time of locoregional/distant tumor recurrence, survival time and expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [6]. Perioperative formalin fixed paraffin embedded (FFPE) tumor material was used for immunohistochemistry (IHC) and fresh frozen tumor material for biochemical assays. An experienced BC pathologist (VS) graded all tumors according to current pathological standards. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Stainings were performed according to previously described standard protocols [7]. For each staining, all sections were stained simultaneously to avoid inter-assay variation. Mouse monoclonal antibodies against p53 protein (M700101 clone D-07: Dako, NL, 0.01M EDTA buffer (pH 8.0)) and Ki67 (M7240 Clone MIB-1: Dako, NL, 0.01M EDTA buffer (pH 8.0)) were used. For active caspase-3 detection an immunohistochemical staining was performed with antibodies directed against cleaved caspase-3 (Anti-Asp175 #9661: Cell Signaling, USA, citrate buffer 0.1M (pH 6.0)). Tonsil and colorectal carcinoma sections served as positive control for p53, Ki67 and active caspase-3 staining respectively. Negative controls underwent the whole immunohistochemical staining without primary antibodies.

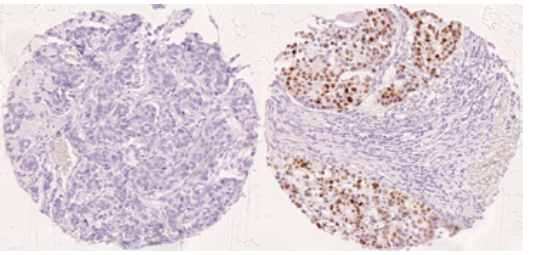
Evaluation of immunostaining

Two independent observers performed quantification of p53-, active caspase-3- and Ki67-positive stained cells in a blinded manner. For p53 the percentage positive stained nuclei of tumor cells were microscopically assessed by determining the mean percentage in all three punches of the TMA. Categorization was made by dividing the mean percentage scores into: wildtype (≤50% positive nuclei in the tumor material) and mutant pattern of staining (>50% expression of tumor nuclei stained positive for p53 (figure 1A)) [8].

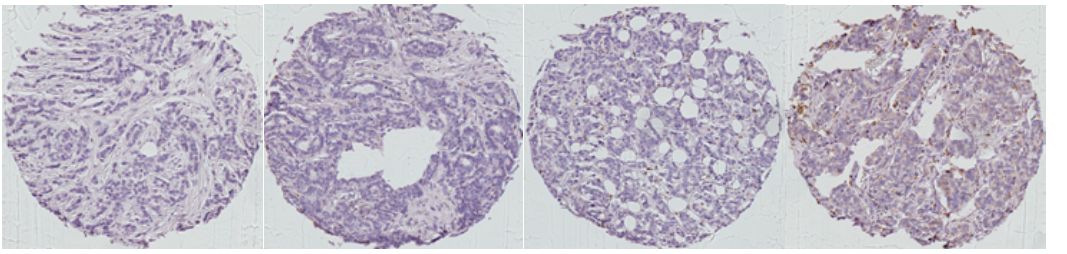
For active caspase-3 the mean expression grade of positively stained cells in the TMA was defined: absent (expression grade: 0-0.49 positive cells), low (expression grade: 0.5-1.49 positive cells), intermediate (expression grade: 1.5-2.49 positive cells) and high scores, corresponding with a mean expression of >2.5 positive cells in the tumor material (figure 1B).

Ki67 expression was divided into absent (0%) and present (>1%) positively stained nuclei, based on the mean percentage of all three-tumor punches per patient (figure 1C).

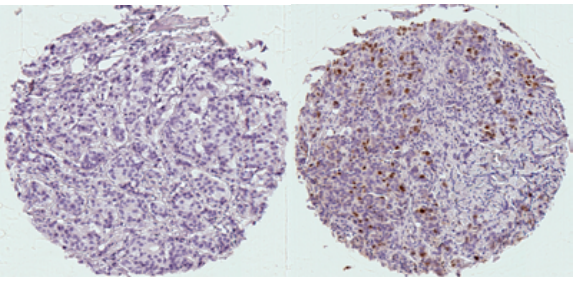
Figure 1:



A: Immunohistochemical p53 staining; left: wildtype staining pattern (≤ 50% of nuclei); right: mutant staining pattern (>50% of the tumor nuclei).



B: Immunohistochemical active caspase-3 staining; from left to right: negative (<0.49 positive cells), low (0.5-1.49 positive cells), intermediate (1.5-2.49 positive cells) and high (>2.49 positive cells) expression in human breast tumor (cut-off points: mean expression of active caspase-3 in three breast cancer tissue cores).



C: Immunohistochemical Ki67 staining; left: absent (0%) and right: present (>1%) staining in human breast tumor.

C2P® risk prediction score assay

C2P® risk prediction scores (C2P®-RS) is a proliferation assay developed by Sysmex Corporation which is based on cyclin-dependent kinase (CDK) 1 and CDK2, both playing a pivotal role in cell cycle regulation [9]. Risk prediction scores are based not only on CDK1 and CDK2 presence in the tumor material but also on the enzyme activity rate [9]. CDK1 and CDK2 assays were performed using frozen tissue samples. Subsequently, the C2P®-RS was calculated using a predetermined formula, after which the tumors were divided into three categories (high, intermediate and low RS groups) [9]. For a detailed assay protocol see manuscript by Kim *et al* [9].

Active caspase-3 assay

Biochemical quantification of active caspase-3 was determined in 18% of the BC patients (126/714). The enzymatic activity of caspase-3 was obtained by lysing ten 10µm thick cryostat sections per sample in 500µL lysis buffer containing 10mM HEPES, pH7.0, 40mM β-glycerophosphate, 50mM NaCl, 2mM MgCl2 and 5mM EGTA, followed by 10 minutes of homogenization using a Polytron homogenisator (PT-MR 2100, Kinematica, Luzern, Switzerland) and four freeze-thaw cycles before storing it at -80 degrees Celsius. Protein concentration was determined using the Bradford method [10]. For measurements of caspase-3 enzyme activity, 50 µL of each sample was incubated with 5 µL of 1mM substrate Ac-DEVD-AFC (A0466-1MG, Sigma Aldrich, USA) in a 100mM HEPES buffer, pH 7.25, containing 10% sucrose, 0.1% (v/v) Nonidet-P40 and 10mM dithiothreitol (DTT; D0632, Sigma Aldrich, USA) for two hours at 37˚C. During incubation at 37˚C, fluorescent AFC was cleaved off by active caspase-3, corresponding with the level of caspase-3-activity per sample. Fluorescent AFC absorbance was monitored in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter at time-point: 00.00 hours and again at time-point: 02.00 hours. Calibration curves were prepared by plotting the values of free-AFC standard absorbance versus concentration in nmol/L.

Caspase-3 activity was indicated in pmolAFC/min/mg protein.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 20.0 IBM SPSS Statistics). Patients with missing data, mostly due to material handling were excluded from statistical analysis. Cohen's kappa coefficient was used to assess the inter-observer agreement in quantification of p53, active Caspase-3 and Ki67 expression. The χ^2 test was used to evaluate associations between various clinicopathological parameters and apoptotic and proliferative markers in the tumor material. The clinical endpoints examined were Relapse-Free Period (RFP), defined as the time from surgery until an event (locoregional recurrence and/or a distant recurrence, whichever came first) and Overall Survival (OS), defined as the time from surgery until death by any reason. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of RFP and OS curves. Cox proportional hazard analysis was used for univariate and multivariable analysis for RFP and OS. Variables with a p-value of < 0.1 in univariate analysis were entered in multivariable analysis.

In order to compare the agreement of the different techniques used for caspase-3 (IHC and biochemical assay) estimation, a Spearman's Rho correlation test was performed.

RESULTS

Patient and tumor characteristics

Perioperative tumor material was available of 87% (714/822) of the patients. The median age of this cohort was 58 years (range= 23-96 years) with a median follow-up of 10 years (range= 0.02-22years) (clinicopathological characteristics: table 1A and 1B). Good inter-observer agreement was seen (≥0.6) using the Cohen's kappa coefficient for quantification of immune-stained markers.

p53 expression

Immunohistochemical data for p53 expression was available for 80% (574/714) of the patients. Mutant p53 was significantly present in patients with more advanced pathological tumor stages (p<0.001), more advanced TNM stage (p=0.033), higher tumor grades (p<0.001) and ductal tumors (p=0.017) (table 1A). Tumors with adverse hormonal characteristics: Estrogen Receptor (ER) negative *(-)*, Progesterone Receptor (PGR) negative *(-)* and Human Epidermal Growth Factor Receptor-2 (HER-2) positive *(+)* are significantly associated with mutant p53 protein (ER: p=0.013; PGR: p=0.004 and HER2: p<0.001) (table 1A).

Analysis of the OS showed a statistical significant association between mutant p53 and survival outcome of patients (p<0.001, Hazard Ratio (HR): 2.150, 95% Confidence Interval (CI): 1.549-2.983; table 2A), also remaining an independent prognostic marker in multivariable analysis (p=0.009, HR: 1.776, 95%CI: 1.158-2.726). The explanation hereof lies in the fact that mutated p53 protein cannot be cleared away in the tumor cell leading to high amounts of inactive p53 stacking which is often seen to a greater extent in more aggressive tumor types since no apoptosis is induced [11]. For relapse free period (RFP) a significant relation was seen for mutant p53 in the univariate analysis only (p=0.002, HR: 1.838, 95%CI: 1.255-2.692) (figure 2A and table 2B).

Active caspase-3 expression

Data of active caspase-3 IHC was available for 80% (575/714) of the BC patients. Tumors in which determination of both active caspase-3 IHC expression and caspase-3 biochemical enzymatic activity was performed (N=106), comparison analyses showed excellent agreement (p=0.011). There was significant association between active caspase-3 expression in IHC and higher pathological tumor stage (p<0.001), more advanced TNM stage (p<0.001), higher tumor grade (p<0.001), ductal tumor histology (p<0.001), and a statistical trend was seen for lymph node involvement (p=0.065) (table 1A). ER negative, PGR negative and HER2 over-expressing tumors are related to high caspase-3 expression with *p*-values of <0.001, p=0.002 and p=0.002 respectively (table 1A). Additional analyses showed a close relationship between caspase-3 expression and Ki67 expression

Table 1A: Clinicopathologic characteristics of the patient population stratified for the tumor suppressor p53 protein and the apoptotic marker active caspase-3

	p53 wildtype		p53 mutant		*p-value*	caspase-3 Negative		caspase-3 Low		caspase-3 Intermediate		caspase-3 High		*p-value*
	N	%	N	%		N	%	N	%	N	%	N	%	
Total	522	100	52	100		177	100	177	100	121	100	100	100	
Age (y)														
<45	106	20.3	8	15.4	0.298	36	20.3	31	17.5	28	23.1	19	19.0	0.617
45-55	128	24.5	10	19.2		51	28.8	39	22.0	23	19.0	24	24.0	
55-65	113	21.6	17	32.7		38	21.5	45	25.4	26	21.5	20	20.0	
>65	175	33.5	17	32.7		52	29.4	62	35.0	44	36.4	37	37.0	
Missing	0		0			0		0		0		0		
Histological type														
Ductal	465	90.1	52	100	**0.017**	146	83.9	164	94.3	112	93.3	97	98.0	**<0.001**
Lobular	51	9.9	0	0		28	16.1	10	5.7	8	6.7	2	2.0	
Missing	6		0			3		3		1		1		
Grade														
I	87	16.9	2	3.8	**<0.001**	51	29.3	25	14.4	10	8.3	3	3.0	**<0.001**
II	266	51.8	10	19.2		92	52.9	98	56.3	53	44.2	31	31.3	
III	161	31.3	40	76.9		31	17.8	51	29.3	57	47.5	65	65.7	
Missing	8		0			3		3		1		1		
Tumor stage														
pT1	216	42.3	11	21.6	**<0.001**	90	52.0	85	48.6	33	27.7	20	20.8	**<0.001**
pT2	245	47.9	26	51.0		69	39.9	76	43.4	73	61.3	55	57.3	
pT3/4	50	9.8	14	27.5		14	8.1	14	8.0	13	10.9	21	21.9	
Missing	11		1			4		2		2		4		
Nodal stage														
pN0	271	53.0	22	44.9	0.276	108	62.1	98	56.6	51	43.2	44	45.8	0.065
pN+	240	47.0	27	55.1		66	37.9	75	43.4	67	56.8	52	54.2	
Missing	11		3			3		4		3		4		
TNM stage														
Stage 0	0	0	0	0	**0.033**	0	0	0	0	0	0	0	0	**<0.001**
Stage I	135	28.7	8	17.0		67	41.4	52	32.7	22	19.6	8	9.4	
Stage IIA	158	33.6	12	25.5		52	32.1	53	33.3	29	25.9	34	40.0	
Stage IIB	112	23.8	13	27.7		25	15.4	35	22.0	42	37.5	23	27.1	
Stage IIIA	24	5.1	4	8.5		7	4.3	7	4.4	9	8.0	5	5.9	
Stage IIIB	15	3.2	5	10.6		2	1.2	6	3.8	5	4.5	6	7.1	
Stage IIIC	26	5.5	5	10.6		9	5.6	6	3.8	5	4.5	9	10.6	
Stage IV	0	0	0	0		0	0	0	0	0	0	0	0	
Missing	14		5			15		18		9		15		
ER receptor														
Negative	210	40.8	30	58.8	**0.013**	54	31.2	55	32.2	57	47.5	63	63.6	**<0.001**
Positive	305	59.2	21	41.2		119	68.8	116	67.8	63	52.5	36	36.4	
Missing	7		1			4		6		1		1		
PGR receptor														
Negative	231	45.7	34	66.7	**0.004**	75	43.6	64	38.1	60	50.0	61	61.6	**0.002**
Positive	275	54.3	17	33.3		97	56.4	104	61.9	60	50.0	38	38.4	
Missing	16		1			5		9		1		1		
HER-2 overexpression														
No overexpression	419	91.3	33	73.3	**<0.001**	129	91.5	147	96.1	95	85.6	77	82.8	**0.002**
Overexpression	40	7.9	12	26.7		12	8.5	6	3.9	16	14.4	16	17.2	
Missing	63		7			36		24		10		7		

Table 1B: Clinicopathologic characteristics of the patient population stratified for the proliferative Ki67 marker and proliferative C2P assay

	Ki67 Low		Ki67 High		*p-value*	C2P® Low		C2P® intermediate		C2P® High		*p-value*
	N	%	N	%		N	%	N	%	N	%	
Total	299	100	257	100		69	100	22	100	83	100	
Age (y)												
<45	52	17.4	53	20.6	0.523	8	11.6	3	13.6	24	28.9	0.064
45-55	71	23.7	60	23.3		11	15.9	5	22.7	19	22.9	
55-65	66	22.1	63	24.5		21	30.4	4	18.2	17	20.5	
>65	110	36.8	81	31.5		29	42.0	10	45.5	23	27.7	
Missing	0		0			0		0		0		
Grade												
I	70	24.0	17	6.6	**<0.001**	8	11.6	2	9.1	9	10.8	**0.004**
II	166	56.8	99	38.7		38	55.1	15	68.2	31	37.4	
III	56	19.2	140	54.7		23	33.3	5	22.7	43	51.8	
Missing	7		1			0		0		0		
Histologic type												
Ductal	251	85.7	245	95.7	**<0.001**	62	89.9	18	81.8	77	92.8	0.326
Lobular	42	14.3	11	4.3		7	10.1	4	18.2	6	7.2	
Missing	6		1				0.0		0.0		0.0	
Tumor stage												
pT1	129	44.2	83	32.8	0.088	19	27.9	7	31.8	21	25.3	0.121
pT2	129	44.2	136	53.8		41	60.3	13	59.1	45	54.2	
pT3/4	34	11.6	34	13.4		8	11.8	2	9.1	17	20.5	
Missing	7		4			1		0		0		
Nodal stage												
pN0	167	57.4	114	45.6	0.102	36	53.7	12	54.5	31	37.8	0.242
pN+	124	42.6	136	54.4		31	46.3	10	45.5	51	62.2	
Missing	8		7			2		0		1		
TNM stage												
Stage 0	0	0	0	0	0.066	0	0	0	0	0	0	0.052
Stage I	80	30.4	53	22.2		12	19.0	4	19.0	11	14.1	
Stage IIA	93	35.4	75	31.4		26	41.3	11	52.4	23	29.5	
Stage IIB	58	22.1	63	26.4		14	22.2	3	14.3	25	32.1	
Stage IIIA	12	4.6	18	7.5		6	9.5	1	4.8	7	9.0	
Stage IIIB	7	2.7	14	5.9		3	4.8	1	4.8	10	12.8	
Stage IIIC	13	4.9	16	6.7		2	3.2	1	4.8	2	2.6	
Stage IV	0	0	0	0		0	0	0	0	0	0	
Missing	36					6		1		5		
ER receptor												
Negative	120	40.5	117	47.2	0.120	29	42.6	6	28.6	44	57.1	**0.049**
Positive	176	59.5	131	52.8		39	57.4	15	71.4	33	42.9	
Missing	3		9			1		1		6		
PGR receptor												
Negative	126	43.2	134	54.3	**0.010**	33	49.3	12	57.1	47	60.3	0.364
Positive	166	56.8	113	45.7		34	50.7	9	42.9	31	39.7	
Missing	7		10			2		1		5		
HER-2 overexpression												
No overexpression	246	92.8	190	86.4	**0.019**	52	85.2	18	94.7	58	84.1	0.251
Overexpression	19	7.2	30	13.6		9	14.8	1	5.3	11	15.9	
Missing	34		37			8		3		14		

in the same tumor material (p=0.001, data not shown), indicating that proliferation and apoptosis are closely linked within the tumor and thus should be accounted for if one seeks optimal prognostic-predictive value determination. Survival analysis showed that a higher caspase-3 expression is significantly associated with worse OS (p<0.001, HR: 1.908, 95%CI: 1.407-2.588, table 2A), however not remaining an independent prognostic factor after multivariate correction (p=0.414). For RFP a significant relation was found for high caspase-3 expression and relapse rate (p<0.001, HR: 1.943, 95%CI: 1.356-2.783, figure 2B), again not maintaining individual prognostic value in the multivariate correction (p=0.366), (table 2B).

Ki67 expression

Ki67 expression data were available for 78% (556/714) of the patients. No relation was seen for Ki67 expression in the tumor and tumor stage or nodal involvement (table 1B). However, for high tumor grades and tumors of ductal histology (both p<0.001), PGR negative (p=0.01) and HER2 over-expressing tumors (p=0.019) a significant association was found with high Ki67 expression, corresponding with a high proliferative rate (table 1B). A statistical trend was seen for TNM stage and high Ki67 expression (p=0.066).

Patients with high Ki67 tumor expression had worse OS (p=0.007, HR: 1.348, 95%CI: 1.086-1.673), however losing its significance in the multivariate correction (p=0.564) (table 2A). A significantly higher relapse rate was noted for high Ki67 expression compared to low proliferation rate in the tumor material (p=0.021, HR: 1.339, 95%CI: 1.045-1.716, figure 2C). High Ki67 did not remain significantly associated with a higher relapse rate in the multivariate correction (p=0.269, table 2B).

C2P® risk prediction score

Data previously published by our group already described the C2P® risk prediction score as a promising prognostic marker in early BC patients [12]. Using the same cohort, 43% (304/714) of the patients had tumor material available for C2P® analyses. Significance was found for high C2P® risk score and tumor grade III scores (p=0.004), young age (<55years of age, p=0.020) and ER positive tumors (p=0.049) (table 1B). A statistical trend was seen for TNM stage (p=0.052).

No statistical relation was seen for C2P® and OS (p=0.263) (table 2A). High C2P® risk scores were significantly associated with higher relapse rates (p=0.026, HR: 1.953, 95%CI: 1.199-3.181), however not remaining its significance in the multivariate correction (figure 2D and table 2B).

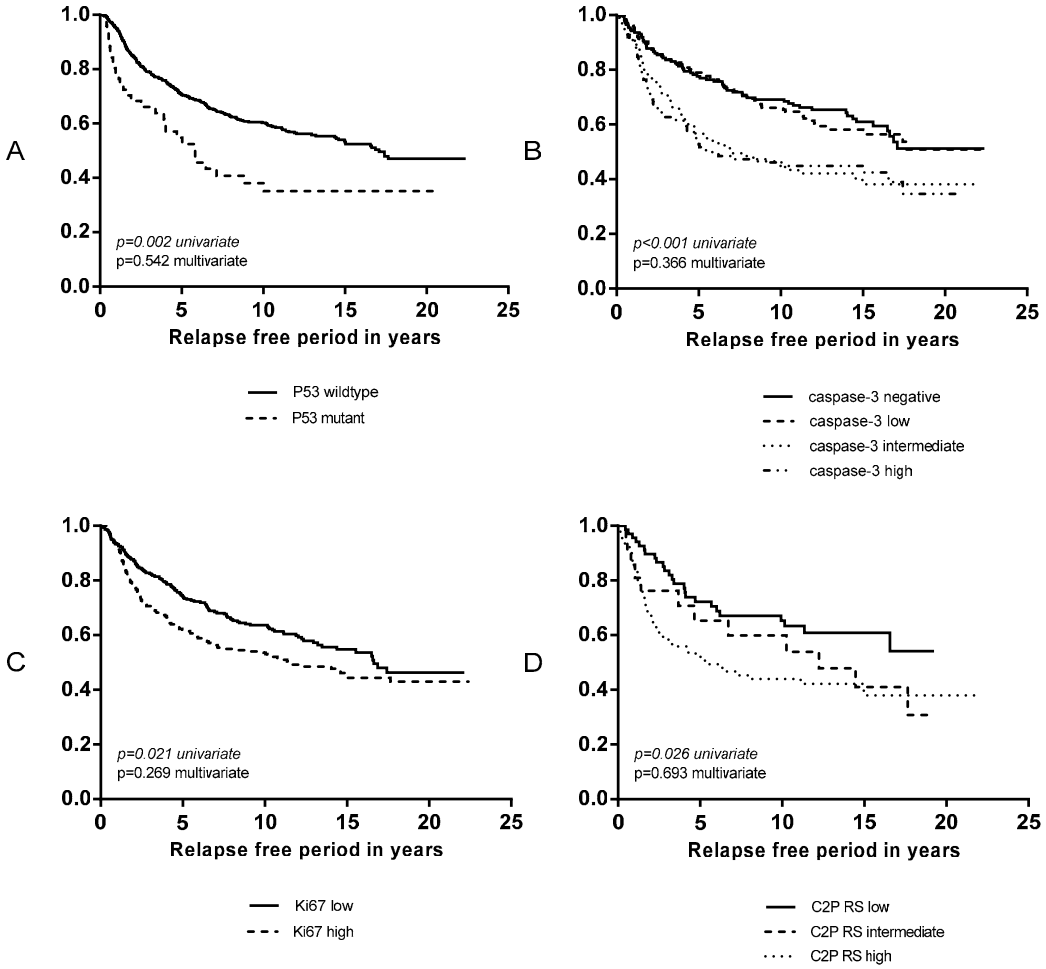


Figure 2: Relapse Free Period (RFP) curves for; **A)** tumor suppressor p53 expression **B)** active caspase-3 expression **C)** proliferative Ki67 expression and **D)** C2P®-Risk Score proliferation assay.

Combined IHC data

P53 - Ki67

From 72% (516/714) of the patients immunohistochemical data was available for both p53 and Ki67, making them eligible for the determination of the prognostic value of a combined p53-Ki67 marker. Significance was found in relation with OS and RFP, where high Ki67 combined with mutant p53 expression had the worse clinical outcome (OS: p<0.001, HR: 2.458, 95%CI: 1.654-3.655 (table 3A) and RFP: p=0.003, HR: 2.307, 95%CI: 1.479-3.598 (table 3B)) compared to a HR of 1.00 in low Ki67 combined with wildtype p53 protein expression. All other combinations of p53 and Ki67 data showed hazard ratios ranging between: >1.00 and <2.396 for the OS and >1.00 and <1.327 for the RFP. However, in the multivariate analysis for OS only the combination low Ki67 expression and mutant p53 remained significant (OS: p=0.037, table 3A).

Table 2A: Multivariable analyses for single apoptotic and proliferative markers in relation to overall survival

Characteristic	Overall Survival							
	Univariate analysis					Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Age								
<45	137	19.2	1.00		**<0.001**	1.00		**<0.001**
45-55	175	24.5	0.789	0.559-0.115		0.696	0.446-1.084	
55-65	157	22.0	1.469	1.062-2.032		1.374	0.910-2.072	
>65	245	34.3	1.914	1.914-3.395		2.185	1.499-3.185	
Grade								
I	116	16.5	1.00		**<0.001**	1.00		0.718
II	342	48.7	1.380	1.012-1.879		1.057	0.679-1.645	
III	244	34.8	1.844	1.345-2.527		1.184	0.721-1.943	
Histological type								
Ductal	638	90.6	1.00		0.125			
Lobular	66	9.4	0.778	0.565-1.072				
Tumor stage								
pT1	289	41.6	1.00		**<0.001**	1.00		**0.003**
pT2	328	47.3	1.836	1.471-2.292		1.354	0.984-1.864	
pT3	44	6.3	2.072	1.390-3.089		1.696	0.986-2.915	
pT4	33	4.8	5.573	3.764-8.251		2.809	1.628-4.847	
Nodal stage								
Negative	381	54.9	1.00		**<0.001**	1.00		**<0.001**
Positive	313	45.1	2.105	1.725-2.568		1.783	1.360-2.338	
ER status								
Negative	288	42.3	1.00		0.266			
Positive	393	57.7	0.892	0.730-1.091				
PGR status								
Negative	316	47.4	1.00		**0.049**	1.00		0.948
Positive	351	52.6	0.818	0.670-0.999		1.009	0.768-1.327	
HER-2 status								
Negative	520	89.8	1.00		**<0.001**	1.00		**0.047**
Positive	59	10.2	1.861	1.359-2.548		1.511	1.006-2.269	
P53								
Wildtype	522	90.9	1.00		**<0.001**	1.00		**0.009**
Mutant	52	9.1	2.150	1.549-2.983		1.776	1.158-2.726	
C2P[*]								
Low	69	39.7	1.00		0.263			
Intermediate	22	12.6	0.951	0.498-1.816				
High	83	47.7	1.355	0.901-2.037				
Caspase3								
Absent	177	30.8	1.00		**<0.001**	1.00		0.414
Low	177	30.8	0.975	0.727-1.306		0.760	0.529-1.091	
Intermediate	121	21.0	1.575	1.167-2.128		0.957	0.668-1.370	
High	100	17.4	1.908	1.407-2.588		0.984	0.669-1.447	
Ki67								
Low	299	53.7	1.00		**0.007**	1.00		0.564
High	257	46.3	1.348	1.086-1.673		1.089	0.816-1.453	

Table 2B: Multivariable analyses for single apoptotic and proliferative markers in relation to relapse free period

Characteristic	Relapse Free Period							
	Univariate analysis					Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Age								
<45	137	19.2	1.00		0.357			
45-55	175	24.5	0.755	0.547-1.042				
55-65	157	22.0	0.898	0.648-1.246				
>65	245	34.3	0.824	0.605-1.122				
Grade								
I	116	16.5	1.00		**<0.001**	1.00		0.845
II	342	48.7	1.460	1.013-2.106		0.927	0.454-1.894	
III	244	34.8	2.158	1.490-3.125		0.816	0.373-1.783	
Histological type								
Ductal	638	90.6	1.00		0.209			
Lobular	66	9.4	1.265	0.877-1.824				
Tumor stage								
pT1	289	41.6	1.00		**<0.001**	1.00		**0.046**
pT2	328	47.3	1.716	1.336-2.203		1.227	0.723-2.081	
pT3	44	6.3	1.955	1.242-3.078		0.767	0.277-2.127	
pT4	33	4.8	4.011	2.476-6.499		3.634	1.521-8.680	
Nodal stage								
Negative	381	54.9	1.00		**<0.001**	1.00		**<0.001**
Positive	313	45.1	2.964	2.349-3.739		2.462	1.519-3.991	
ER status								
Negative	288	42.3	1.00		0.377			
Positive	393	57.7	0.901	0.716-1.135				
PGR status								
Negative	316	47.4	1.00		0.235			
Positive	351	52.6	0.870	0.691-1.095				
HER-2 status								
Negative	520	89.9	1.00		**0.002**	1.00		0.811
Positive	59	10.1	1.772	1.229-2.555		0.909	0.417-1.981	
P53								
Wildtype	522	90.9	1.00		**0.002**	1.00		0.542
Mutant	52	9.1	1.838	1.255-2.692		1.288	0.571-2.906	
C2P*								
Low	69	39.7	1.00		**0.026**	1.00		0.693
Intermediate	22	12.6	1.638	0.822-3.264		0.807	0.443-1.468	
High	83	47.7	1.953	1.199-3.181		1.363	0.550-3.377	
Caspase3								
Absent	177	30.8	1.00		**<0.001**	1.00		0.366
Low	177	30.8	1.060	0.754-1.489		1.208	0.613-2.381	
Intermediate	121	21.0	1.860	1.323-2.615		1.564	0.815-3.004	
High	100	17.4	1.943	1.356-2.783		1.865	0.849-4.099	
Ki67								
Low	299	53.7	1.00		**0.021**	1.00		0.269
High	257	46.3	1.339	1.045-1.716		1.304	0.815-2.087	

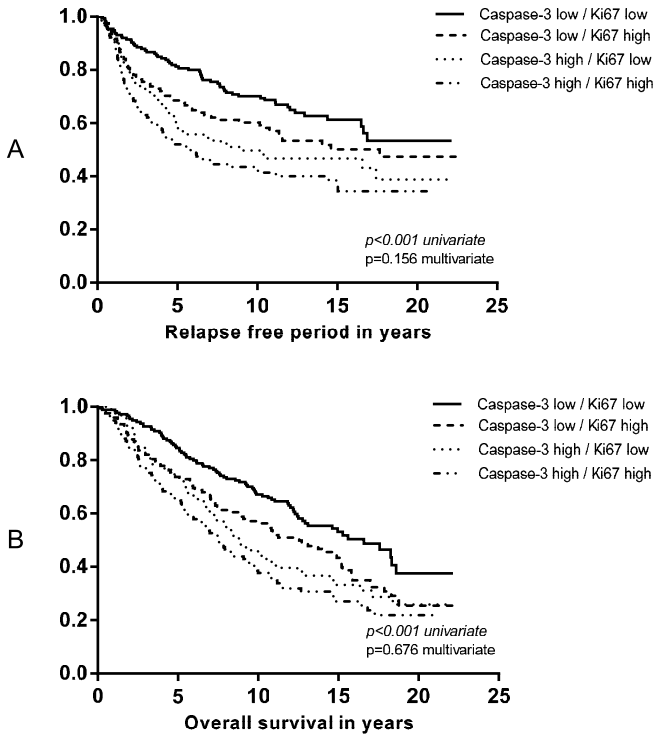


Figure 3: A) Relapse Free Period (RFP) curves for combined analysis of active caspase-3 and the proliferative marker Ki67. Both single markers were grouped into low or high expression in the tumor tissue (for active caspase-3 the division was made based on the RFP curve seen in Figure 2B) after which they were combined. **B)** The same was done for the Overall Survival (OS) curves for this combined marker.

When we compared the highest hazard ratios of the single markers for p53 (OS HR: 2.150 and RFP HR: 1.838) and Ki67 (OS HR: 1.348 and RFP HR: 1.339), we concluded that by combining these two markers in one combination (p53-Ki67) we induce additive strength to his prognostic-predictive marker, leading to a higher hazard ratio (OS HR: 2.458 and RFP HR: 2.307) than the single biomarker hazard ratios (table 4A and 4B).

P53 - active caspase-3

Seventy four percent (529/714) of the patients had both p53 and active caspase-3 IHC data available. Again for both OS and RFP significance was found with the combined p53-caspase-3 biomarker. Mutant p53 protein expression combined with high active caspase-3 expression resulted in the highest HR for death in OS (p<0.001, HR: 3.012, 95%CI: 2.044-4.439, table 3A) and the RFP (p<0.001, HR: 2.673, 95%CI: 1.703-4.195, table 3B). For the OS this remained an independent prognostic biomarker after multivariate correction (p=0.037, HR: 2.008, 95%CI: 1.241-3.249, table 3A).

Again a higher hazard ratio (HR OS: 3.012 and HR RFP: 2.673) was seen when patients with the clinically most adverse expression pattern of single markers p53 (OS HR: 2.150 and RFP HR: 1.838) and active caspase-3 (OS HR: 1.908 and RFP HR: 1.943) were compared to the HR of the combined p53-caspase-3 marker, indicating the probability of an additive quality (table 4A and 4B).

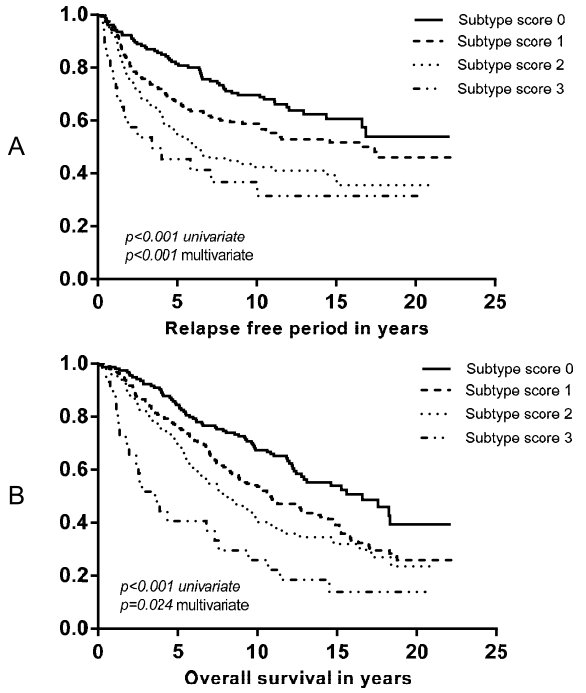


Figure 4: Apoptotic-proliferative tumor subtypes: all curves and the univariate p-values are based on the entire patient population in whom all markers (p53, active caspase-3 and Ki67) are known. *Multivariate p-values are based on only grade I breast tumors from this cohort.

Ki67 - active caspase-3

Data of both Ki67 and active capase-3 expression was available from 33% (239/714) of the patients of this cohort. Both high expression of Ki67 and active caspase-3 had a significant worse OS (p<0.001, HR: 3.012, 95%CI: 2.044-4.439 (table 3A)) and RFP (p<0.001, HR: 2.258, 95%CI: 1.599-3.189 (table 3B)) compared to low Ki67 with low Caspase-3 expression (figure 3A and 3B). In the multivariate analyses, neither the RFP (p=0.156) nor the OS (p=0.676) remained an individual prognostic marker. Again additive properties were seen for the combined biomarker: Ki67-active-caspase3 (OS HR: 2.137 and RFP HR: 2.258), compared to the single biomarkers (Ki67**:** HR-OS: 1.348 and HR-RFP: 1.339; Caspase-3: HR-OS: 1.908 and HR-RFP: 1.943) (table 4A and 4B).

C2P® in combination with p53 or active caspase-3 or Ki67

Neither p53 (20% (142/714)), active caspase-3 (21% (147/714)) nor Ki67 (21% (150/714)) combined with C2P®-RS showed a statistical significant relation with outcome.

Apoptotic - proliferative tumor subtype

Due to the supporting outcome of the combined markers, we constructed a prognostic model based on the expression pattern of the three risk contributing markers: p53, active caspase-3 and Ki67(488/714, 68%). C2P® was not included in this model due to the limited number of patients in whom this marker was determined (frozen tumor tissue was needed), leading to lack of power in the combined analysis. Expression scores of

Table 3A: Multivariable analyses for combined apoptotic and proliferative markers in relation to overall survival

Characteristic	Overall Survival combination(s)							
			Univariate analysis			Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Ki67 - p53 *								
Low-wildtype	259	50.2	1.00		**<0.001**	1.00		**0.037**
Low-mutant	12	2.3	2.396	1.259-4.561		2.377	1.113-5.079	
High-wildtype	207	40.1	1.296	1.019-1.646		1.081	0.813-1.437	
High-mutant	38	7.4	2.458	1.654-3.655		1.717	1.033-2.852	
Ki67 - caspase3*								
low-negative	86	36.0	1.00		**<0.001**	1.00		0.676
high-negative	56	23.4	1.492	1.094-2.035		1.111	0.752-1.643	
low-positive	40	16.7	1.737	1.249-2.415		1.160	0.782-1.720	
high-positive	57	23.9	2.137	1.575-2.899		1.282	0.855-1.923	
Caspase3 - p53*								
negative-wildtype	300	56.7	1.00		**<0.001**	1.00		**0.037**
negative-mutant	12	2.3	1.694	0.831-3.451		1.480	0.594-3.689	
positive-wildtype	179	33.8	1.580	1.242-2.009		1.095	0.834-1.439	
positive-mutant	38	7.2	3.012	2.044-4.439		2.008	1.241-3.249	
C2P® - p53								
Low-wildtype	49	34.5	1.00		0.313			
Low-mutant	8	5.6	1.865	0.765-4.548				
Intermed-wildtype	14	9.9	1.122	0.508-2.479				
Intermed-mutant	2	1.4	0.944	0.128-6.963				
High-wildtype	65	45.8	1.338	0.820-2.185				
High-mutant	4	2.8	3.612	1.089-11.984				
Caspase3 - C2P®								
negative-low	30	20.4	1.0		0.697			
negative-intermediate	15	10.2	1.267	0.579-2.772				
negative-high	32	21.8	1.096	0.568-2.112				
positive-low	29	19.7	0.995	0.507-1.950				
positive-intermediate	4	2.7	0.371	0.049-2.791				
positive-high	37	25.2	1.395	0.753-2.584				
Ki67 - C2P®								
low-low	29	19.3	1.00		0.280			
high-low	30	20.0	1.679	0.834-3.381				
low-intermediate	13	8.7	1.573	0.652-3.796				
high-intermediate	7	4.7	0.571	0.128-2.536				
low-high	32	21.3	1.801	0.901-3.601				
high-high	39	26.0	1.947	0.999-3.793				

Table 3A: (continued)

Characteristic	Overall Survival combination(s)							
			Univariate analysis			Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
CDK1 - caspase3*								
<median of the ratio	54	50.5	1.00		**0.014**	1.00		**0.015**
>median of the ratio	53	49.5	1.877	1.134-3.108		2.137	1.161-3.934	
CDK2 - caspase3								
<median of the ratio	58	50.4	1.00		0.179			
>median of the ratio	57	49.6	1.407	0.856-2.313				
CDK1&2 - caspase3								
<median of the ratio	50	47.6	1.00		0.124			
>median of the ratio	55	52.4	1.504	0.894-2.530				
Subtype**								
Score 0	46	65.7	1.00		**0.050**	1.00		**0.024**
Score 1	20	28.6	1.964	0.879-4.387		0.903	0.277-2.947	
Score 2	4	5.7	3.529	1.156-10.772		7.344	1.538-35.066	
Score 3	0	0.0	-	-		-	-	
Subtype***								
Score 0	52	45.2	1.00		**0.002**	1.00		**0.056**
Score 1	47	40.9	1.606	0.815-3.165		0.986	0.460-2.111	
Score 2	13	11.3	1.238	0.444-3.454		0.802	0.234-2.751	
Score 3	3	2.6	11.711	3.271-41.925		8.107	1.694-38.805	
Subtype****								
Score 0	46	32.1	1.00	0.955-2.697	**0.043**	1.00		0.255
Score 1	60	42.0	1.605	0.926-3.119		1.064	0.610-1.858	
Score 2	30	21.0	1.700	1.384-8.512		1.058	0.555-2.018	
Score 3	7	4.9	3.433			2.670	0.992-7.187	

*All adjusted for age, grade, pathological tumor stage, nodal stage, PGR and HER-2
****Subtypes** only for grade I tumors, adjusted for age, pathological tumor stage, nodal stage, PGR and HER-2
***** **Subtypes** only for TNM stage I patients, adjusted for age, PGR and HER-2
****** **Subtype** only for TNM stage IIA patients, adjusted for age, PGR and HER-2
All combinations were tested in separate models

these markers were dichotomized. For all patients one point was allocated for each marker expressed, indicating one risk factor present; resulting in a score of zero for patients without expression of any marker and a score of three for patients with all markers highly expressed. The apoptotic-proliferative subtype model was significantly associated with the molecular subtype of the tumor, in which higher apoptotic-proliferative scores were related to more aggressive molecular tumor subtypes (HER2+ type and Basal

Table 3B: Multivariable analyses for combined apoptotic and proliferative markers in relation to relapse free period

Characteristic	Relapse Free Period Combination(s)							
			Univariate analysis			Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Ki67 - p53*								
Low-wildtype	259	50.2	1.00		**0.003**	1.00		0.538
Low-mutant	12	2.3	1.327	0.541-3.256		1.503	0.591-3.820	
High-wildtype	207	40.1	1.257	0.954-1.657		0.963	0.700-1.326	
High-mutant	38	7.4	2.307	1.479-3.598		1.356	0.760-2.419	
Ki67 - caspase3*								
low-negative	86	36.0	1.00		**<0.001**	1.00		0.156
high-negative	56	23.4	1.437	0.998-2.069		2.363	1.049-5.325	
low-positive	40	16.7	1.804	1.238-2.628		1.283	0.506-3.253	
high-positive	57	23.9	2.258	1.599-3.189		1.942	0.890-4.240	
Caspase3 - p53*								
negative-wildtype	300	56.7	1.00		**<0.001**	1.00		0.075
negative-mutant	12	2.3	1.653	0.726-3.762		1.304	0.405-4.195	
positive-wildtype	179	33.8	1.811	1.379-2.378		1.353	0.992-1.844	
positive-mutant	38	7.2	2.673	1.703-4.195		1.943	1.121-3.368	
C2P® - p53								
Low-wildtype	49	34.5	1.00		0.331			
Low-mutant	8	5.6	1.698	0.572-5.039				
Intermed-wildtype	14	9.9	1.758	0.764-4.045				
Intermed-mutant	2	1.4	-					
High-wildtype	65	45.8	1.755	0.992-3.104				
High-mutant	4	2.8	3.828	0.883-16.592				
Caspase3 - C2P®								
Negative-low	30	20.4	1.00		0.226			
Negative-intermediate	15	10.2	1.297	0.510-3.299				
Negative-high	32	21.8	1.269	0.593-2.716				
Positive-low	29	19.7	0.791	0.341-1.831				
Positive-intermediate	4	2.7	1.670	0.471-5.927				
Positive-high	37	25.2	1.935	0.957-3.915				
Ki67 - C2P®*								
low-low	29	19.3	1.00		0.069	1.00		0.202
high-low	30	20.0	3.704	1.366-10.045		4.257	1.413-12.822	
low-intermediate	13	8.7	3.431	1.047-11.249		3.919	1.062-14.459	
high-intermediate	7	4.7	2.973	0.794-11.127		3.627	0.692-18.993	
low-high	32	21.3	3.991	1.471-10.831		3.098	1.083-8.865	
high-high	39	26.0	4.770	1.804-12.614		3.130	1.043-9.398	

Table 3B: (continued)

Characteristic	Relapse Free Period Combination(s)							
	Univariate analysis					Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
CDK1 - caspase3*								
<median of the ratio	54	50.5	1.00		**0.016**	1.00		**0.009**
>median of the ratio	53	49.5	2.071	1.144-3.748		2.460	1.248-4.849	
CDK2 - caspase3*								
<median of the ratio	58	50.4	1.00		**0.003**	1.00		**0.012**
>median of the ratio	57	49.6	2.560	1.385-4.731		2.501	1.228-5.096	
CDK1&2 - caspase3*								
<median of the ratio	50	47.6	1.00		**0.049**	1.00		0.121
>median of the ratio	55	52.4	1.842	1.003-3.383		1.818	0.854-3.869	
Subtype**								
Score 0	46	65.7	1.00		0.125	1.00		**0.001**
Score 1	20	28.6	1.573	0.626-3.958		1.119	0.316-3.964	
Score 2	4	5.7	3.609	1.016-12.820		21.396	4.111-111.351	
Score 3	0	0.0	-	-		-	-	
Subtype***								
Score 0	52	45.2	1.00		0.059			
Score 1	47	40.9	1.796	0.805-4.007				
Score 2	13	11.3	1.485	0.463-4.767				
Score 3	3	2.6	7.956	1.717-36.863				
Subtype****								
Score 0	46	32.1	1.00		0.259			
Score 1	60	42.0	1.671	0.913-3.057				
Score 2	30	21.0	1.513	0.738-3.101				
Score 3	7	4.9	2.503	0.836-7.499				

*all adjusted for grade, pathological tumor stage, nodal stage and HER-2

****Subtypes** only for grade I tumors, adjusted for pathological tumor stage, nodal stage and HER-2

***** **Subtypes** only for TNM stage I patients, adjusted for age and HER-2

****** **Subtype** only for TNM stage IIA patients, adjusted for age and HER-2

All combinations were tested in separate models

like) and negative to low apoptotic-proliferative scores to the less aggressive Luminal A and Luminal B molecular tumor subtypes (p<0.001).

For the OS (p<0.001, *score 1*: HR 1.569 (95%CI: 1.171-2.103); *score 2*: HR 1.922, 95%CI: 1.386-2.667); *score 3*: HR 3.657 (95%CI: 2.297-5.822)) and RFP (p<0.001, *score 1*: HR 1.468 (95%CI: 1.046-2.061); *score 2*: HR 2.122 (95%CI: 1.473-3.059); *score 3*: HR 3.058 (95%CI: 1.792-5.218) significant univariate association was found (figure 4). When the cohort was split on tumor grade, we found a significant association in the multivariate corrected

analyses for both the OS (p=0.024) and RFP (p=0.001) for only grade I tumors (figure 4 and table 3A and B). When the cohort was split on TNM stage, we found that only stage I and IIA patients had a significant outcome in the univariate OS analysis for the apoptotic-proliferative subtype model. This remained borderline significant in the multivariate corrected analysis for OS in TNM stage I patients (p=0.056, table 3A).

Biochemical assay active caspase-3

Eighteen percent (126/714) of the patients had frozen material available for a biochemical caspase-3 assay. For analysis, outcomes were converted into a categorical parameter (< and > the median value (2.74 pmol AFC/min/mg protein)). In the univariate analyses, neither for OS (p=0.7) or RFP (p=0.5) a significant relation was found herewith.

When caspase-3 assay data were combined with the C2P® data (75/714,10.5%), a significant association was found for the C2P® risk prediction and the dichotomized biochemical caspase-3 expression (low/high). Results showed that high C2P® was significantly associated with high biochemical caspase-3 expression. However, there was no significant relation regarding OS (p=0.670) or RFP (p=0.628) for this combination (data not shown).

Next, we calculated the ratio between CDK-1activity, a crucial contributor of the C2P® biomarker, and biochemical caspase-3 (107/714,15%). The ratio was transformed in a dichotomous variable by use of the median value due to a skewed distribution. Significant associations, in the favor of weaker proliferative characteristics of the tumor, were seen in the RFP (p=0.016) and OS (p=0.014), both maintaining their significance in the multivariable analyses (RFP: p=0.009, HR 2.460, 95%CI: 1.248-4.849 and OS: p=0.015, HR 2.137, HR1.161-3.934 (table 3A and 3B respectively). Combined CDK-2 and biochemical

Table 4A: Single marker and combined marker hazard ratios for overall survival

Marker-1	HR	*p-value*	Marker-2	HR	*p-value*	Combined	HR	*p-value*	95% CI
P53	2.2	<0.001	**Ki67**	1.3	0.007	**P53-Ki67**	2.5	<0.001	1.7-3.7
P53	2.2	<0.001	**Caspase-3**	1.9	<0.001	**P53-caspase-3**	3.0	<0.001	2.0-4.4
Ki67	1.3	0.007	**Caspase-3**	1.9	<0.001	**Ki67-caspase-3**	2.1	<0.001	1.6-2.9

An overview of single (Marker 1 and 2) and combined marker hazard ratios (HR), as seen in tables 3B and 4B. All hazard ratios and p-values shown in this table are univariate results.

Table 4B: Single marker and combined marker hazard ratios for relapse free period

Marker-1	HR	*p-value*	Marker-2	HR	*p-value*	Combined	HR	*p-value*	95% CI
P53	1.8	0.002	**Ki67**	1.3	0.021	**P53-Ki67**	2.3	0.003	1.5-3.6
P53	1.8	0.002	**Caspase-3**	1.9	<0.001	**P53-caspase-3**	2.7	<0.001	1.7-4.2
Ki67	1.3	0.021	**Caspase-3**	1.9	<0.001	**Ki67-caspase-3**	2.3	<0.001	1.6-3.2

An overview of single (Marker 1 and 2) and combined marker hazard ratios (HR), as seen in tables 3A and 4A. All hazard ratios and p-values shown in this table are univariate results.

caspase-3 (115/174, 16.1%) only showed a significant association in the RFP (p=0.003) in favor of a higher apoptotic rate, remaining an independent factor after multivariate correction with p-value=0.012, HR 2.501, 95%CI: 1.228-5.096 (table 3B).

DISCUSSION

Over the last few years the impact of single apoptotic and proliferative markers on tumor progression and patient outcome in BC was thoroughly investigated but often showed contradictory results [13-15]. An explanation could be the misinterpretation that emanates from single apoptotic and proliferative marker expression due to the fact that they do not reflect the interaction with one another. In this manuscript we circumvented this shortcoming by combining dual markers and constructed a apoptotic-proliferative subtype model, in which all important markers were incorporated to prevent misinterpretation of these closely linked pathways.

It is hypothesized that imbalanced presence of apoptosis and proliferation is a hallmark for tumor aggressiveness. Consequently, this apoptotic-proliferative misbalance results in either progression or inhibition of tumor growth, depending on the direction of the outcome of the balance.

For both single and combined markers, independent of being a proliferative or apoptotic marker, high expression rates are associated with higher hazard ratios, in which the majority of combined markers have an additive effect on one another leading to higher hazard ratios.

For active caspase-3 our data showed counter intuitive worse clinical outcome when highly expressed, thus corresponding with a high apoptotic rate in the tumor [2]. Combined analyses demonstrated that this poor outcome was associated with high proliferative Ki67 presence in the breast tumor, being a good example of how single marker experiments can be misinterpreted. It should be clear that the high proliferative Ki67 marker apparently dominates the clinical outcome of these high active caspase-3 expressing tumors. It could be considered that the apoptotic marker can merely keep up with the high proliferation rate of the tumor, resulting in excess proliferation, consequently leading to progression of the BC. Nevertheless, this difference in apoptosis induction in tumors expressing high levels of Ki67 is also a tumor characteristic worthy of observation and serves as an excellent marker for more accurate prognostication. The combined high apoptosis - high proliferation relation seen in this study was also seen in work done by Parton *et al* [16].

Biochemical assay data retrieved from this study strengthens the conclusion found in IHC focusing on combined marker analyses. Our assay results are supported by data

from Zeestraten *et al.* whom also showed the high prognostic value of CDK1 in stage II colon cancer patients [17].

By constructing an apoptotic-proliferative tumor subtyping model, we demonstrated that the combination of the expression rates of all relevant apoptotic and proliferative markers leads to a valuable prognostic indicator in grade I breast tumors. To the best of our knowledge, we are the first group providing such detailed insight in the tumor apoptosis and proliferation ratio in BC, showing that this cell proliferative and death ratio is of crucial value compared to single marker interpretation in the control of tumor progression and therefore in determining patient prognosis. Results of this study lead to assume that apoptotic-proliferative subtyping in grade I tumors could be of crucial importance in identifying patients with a low tumor grade with an increased risk of poor prognosis, being those containing the most detrimental apoptotic-proliferative marker combination. With the increased tendency of earlier diagnosis due to better BC awareness and the introduction of population based screening, it comes as no surprise that the BC incidence has tilted to more early stage, low grade breast tumors [18]. Introducing our newly designed apoptotic-proliferative tumor subtyping model will lead to targeted selection of the grade I BC patients that would truly benefit of an aggressive therapeutic regime due to an adverse apoptotic-proliferative balance. In the current state of affairs, where over- and under- treatment leads to considerable debate in clinical practice, identification of patient groups for implementation of personalized therapy will become increasingly important.

This cohort consisted of BC patients diagnosed and treated between 1985 and 1996, this time frame also marking the beginning of adjuvant hormonal therapy which led to less protocolled regimes and documentation hereof. Also, the chemotherapy given at that time point clearly does not meet today's standards and therefore no clinical consequence could be deduced. Despite these shortcomings, this study clearly states high prognostic value. Further research should validate our findings and focus on the predictive value in light of today's therapeutic standards.

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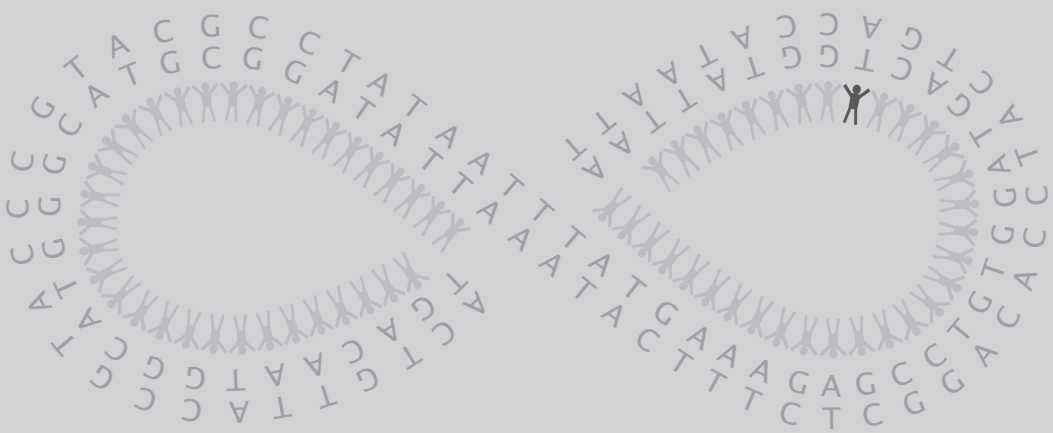

Chapter 3

Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients

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ABSTRACT

Introduction

There is strong evidence that the host's cellular immune response is linked to tumor progression, however its impact on patient outcome in breast cancer is poorly understood. The purpose of this study is to define tumor immune subtypes, focusing on cellular immune responses and investigate their prognostic effect in breast cancer patients.

Methods

Our training (n=440) and validation cohort (n=382) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1996. Tumor tissue sections were immunohistochemically stained for CD8 (CTL) and PEN5 (NK cells). Tumor expression of classical and non-classical HLA class I, and tumor-infiltrating Tregs were previously determined. Tumor immune subtypes were constructed based on quantification of these markers and biological rationale.

Results

High, intermediate and low immune susceptible tumor immune subtypes were found in respectively 16%, 63% and 20% of patients in the training cohort and 16%, 71% and 13% in the validation cohort. The subtypes showed to be statistically significant prognostic in multivariate analyses for relapse free period (RFP) ($p<0.0001$, intermediate versus high: hazard ratio (HR) 1.95; low versus high HR 2.98) and relative survival (RS) ($p=0.006$, intermediate versus high HR 3.84; low versus high: HR 4.26). Validation of these outcome analyses confirmed the independent prognostic associations: RFP ($p=0.025$) and RS ($p=0.040$).

Conclusion

The tumor immune subtypes that we present represent a prognostic profile with solid underlying biological rationale and with high discriminative power confirmed in an independent validation cohort. Our results emphasize the importance of tumor immune surveillance in the control of tumor development and, therefore, in determining patient prognosis. Tumor immune subtype profiling is promising for prognosis prediction and the achievement of tailored treatment for breast cancer patients.

INTRODUCTION

Breast cancer is the most commonly diagnosed female cancer and is the leading cause of death from cancer in women in the western world [1]. Decisions regarding use of systemic therapy in primary non-metastasized breast cancer patients are mainly based on prognostic and predictive factors like lymph node status, tumor size, grade, hormone receptor and human epidermal growth factor receptor 2 (HER2) expression [2]. However, currently these do not provide optimal risk-stratification. Therefore, additional prognostic and predictive information is sought in order to improve tailored treatment for patients with breast cancer.

There is strong evidence that a host's cellular immune response is able to control tumor progression [3]. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from such immune recognition [4]. Various interactions underlie the balance between immune control and tumor escape (Figure 1). Cytotoxic T-lymphocytes (CTL) are capable of recognizing tumor-associated antigens presented by classical human leukocyte antigen (HLA) class I (HLA-A, HLA-B, HLA-C) on the tumor cell surface. In order to avoid immune recognition from CTL, cancer cells may lose expression of classical HLA class I [5]. However, this makes them prone to natural killer (NK) cell recognition [6]. Non-classical HLA class I molecules (HLA-E, HLA-G) play a crucial role in immune surveillance by NK-cells. Expression of these molecules on the cell surface causes an inhibitory effect on NK-cell attack [6-8]. Another tumor escape mechanism from immunosurveillance is attraction and induction of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment [9].

A variety of immune reactions have been found to date in breast cancer. Studies have indicated that breast cancer is highly immunogenic and often shows high numbers of tumor-infiltrating lymphocytes [10;11]. However, as previously reported by our group and others, loss of classical HLA class I expression, upregulation of non-classical HLA-E and HLA-G expression [12-14] and induction and infiltration of Treg in the tumor microenvironment [13;15-17] are frequent events in breast cancer, indicating that breast tumors are also capable of evading immune recognition. Together, this suggests that complex interactions take place between breast tumor cells and cells from the immune system [18]. Therefore, to get a good perspective on the effects of the immune system on tumor progression and patient outcome, such interactions should be accounted for. Indeed, previous studies of our group and others showed interactions between classical HLA class I and Treg, where loss of HLA class I in combination with presence of Treg in the tumor microenvironment resulted in a worse patient's outcome [16;18]. This was also the case for classical HLA class I and HLA-E and HLA-G tumor expression, where HLA-E and HLA-G expression resulted in a worse patient outcome exclusively in patients with loss

of tumor expression of classical HLA class I [12]. Together, this emphasizes the importance of research on combinations of markers of immune surveillance together with markers of tumor immune escape.

We defined tumor immune subtypes, with focus on cellular immune responses, based on tumor expression of classical HLA class I, HLA-E and HLA-G, and tumor infiltration of CTL, NK cells, and Treg. The aim was to investigate the distribution and prognostic effect of the different immune subtypes in a large cohort of breast cancer patients and subsequently validate these effects on a second cohort of breast cancer patients.

PATIENTS AND METHODS

Patients and tumors

The total patient population comprised all retrospectively assessed primary non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1996 (n=822). Patients with bilateral tumors or a prior history of cancer (other than basal cell carcinoma or cervical carcinoma *in situ*) were excluded. The following data were known: age, tumor grade, histological type, TNM stage, local and systemic therapy, time of locoregional/distant tumor recurrence, survival time, and expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [19]. All tumors were graded according to current pathological standards by an experienced breast cancer pathologist. Approval for the study was obtained from the Leiden University Medical Center Medical Ethics Committee. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). The REMARK criteria were respected for analyses of the immune subtypes and writing of this article [32]. No statistically significant differences were found in patient or tumor characteristics between the training cohort (1985-1990 (n=440)) and a validation set (1990-1996 (n=382)).

Immunohistochemistry

Mouse antibody against CD8 (ab17147 clone 144B: AbCam, UK) and PEN5 (IM2354, clone 5H10.21.5: Beckman Coulter, NL) were used for immunohistochemical staining of respectively CTL and NK cells in tissue sections cut from intra-operatively derived FFPE tumor material according to previously described standard protocols [16]. Previously described were immunohistochemical stainings for expression of classical HLA class (anti-HLA-A and anti-HLAB/C; Dr. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands, HLA-E (ab2216 clone MEM-E/02: AbCam, UK), HLA-G (kindly provided

by Prof. Dr. P.J. Van de Elsen) and Treg infiltration (FoxP3, ab20034 clone 236A/E7: AbCam, UK) [12;16].

Evaluation of immunostaining

Quantification of CD8-positive stained cells and PEN5-positive stained cells in microscopical fields containing tumor was performed by two independent observers in a blinded manner in both training and validation cohorts. CD8 tumor infiltration was classified in two groups: (1) low CTL infiltration, 0-100 CD8 tumor infiltrating cells/mm^2; (2) high CTL infiltration, 100-3000 CD8 infiltrating cells/mm^2. For PEN5, only few positive infiltrating cells were seen. Therefore, any versus none PEN5-positive infiltrating cell were considered as presence and absence of NK cell infiltration respectively. Expression of classical HLA class I, HLA-E and HLA-G and Treg infiltration were previously categorized respectively as loss versus expression, no expression versus expression and absent versus present infiltration [12;16].

Statistical analysis

Statistical analyses were performed using the statistical packages SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA) and Stata (version 10.0 for Windows, StataCorp, College Station, TX, USA). Cohen's kappa coefficient represented the inter-observer agreement. The χ^2 test evaluated associations between clinicopathological parameters and tumor immune subtypes. Relapse-free period was defined as the time from date of surgery until any recurrence and was reported as cumulative incidence function, after accounting for death as competing risk. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of curves. Cox proportional hazard analysis calculated univariate and multivariable analysis for relapse-free period. Relative survival was calculated by the Hakulinen method as the ratio of the survival observed among the cancer patients and the survival that would have been expected based on the corresponding (age, sex, and year) general population. National life tables were used to estimate expected survival. Relative excess risks of death were estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times. Hazard ratios and relative risks served as indications for respectively risk of relapse and relative risk of survival. Variables with a P-value of < 0.10 in univariate analysis were entered in multivariable analysis.

RESULTS

Patient and tumor characteristics

Tumor material was available of 86% (380/440) and 87% (334/382) of the patients in the training cohort and validation cohort respectively. For the training cohort the median age of patients was 58 years (range= 23-96 years) and the median follow-up was 19 years (range= 0.1-22). For the validation cohort the median age and follow-up of patients were respectively 58 years (range= 32-90) and 13 years (range= 0.2-17). Clinicopathological and treatment characteristics are shown in supplementary tables (training cohort table 1A, 1B; validation cohort table 2A, 2B).

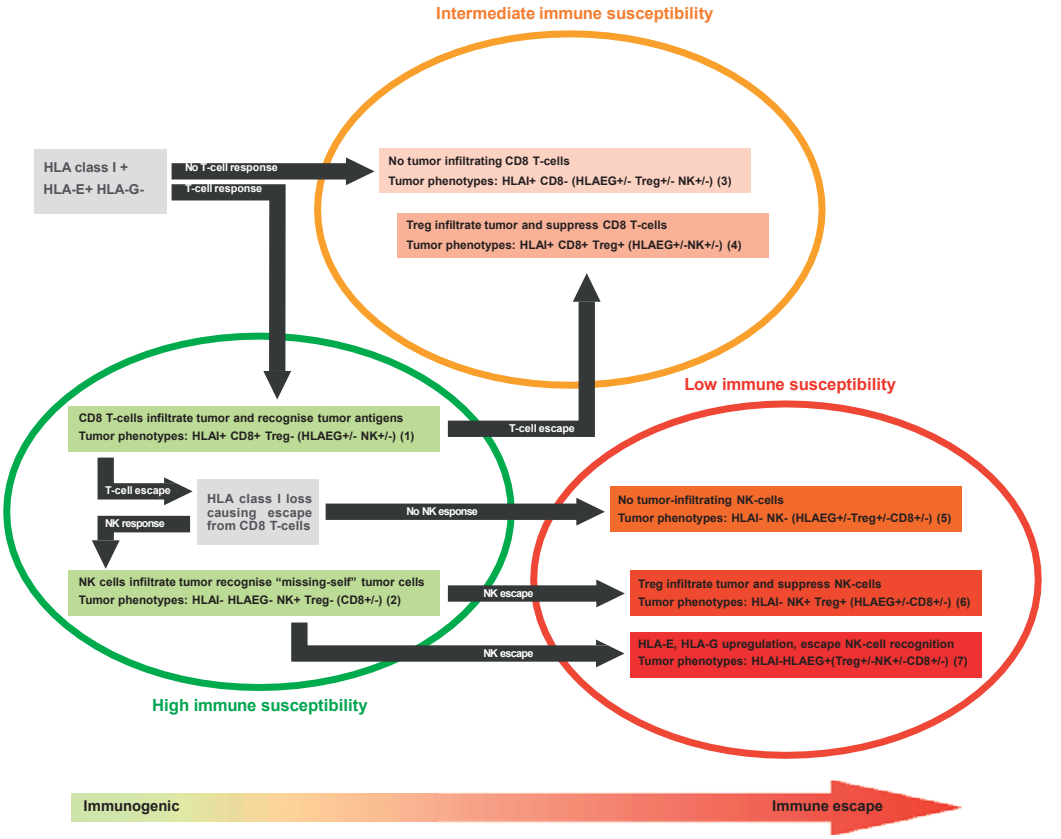

Figure 1: Tumor immune subtypes showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 tumor immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape and low immune susceptibility (red), concerning combinations of CTL infiltration, NK cell infiltration, Treg infiltration, classical HLA class I tumor expression and HLA-EG tumor expression. Tumor immune subtypes were clustered by combining from the original tumor immune subtypes groups as shown in by encircled groups (high immune susceptible) clustered (1) and (2)(green circle), (intermediate immune susceptible) clustered (3) and (4)(orange circle), (low immune susceptible) clustered (5), (6) and (7) (red circle).

Table 1: Cox univariate and multivariate analysis in the training cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 3 groups that are described in the results section.

Characteristic	N	Relapse Free Period						Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
		HR	95% CI	*P*	HR	95% CI	*P*	RER	95% CI	*P*	RER	95% CI	*P*
Age													
<40	74	1.00		0.354				1.00		**0.048**	1.00		**0.031**
40-50	92	0.87	0.58-1.33					0.79	0.49-1.28		0.60	0.32-1.12	
50-60	81	1.24	0.82-1.88					1.51	0.96-2.38		1.49	0.83-2.65	
>60	133	0.95	0.64-1.42					1.20	0.71-2.03		1.05	0.54-2.05	
Grade													
I	53	1.00		**0.030**	1.00		0.293	1.00		**0.005**	1.00		**0.023**
II	186	1.38	0.86-2.22		1.30	0.73-2.31		1.74	0.82-3.68		0.62	0.30-1.30	
III	136	1.83	1.13-2.96		1.55	1.55-0.86		2.73	1.29-5.75		1.20	0.60-2.41	
Histological type													
Ductal	345	1.00		0.405				1.00		0.333			
Other	31	1.23	0.76-2.00					1.34	0.74-2.40				
Tumor stage													
pT1	127	1.00		**0.001**	1.00		**0.045**	1.00		**<0.001**	1.00		**0.003**
pT2	198	1.34	0.97-1.86		1.03	0.70-1.51		1.84	1.18-2.86		1.90	1.10-3.29	
pT3/4	45	2.56	1.51-3.69		1.75	1.06-2.88		3.69	2.18-6.24		3.40	1.68-6.89	
Nodal stage													
Negative	199	1.00		**<0.001**	1.00		**<0.001**	1.00		**<0.001**	1.00		**<0.001**
Positive	171	3.09	2.30-4.16		2.78	1.97-3.92		2.97	2.04-4.33		2.30	1.48-3.56	
ER status													
Negative	133	1.00		0.890				1.00		0.157			

Table 1: (continued)

Characteristic	N	Relapse Free Period						Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
		HR	95% CI	*P*	HR	95% CI	*P*	RER	95% CI	*P*	RER	95% CI	*P*
Positive	229	1.02	0.76-1.38					0.77	0.54-1.10				
PGR status													
Negative	155	1.00		0.765				1.00		0.248			
Positive	201	1.05	0.78-1.41					0.81	0.56-1.16				
HER-2 status													
Negative	271	1.00		0.166				1.00		**0.004**	1.00		0.154
Positive	32	1.42	0.87-2.32					2.03	1.25-3.30		1.59	0.84-3.00	
Immune phenotype													
High immune susceptibility	48	1.00		**0.005**	1.00		**<0.001**	1.00		0.098	1.00		**0.006**
Intermediate immune susceptibility	186	1.80	1.06-3.05		1.95	1.13-3.39		1.95	0.98-3.98		3.84	1.62-9.09	
Low immune susceptibility	59	2.56	1.44-4.57		2.98	1.62-5.48		2.02	0.97-4.53		4.26	1.70-10.70	

Abbreviations: N: number of patients; HR: hazard ratio; 95%CI: 95% Confidence Interval; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; ET: endocrine therapy; CT: chemotherapy

Table 2: Cox univariate and multivariate analysis in the validation cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 3 groups that are described in the results section.

Characteristic	N	Relapse Free Period						Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
		HR	95% CI	*P*	HR	95% CI	*P*	RER	95% CI	*P*	RER	95% CI	*P*
Age													
<40	63	1.00		0.147				1.00		0.431			
40-50	83	0.62	0.38-1.03					0.58	0.30-1.10				
50-60	76	0.57	0.33-0.97					0.80	0.42-1.53				
>60	112	0.68	0.42-1.10					0.77	0.35-1.69				
Grade													
I	63	1.00		**0.001**	1.00		0.433	1.00		**0.026**	1.00		0.603
II	156	1.45	0.82-2.59		1.68	0.68-4.16		1.83	0.64-5.28		1.99	0.50-7.99	
III	108	2.54	1.43-4.52		1.86	0.72-4.79		3.27	1.16-9.21		1.69	0.40-7.14	
Histological type													
Ductal	293	1.00		0.298				1.00		0.300			
Other	35	1.35	0.77-2.35					1.46	0.71-3.01				
Tumor stage													
pT1	162	1.00		**<0.001**	1.00		0.171	1.00		**0.002**	1.00		0.227
pT2	130	2.18	1.46-3.23		1.78	0.98-3.26		2.57	1.34-4.90		1.96	0.85-4.52	
pT3/4	32	2.46	1.34-4.51		1.54	0.63-3.77		4.30	1.86-9.96		2.30	0.78-6.79	
Nodal stage													
Negative	182	1.00		**<0.001**	1.00		**0.01**	1.00		**<0.001**	1.00		0.208
Positive	142	2.81	1.93-4.08		2.06	1.19-3.57		3.09	1.73-5.13		1.59	0.77-3.25	

Table 2: (continued)

Characteristic	N	Relapse Free Period						Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
		HR	95% CI	*P*	HR	95% CI	*P*	RER	95% CI	*P*	RER	95% CI	*P*
ER status													
Negative	155	1.00		**0.034**	1.00		0.889	1.00		**0.008**	1.00		0.488
Positive	164	0.67	0.46-0.97		1.04	0.60-1.82		0.44	0.24-0.81		0.78	0.39-1.57	
PGR status													
Negative	161	1.00		**0.006**	1.00		0.184	1.00		**0.028**	1.00		0.232
Positive	150	0.59	0.40-0.86		0.68	0.38-1.20		0.54	0.31-0.93		0.65	0.31-1.38	
HER-2 status													
Negative	249	1.00		**0.002**	1.00		0.934	1.00		**<0.001**	1.00		0.232
Positive	27	2.36	1.36-4.09		0.97	0.42-2.22		3.52	1.91-6.49		1.71	0.71-4.10	
Immune phenotype													
High immune susceptibility	34	1.00		**0.005**	1.00		**0.025**	1.00		0.089	1.00		**0.040**
Intermediate immune susceptibility	156	2.66	1.15-6.16		2.45	0.87-6.89		5.31	0.64-31.33		5.47	0.72-41.70	
Low immune susceptibility	29	4.72	1.83-12.18		4.73	1.48-15.06		11.12	1.12-55.41		10.95	1.31-91.63	

Abbreviations: N: number of patients; HR: hazard ratio; 95%CI: 95% Confidence Interval; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; ET: endocrine therapy; CT: chemotherapy

Tumor immune subtypes

The Cohen's kappa coefficient for inter-observer agreement of CTL and PEN5 quantification all reached a coefficient of 0.82 or higher. Missing immunohistochemical data was due to tissue damage.

Tumor immune subtypes, representing tumor adaptive immune escape variants were constructed from available data (Figure1). The defined tumor immune subtypes were in ascending order from high immune susceptibility to low immune susceptibility: (1) CTL are able to recognize tumor-associated antigens (TAA) presented by classical HLA class I and anti-tumor immune reaction can take place: Tumors with expression of classical HLA class I, high infiltration of CTL and absence of infiltration of Treg; (2) Tumors with a lack of classical HLA class I expression can escape CTL recognition, but NK cells are able to recognize these cells and anti-tumor immune reaction can take place: Tumors with loss of expression of classical HLA class I, no expression of HLA-EG, present infiltration of NK cells and absent infiltration of Treg; (3) Classical HLA class I present TAA and could be recognized by CTL, but a low infiltration of CTL results in a limited anti-tumor immune reaction: Tumors with expression of classical HLA class I but low CTL infiltration; (4) Classical HLA class I present TAA and could be recognized by CTL, but immunosuppressive Treg weaken CTL function, resulting in a limited anti-tumor immune reaction: Tumors with expression of classical HLA class I, high infiltration of CTL, but also present infiltration of Treg; (5) Tumors with lack of classical HLA class I escape CTL recognition, but could be recognized by NK cells, which however are not present, resulting in failure of anti-tumor immune reaction: Tumors with loss of expression of classical HLA class I and absent NK cell infiltration; (6) Tumors with lack of classical HLA class I expression escape CTL recognition, but could be recognized by NK cells, however immunosuppressive Treg weaken NK cell function [19], resulting in failure of anti-tumor immune reaction: Tumors with loss of expression of classical HLA class I, present NK cell infiltration, but also present Treg infiltration; (7) Tumor with lack of classical HLA class I expression but expression of non-classical HLA-EG escape from both CTL recognition and NK cell recognition: Tumor with loss of expression of classical HLA class I and expression of HLA-EG.

A more simplified tumor immune subtype variable was constructed by joining together tumor immune subtypes: High (subtypes 1-2), intermediate (subtypes 3-4) and low (subtypes 5-7) immune susceptibility (Figure1, clustered groups shown by circles).

Associations between clinicopathological patient and tumor characteristics and tumor immune subtypes classified into 7 groups and into 3 groups are shown in supplementary tables 1A, B and 2A, B. No statistically significant validated association was found between patient and tumor characteristics and tumor immune subtypes classified into 7 groups or into 3 groups.

Tumor immune subtypes classified into 7 groups

Distribution in patient training and validation cohort

The tumor immune subtypes classified into 7 groups could be determined for patients with data available for all immune markers: 77% (293/380) of patients in the training cohort; 66% validation cohort. Distributions of immune subtypes and associations with known clinicopathological parameters are shown in supplementary tables (training cohort Table 1A; validation cohort Table 2A).

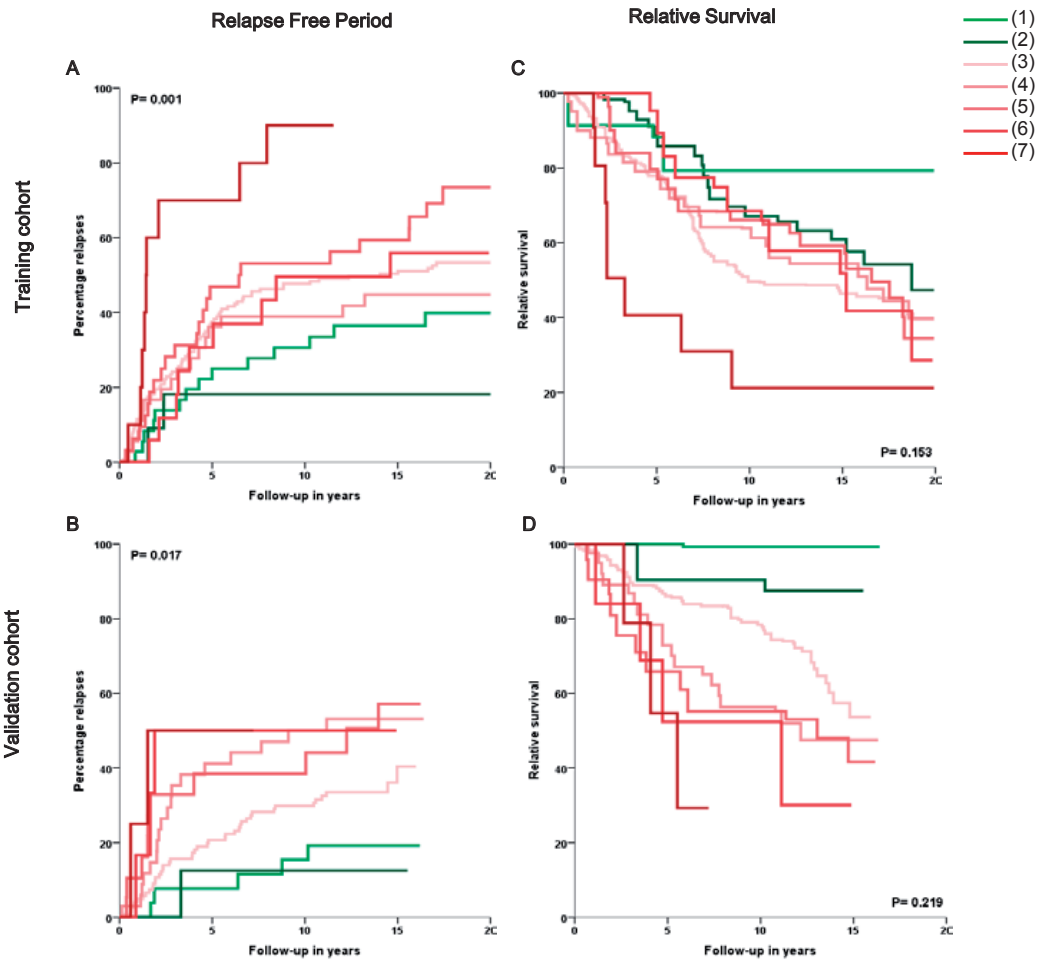

Figure 2: Outcome analyses by tumor immune subtypes for Relapse free period (RFP) (A, B) and relative survival (RS) (C, D) according to the 7 tumor immune subtypes that are described in the Results section for training cohort patients (A, C), and for validation cohort patients (B, D). Tumor immune subtypes representative for more tumor immune escape resulted in an unfavourable patient outcome concerning RFP and RS compared to more immunogenic tumor immune subtypes. Log-rank P-values are shown in each graph.

Prognostic associations with patient outcome

The association of tumor immune subtypes classified into 7 groups in the training cohort with relapse-free period and relative survival are shown in Figure 2. Analysis of relapse-free period showed a statistically significant association between the 7 tumor immune subtypes and clinical outcome of patient (RFP p=0.001, Figure 2 A). Tumors that were expected to show lower immune susceptibility resulted in more patient relapses over time compared to tumors that were expected to show higher immune susceptibility. A similar though not significant trend was seen for the association between the 7 immune subtypes and relative survival outcome of patients (RS p=0.153, Figure 2 C). Results for outcome analyses were confirmed in the validation cohort (RFP p=0.017, Figure 2B and RS p=0.219, Figure 2D). Multivariable analyses demonstrated that these 7 tumor immune subtypes were a statistically significant independent prognostic factor in breast cancer patients for both RFP and RS (supplementary Table 3). Though statistical significance was lost in multivariable analyses in the validation cohort, a statistical trend remained for the association between 7 tumor immune subtypes and patient outcome concerning RFP (p=0.055, supplementary Table 4).

Tumor immune subtypes classified into 3 groups

Distribution in patient training and validation cohort

The tumor immune subtypes, consisting of three groups as described above showed the following distribution in the training and validation cohort respectively: High immune susceptible, 16% (48/293) and 16% (34/219); Intermediate immune susceptible, 63% (186/293) and 71% (156/219); Low immune susceptible, 20% (59/293) and 13% (29/219). Associations with known clinicopathological parameters are shown in supplementary tables (training cohort Table 1B; validation cohort Table 2B).

Prognostic associations with patient outcome

The association of the tumor immune subtypes classified into 3 groups with relapse-free period and relative survival is shown in Figure 3. Analysis of relapse-free period showed a significant association between tumor immune subtype and clinical outcome of patients (RFP p=0.004, Figure 3A). Lower immune susceptible tumor subtypes, resulted in more relapses over time compared to higher immune susceptible tumor subtypes. Again, though not significant a similar associative trend was seen for relative survival outcome of patient and tumor immune subtype (RS p=0.146, Figure 3C). Results of outcome analyses in the validation cohort were similar to the results found in the training cohort (RFP p=0.003, Figure 3B and RS p=0.112, Figure 3D).

Multivariable analyses demonstrated that the tumor immune subtypes were a statistically significant independent prognostic factor in breast cancer patients for both RFP

(p<0.001, Table 1B) and RS (p=0.006, Table 1B) with high discriminative power; compared to patients with high immune susceptible tumors, patients with intermediate immune susceptible tumors showed an almost twice elevated risk (HR 1.95, 95%CI 1.13-3.39) for

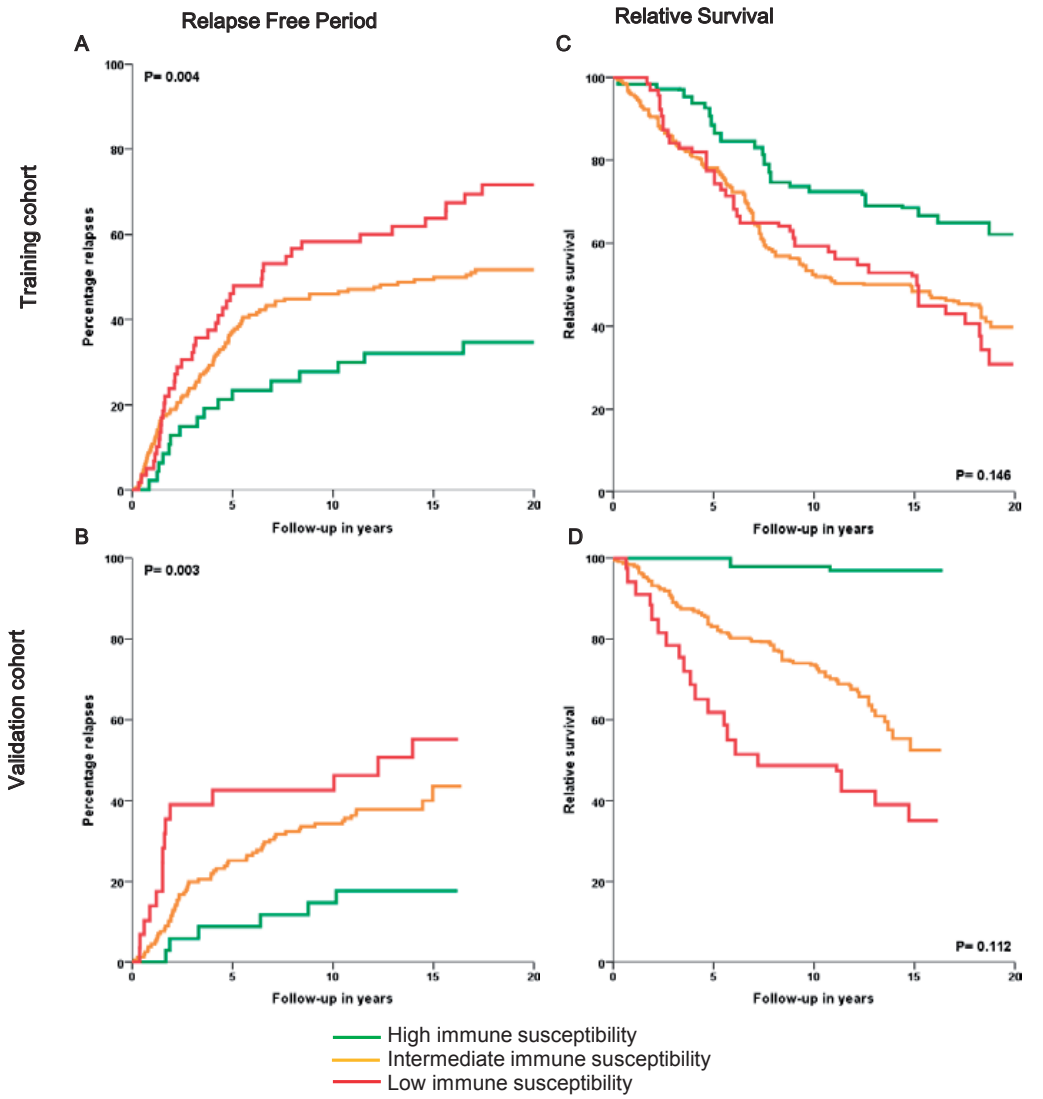

Figure 3: Outcome analyses by tumor immune subtypes for Relapse free period (RFP) (A, B) and relative survival (RS) (C, D) according to the 3 tumor immune subtypes that are described in the Results section for training cohort patients (A, C), and for validation cohort patients (B, D). Tumor immune subtypes representative for more tumor immune escape resulted in an unfavourable patient outcome concerning RFP and RS compared to more immunogenic tumor immune subtypes. Log-rank P-values are shown in each graph.

developing relapses over time and an almost four times higher relative risk for survival (RR 3.84, 95% CI 1.62-9.09), while patients with low immune susceptible tumors showed an almost three times elevated risk on relapses over time (HR 2.98, 95%CI 1.62-5.48) and

a more than four times higher relative risk for survival (RR 4.26, 95%CI 1.70-10.70) (Table 1B). Results of the validation cohort confirmed the associations found in multivariable analyses (RFP p=0.025, Table 2B and RS p=0.040, Table 2B)

DISCUSSION

The impact of the immune response and subsequent tumor immune evasion on tumor progression and patient outcome in breast cancer is poorly understood. Most studies focus on the effect of single parameters, like tumor expression of HLA class I or immune cell tumor infiltration, but separately these do not reflect the multifaceted interaction between immune cells and tumor cells. In order to get a good perspective on the processes involved in these interactions, we defined tumor immune subtypes. These subtypes were defined based on tumor susceptibility for cellular immune responses using expression of key factors in these responses that reflect local presence of CTL, NK cells, and Treg and tumor expression of classical HLA class I and HLA-E and -G. Outcome analyses of the immune subtypes revealed strong associations with patient outcome where tumors defined as being highly susceptible to immune system attack showed a favorable outcome for breast cancer patients compared to patients with tumors defined having a low immune susceptible profile. These prognostic effects were shown in this study to be independent of known clinicopathological prognostic parameters and were additionally validated in an independent breast cancer patient cohort confirming the high discriminative power on patient outcome stratification.

Prior studies by our group and others have focused on a cellular immune response and its effect on tumor progression and patient outcome in breast cancer [11-16]. DeNardo *et al.* even provides evidence that treatment response is in part regulated by the immune microenvironment [20], again urging the importance of comprehensive determination of the tumor immune status. High tumor infiltration of CD8+ lymphocytes, representative for CTL infiltration, has been found to result in a favorable patient prognosis in one study [11]. However, another study reported high CTL infiltration to be associated with a worse patient outcome [21]. Yet another study could not find a statistically significant prognostic effect for CTL [10]. High Treg infiltration resulted in an unfavorable prognostic factor in a variety of studies [10;15;22], while it did not show a statistically significant association with patient outcome in a previous study of our group [16]. Loss of expression of classical HLA class I showed to be a favorable [23] as well as an unfavorable [16] prognostic factor in two different studies and revealed no statistically significant associations with patient outcome in two other studies [24;25]. Concerning non-classical HLA-E and HLA-G, one study could not find a statistically significant relation with patient prognosis for HLA-G [13;25]

while a study of our group showed tumor expression of HLA-E and HLA-G resulted to be a statistically significant unfavorable prognostic parameter [12]. To our knowledge, the prognostic impact of NK cell infiltration has not been studied in breast cancer, but NK cell presence in the tumor microenvironment has been shown to result in a favorable patient outcome in colorectal cancer [26].

Taken together, these reports show contradictory results and, therefore, do not draw a clear picture of the interaction between breast cancer cells and the immune system. Our present study shows that this may be explained by the simple fact that a successful anti-tumor immune response depends not only on the level of expression of a single marker such as classical HLA class I, but on the variety of factors involved in the multifaceted immune response. Due to the complexity of the balance between immune surveillance and tumor immune escape, it is not a single marker that is able to reflect outcome of the interaction, but a set of key markers. In this study we analyzed a set of such crucial immune markers and defined tumor immune subtypes based on these markers. We demonstrated that a profile that represents tumors that may be more immune susceptible is predictive for a more favorable clinical outcome for patients with breast cancer. In addition, the prognostic impact with high discriminative power that we found for these tumor immune subtypes, suggests that previous single marker studies are understating or even confounding the impact of the immune system on tumor control. The results found for the tumor immune subtypes are not only concordant with prior evidence on tumor immune biology in breast cancer [4;18], but additionally join together the conclusions of prior studies by linking single tumor-immune markers to functional tumor-immune interaction. This is the first study providing detailed insight in tumor immune biology in breast cancer, showing that tumor immune surveillance is of crucial importance in the control of tumor progression and therefore in determining patient prognosis.

Many prognostic factors have been identified for breast cancer. Of these, the ASCO guidelines advised the use in clinical practice of urokinases plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1) and gene profiles detected with multiparameter gene expression assays [27]. The clinical value of microarray-based prognostic tools, like the MammaPrint, a 70-gene expression profile, and Oncotype DX, a 21-gene expression profile is currently being debated [28;29]. One major critique is that these gene prints were constructed using top-down analyses and were not defined based on a biological rationale. Therefore, it is unclear what tumor types are represented by the various patient risk-groups [30]. Contrary to these top-down analyses, the tumor immune subtypes we defined are based on well-founded biological hypotheses. Future research will further improve this function-based approach of prognostic profiling in breast cancer.

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SUPPLEMENTARY TABLES

Supplementary Table 1: Correlations between tumor immune subtypes into 7 groups that are described in the results section in the training cohort of patients (**A**) and well-established prognostic factors using chi-square test (**B**).

A	N	%	(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	
<40	74	19.5	11	30.6	1	8.3	21	14.1	10	27.0	8	25.0	3	17.6	4	40.0	**0.047**
40-50	92	24.2	7	19.4	4	33.3	44	29.5	6	16.2	3	9.4	5	29.4	1	10.0	
50-60	81	21.3	6	16.7	2	16.7	29	19.5	6	16.2	13	40.6	2	11.8	4	40.0	
>=60	133	35.0	12	33.3	5	41.7	55	36.9	15	40.5	8	25.0	7	41.2	1	10.0	
Grade																	
I	53	14.1	4	11.1	4	36.4	17	11.4	1	2.7	6	18.8	4	23.5	3	30.0	**0.033**
II	186	49.6	16	44.4	5	45.5	78	52.3	19	51.4	17	53.1	10	58.8	1	10.0	
III	136	36.3	16	44.4	2	18.2	54	36.2	17	45.9	9	28.1	3	17.6	6	60.0	
Histological type																	
Ductal	345	91.8	31	86.1	10	90.9	141	94.6	33	89.2	29	90.6	16	94.1	10	100.0	0.578
Lobular	31	8.2	5	13.9	1	9.1	8	5.4	4	10.8	3	9.4	1	5.9	0	0.0	
T-status																	
T1	127	34.3	6	16.7	6	50.0	52	35.4	10	27.8	13	40.6	7	43.8	2	20.0	0.305
T2	198	53.5	25	69.4	5	41.7	70	47.6	22	61.1	14	43.8	9	56.2	6	60.0	
T3/4	45	12.2	5	13.9	1	8.3	25	17.0	4	11.1	5	15.6	0	0.0	2	20.0	
N-status																	
N0	199	53.8	20	57.1	6	50.0	83	57.2	16	44.4	16	51.6	10	58.8	2	20.0	0.321
N1-3	171	46.2	15	42.9	6	50.0	62	42.8	20	55.6	15	48.4	7	41.2	8	80.0	
ER-status																	

Supplementary Table 1: (continued)

A	N	%	(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Negative	133	36.7	18	50.0	5	41.7	58	39.2	14	37.8	5	16.1	4	23.5	6	60.0	0.057
Positive	229	63.3	18	50.0	7	58.3	90	60.8	23	62.2	26	83.9	13	76.5	4	40.0	
PGR-status																	
Negative	155	43.5	19	52.8	5	41.7	61	41.2	18	48.6	6	20.7	9	52.9	6	60.0	0.131
Positive	201	56.5	17	47.2	7	58.3	87	58.8	19	51.4	23	79.3	8	47.1	4	40.0	
HER-2-status																	
Overexpression -	271	89.4	26	86.7	11	100.0.0	105	85.4	28	93.3	25	96.2	16	100.0	8	100.0	0.206
Overexpression +	32	10.6	4	13.3	0		18	14.6	2	6.7	1	3.8	0	0.0	0	0.0	
Local Therapy																	
MAST-RT	132	34.7	11	30.6	6	50.0	55	36.9	15	40.5	11	34.4	7	41.2	1	10.0	0.714
MAST+RT	80	21.1	10	27.8	1	8.3	31	20.8	5	13.5	6	18.8	2	11.8	4	40.0	
BCS	168	44.2	15	41.7	5	41.7	63	42.3	17	45.9	15	46.9	8	47.1	5	50.0	
Systemic therapy																	
CT alone	78	20.5	11	30.6	1	8.3	35	23.5	9	24.3	2	6.2	2	11.8	4	40.0	0.273
HT alone	27	7.1	3	8.3	0	0.0	11	7.4	1	2.7	3	9.4	1	5.9	0	0.0	
CT&HT	4	1.1	0	0.0	0	0.0	1	0.7	1	2.7	2	6.2	0	0.0	0	0.0	
None	271	71.3	22	61.1	11	91.7	102	68.5	26	70.3	25	78.1	14	82.4	6	60.0	
Total	**380**	**100**	**36**	**100**	**12**	**100**	**149**	**100**	**37**	**100**	**32**	**100**	**17**	**100**	**10**	**100**	

B	N	%	High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
			N	%	N	%	N	%	
Age									
<40	74	19.5	12	25.0	31	16.7	15	25.4	0.094
40-50	92	24.2	11	22.9	50	26.9	9	15.3	
50-60	81	21.3	8	16.7	35	18.8	19	32.2	
>=60	133	35.0	17	35.4	70	37.6	16	27.1	
Grade									
I	53	14.1	8	17.0	18	9.7	13	22.0	0.138
II	186	49.6	21	44.7	97	52.2	28	47.5	
III	136	36.3	18	38.3	71	38.2	18	30.5	
Histological type									
Ductal	345	91.8	41	87.2	174	93.5	55	93.2	0.332
Lobular	31	8.2	6	12.8	12	6.5	4	6.8	
T-status									
T1	127	34.3	12	25.0	62	33.9	22	37.9	0.534
T2	198	53.5	30	62.5	92	50.3	29	50.0	
T3/4	45	12.2	6	12.5	29	15.8	7	12.1	
N-status									
N0	199	53.8	26	55.3	99	54.7	28	48.3	0.669
N1-3	171	46.2	21	44.7	82	45.3	30	51.7	
ER-status									
Negative	133	36.7	23	47.9	72	38.9	15	25.9	0.058
Positive	229	63.3	25	52.1	113	61.1	43	74.1	
PGR-status									
Negative	155	43.5	24	50.0	79	42.7	21	37.5	0.437
Positive	201	56.5	24	50.0	106	57.3	35	62.5	
HER-2-status									
Overexpression -	271	89.4	37	90.2	133	86.9	49	98.0	0.081
Overexpression +	32	10.6	4	9.8	20	13.1	1	2.0	
Local Therapy									
MAST-RT	132	34.7	17	35.4	70	37.6	19	32.2	0.928
MAST+RT	80	21.1	11	22.9	36	19.4	12	20.3	
BCS	168	44.2	20	41.7	80	43.0	28	47.5	
Systemic therapy									
CT alone	78	20.5	12	25.0	44	23.7	8	13.6	0.508
HT alone	27	7.1	3	6.2	12	6.5	4	6.8	
CT&HT	4	1.1	0	0.0	2	1.1	2	3.4	
None	271	71.3	33	68.8	128	68.8	45	76.3	
Total	**380**	**100**	**48**	**100**	**186**	**100**	**59**	**100**	

Abbreviations: N: number of patients; %: percentage; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; MAST: mastectomy; RT: radiotherapy; BCS: breast conservative surgery; ET: endocrine therapy; CT: chemotherapy

Supplementary Table 2: Correlations between tumor immune subtypes into 7 groups that are described in the results section in the validation cohort of patients (**A**) and well-established prognostic factors using chi-square test (**B**).

A	N	%	(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	
<40	63	18.9	6	23.1	2	25.0	25	20.5	10	29.4	5	26.3	1	16.7	1	25.0	0.794
40-50	83	24.9	6	23.1	2	25.0	29	23.8	12	35.3	4	21.1	1	16.7	0	0.0	
50-60	76	22.8	5	19.2	3	37.5	25	20.5	5	14.7	4	21.1	3	50.0	2	50.0	
>=60	112	33.5	9	34.6	1	12.5	43	35.2	7	20.6	6	31.6	1	16.7	1	25.0	
Grade																	
I	63	19.3	6	24.0	2	25.0	25	20.7	2	6.1	2	11.1	1	16.7	1	33.3	0.420
II	156	47.7	8	32.0	4	50.0	62	51.2	14	42.4	9	50.0	2	33.3	1	33.3	
III	108	33.0	11	44.0	2	25.0	34	28.1	17	51.5	7	38.9	3	50.0	1	33.3	
Histological type																	
Ductal	293	89.3	22	88.0	8	100.0.0	111	91.7	29	87.9	13	72.2	4	66.7	3	100.0	0.109
Lobular	35	10.7	3	12.0	0		10	8.3	4	12.1	5	27.8	2	33.3	0	0.0	
T-status																	
T1	162	50.0	14	56.0	5	62.5	54	45.4	15	45.5	4	25.0	2	33.3	2	50.0	0.541
T2	130	40.1	10	40.0	2	25.0	52	43.7	5	45.5	8	50.0	2	33.3	2	50.0	
T3/4	32	9.1	1	4.0	1	12.5	13	10.9	3	9.1	4	25.0	2	33.3	0	0.0	
N-status																	
N0	182	56.2	17	68.0	5	62.5	61	51.3	18	54.5	9	50.0	3	50.0	1	33.3	0.779
N1-3	142	43.8	8	32.0	3	37.5	58	48.7	15	45.5	9	50.0	3	50.0	2	66.7	
ER-status																	
Negative	155	48.6	13	54.2	3	37.5	46	38.0	20	58.8	8	42.1	3	50.0	2	50.0	0.411
Positive	164	51.4	11	45.8	5	62.5	75	62.0	14	41.2	11	57.9	3	50.0	2	50.0	

Supplementary Table 2: (continued)

A	N	%	(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	
PGR-status																	
Negative	161	51.8	15	62.5	2	25.0	52	42.6	24	70.6	8	44.4	4	66.7	2	50.0	**0.046**
Positive	150	48.2	9	37.5	6	75.0	70	57.4	10	29.4	10	55.6	2	33.3	2	50.0	
HER-2-status																	
Overexpression -	249	90.2	15	83.3	6	100.0.0	99	92.5	28	90.3	15	93.8	4	66.7	4	100.0	0.316
Overexpression +	27	9.8	3	16.7	0		8	7.5	3	9.7	1	6.2	2	33.3	0	0.0	
Local Therapy																	
MAST-RT	153	45.8	13	50.0	4	50.0	55	45.1	14	41.2	9	47.4	3	50.0	3	75.0	0.807
MAST+RT	52	15.6	5	19.2	1	12.5	19	15.6	7	20.6	6	31.6	2	33.3	0	0.0	
BCS	129	38.6	8	30.8	3	37.5	48	39.3	13	38.2	4	21.1	1	16.7	1	25.0	
Systemic therapy																	
CT alone	49	14.7	2	7.7	1	12.5	18	14.8	6	17.6	7	36.8	1	16.7	1	25.0	0.594
HT alone	86	25.7	8	30.8	2	25.0	34	27.9	6	17.6	4	21.1	2	33.3	1	25.0	
CT&HT	23	6.9	0	0.0	1	12.5	11	9.0	5	14.7	0	0.0	0	0.0	0	0.0	
None	176	52.7	16	61.5	4	50.0	59	48.4	17	50.0	8	42.1	3	50.0	2	50.0	
Total	**334**	**100**	**26**	**100**	**8**	**100**	**122**	**100**	**34**	**100**	**19**	**100**	**6**	**100**	**4**	**100**	

B	N	%	High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
			N	%	N	%	N	%	
Age									
<40	63	18.9	8	23.5	35	22.4	7	24.1	0.842
40-50	83	24.9	8	23.5	41	26.3	5	17.2	
50-60	76	22.8	8	23.5	30	19.2	9	31.0	
>=60	112	33.5	10	29.4	50	32.1	8	27.6	
Grade									
I	63	19.3	8	24.2	27	17.5	4	14.8	0.649
II	156	47.7	12	36.4	76	49.4	12	44.4	
III	108	33.0	13	39.4	51	33.1	11	40.7	
Histological type									
Ductal	293	89.3	30	90.9	140	90.9	20	74.1	**0.035**
Lobular	35	10.7	3	9.1	14	9.1	7	25.9	
T-status									
T1	162	50.0	19	57.6	69	45.4	8	30.8	0.148
T2	130	40.1	12	36.4	67	44.1	12	46.2	
T3/4	32	9.1	2	6.1	16	10.5	6	23.1	
N-status									
N0	182	56.2	22	66.7	79	52.0	13	48.1	0.253
N1-3	142	43.8	11	33.3	73	48.0	14	51.9	
ER-status									
Negative	155	48.6	16	50.0	66	42.6	13	44.8	0.740
Positive	164	51.4	16	50.0	89	57.4	16	55.2	
PGR-status									
Negative	161	51.8	17	53.1	76	48.7	14	50.0	0.901
Positive	150	48.2	15	46.9	80	51.3	14	50.0	
HER-2-status									
Overexpression -	249	90.2	21	87.5	127	92.0	23	88.5	0.691
Overexpression +	27	9.8	3	12.5	11	8.0	3	11.5	
Local Therapy									
MAST-RT	153	45.8	17	50.0	69	44.2	15	51.7	0.345
MAST+RT	52	15.6	6	17.6	26	16.7	8	27.6	
BCS	129	38.6	11	32.4	61	39.1	6	20.7	
Systemic therapy									
CT alone	49	14.7	3	8.8	24	15.4	9	31.0	0.104
HT alone	86	25.7	10	29.4	40	25.6	7	24.1	
CT&HT	23	6.9	1	2.9	16	10.3	0	0.0	
None	176	52.7	20	58.8	76	48.7	13	44.8	
Total	**334**	**100**	**34**	**100**	**156**	**100**	**29**	**100**	

Abbreviations: N: number of patients; %: percentage; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; MAST: mastectomy; RT: radiotherapy; BCS: breast conservative surgery; ET: endocrine therapy; CT: chemotherapy

Supplementary Table 3: Cox univariate and multivariate analysis in the training cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 7 groups that are described in the results section.

Characteristic	Relapse Free Period							Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age													
<40	74	1.00		0.354				1.00		**0.048**	1.00		**0.006**
40-50	92	0.87	0.58-1.33					0.79	0.49-1.28		0.50	0.27-0.96	
50-60	81	1.24	0.82-1.88					1.51	0.96-2.38		1.52	0.84-2.72	
>60	133	0.95	0.64-1.42					1.20	0.71-2.03		1.00	0.49-2.04	
Grade													
I	53	1.00		**0.030**	1.00		0.384	1.00		**0.005**	1.00		**0.043**
II	186	1.38	0.86-2.22		1.35	0.76-2.41		1.74	0.82-3.68		0.59	0.28-1.24	
III	136	1.83	1.13-2.96		1.51	0.84-2.73		2.73	1.29-5.75		1.11	0.56-2.23	
Histological type													
Ductal	345	1.00		0.405				1.00		0.333			
Other	31	1.23	0.76-2.00					1.34	0.74-2.40				
Tumor stage							0.153						
pT1	127	1.00		**0.001**	1.00			1.00		**<0.001**	1.00		**0.002**
pT2	198	1.34	0.97-1.86		1.01	0.69-1.49		1.84	1.18-2.86		2.11	1.21-3.68	
pT3/4	45	2.56	1.51-3.69		1.57	0.94-2.61		3.69	2.18-6.24		3.62	1.77-7.41	
Nodal stage													
Negative	199	1.00		**<0.001**	1.00		**<0.001**	1.00		**<0.001**	1.00		**<0.001**
Positive	171	3.09	2.30-4.16		2.81	1.98-3.99		2.97	2.04-4.33		2.30	1.47-3.60	
ER status													
Negative	133	1.00		0.890				1.00		0.157			
Positive	229	1.02	0.76-1.38					0.77	0.54-1.10				

Supplementary Table 3: (continued)

Characteristic	Relapse Free Period							Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
	N	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
PGR status													
Negative	155	1.00		0.765				1.00		0.248			
Positive	201	1.05	0.78-1.41					0.81	0.56-1.16				
HER2 status													
Negative	271	1.00		0.166				1.00		**0.004**	1.00		0.135
Positive	32	1.42	0.87-2.32					2.03	1.25-3.30		1.62	0.86-3.07	
Immune phenotype													
(1)	36	1.00		**0.002**	1.00		**0.010**	1.00		0.098	1.00		**0.002**
(2)	12	0.43	0.10-1.91		0.53	0.12-2.38		0.12	0.00-62.27		0.001	0-∞	
(3)	149	1.60	0.90-2.82		1.82	1.00-3.32		1.54	0.80-2.97		3.43	1.41-8.32	
(4)	37	1.34	0.65-2.75		1.40	0.67-2.94		1.26	0.54-2.92		2.40	0.86-6.67	
(5)	32	2.15	1.11-4.18		2.45	1.20-4.99		1.39	0.62-3.13		2.33	0.84-6.51	
(6)	17	1.48	0.64-3.41		2.18	0.91-5.22		1.03	0.33-3.21		4.26	1.28-14.15	
(7)	10	5.09	2.19-11.82		4.41	1.83-10.62		3.68	1.44-9.40		11.84	3.86-36.34	

Abbreviations: N: number of patients; HR: hazard ratio; 95%CI: 95% Confidence Interval; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; ET: endocrine therapy; CT: chemotherapy

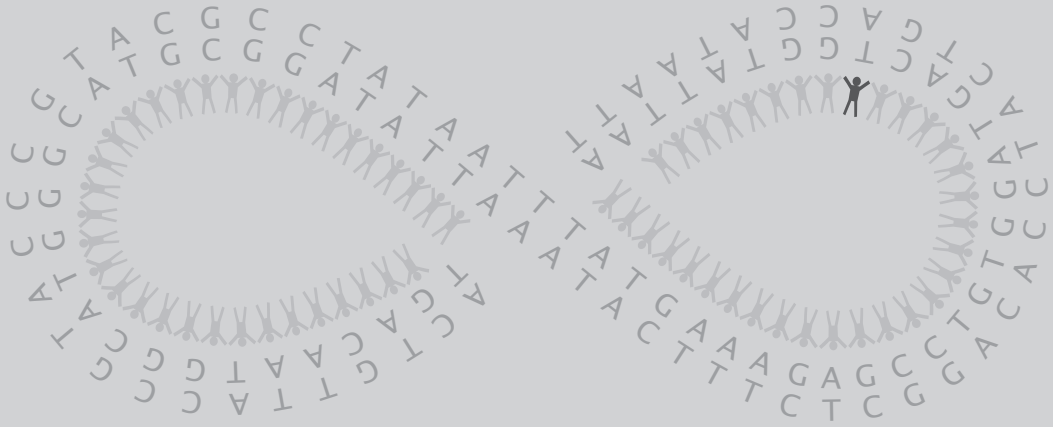
Supplementary Table 4: Cox univariate and multivariate analysis in the validation cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 7 groups that are described in the results section.

Characteristic	Relapse Free Period							Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
	N	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Age													
<40	63	1.00		0.147				1.00		0.431			
40-50	83	0.62	0.38-1.03					0.58	0.30-1.10				
50-60	76	0.57	0.33-0.97					0.80	0.42-1.53				
>60	112	0.68	0.42-1.10					0.77	0.35-1.69				
Grade													
I	63	1.00		**0.001**	1.00		0.61	1.00		**0.026**			
II	156	1.45	0.82-2.59		1.55	0.62-3.89		1.83	0.64-5.28				
III	108	2.54	1.43-4.52		1.62	0.61-4.30		3.27	1.16-9.21				
Histological type													
Ductal	293	1.00		0.298				1.00		0.300			
Other	35	1.35	0.77-2.35					1.46	0.71-3.01				
Tumor stage													
pT1	162	1.00		**<0.001**	1.00		0.113	1.00		**0.002**			
pT2	130	2.18	1.46-3.23		1.93	1.04-3.56		2.57	1.34-4.90				
pT3/4	32	2.46	1.34-4.51		1.79	0.73-4.39		4.30	1.86-9.96				
Nodal stage													
Negative	182	1.00		**<0.001**	1.00		**0.014**	1.00		**<0.001**			
Positive	142	2.81	1.93-4.08		2.03	1.16-3.56		3.09	1.73-5.13				
ER status													
Negative	155	1.00		**0.034**	1.00		0.728	1.00		**0.008**			
Positive	164	0.67	0.46-0.97		1.11	0.62-1.97		0.44	0.24-0.81				

Supplementary Table 4: (continued)

Characteristic	Relapse Free Period							Relative Survival					
		Univariate analysis			Multivariable analysis			Univariate analysis			Multivariable analysis		
	N	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
PGR status													
Negative	161	1.00		**0.006**	1.00		0.243	1.00		**0.028**			
Positive	150	0.59	0.40-0.86		0.70	0.39-1.27		0.54	0.31-0.93				
HER2 status													
Negative	249	1.00		**0.002**	1.00		0.815	1.00		**<0.001**			
Positive	27	2.36	1.36-4.09		1.11	0.46-2.66		3.52	1.91-6.49				
Immune phenotype													
(1)	26	1.00		**0.031**	1.00		0.055	1.00		0.219			
(2)	8	0.58	0.07-4.94		0.77	0.08-7.67		$5.2^{E}5$	0-∞				
(3)	122	2.10	0.83-5.31		2.04	0.61-6.89		$1.5^{e}6$	0-∞				
(4)	34	3.45	1.28-9.28		3.06	0.85-10.97		$2.5^{e}6$	0-∞				
(5)	19	4.09	1.39-12.01		3.67	0.91-1479		$2.6^{e}6$	0-∞				
(6)	6	3.82	0.91-16.02		4.16	0.81-21.44		$3.7^{e}6$	0-∞				
(7)	4	5.91	1.14-30.67		13.4	2.12-84.86		$6.5^{e}6$	0-∞				

Abbreviations: N: number of patients; HR: hazard ratio; 95%CI: 95% Confidence Interval; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; ET: endocrine therapy; CT: chemotherapy



Chapter 4

Immunological subtypes in breast cancer are prognostic for invasive ductal but not for invasive lobular breast carcinoma

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ABSTRACT

Background

Classical patient and tumor characteristics are the benchmark of personalized breast cancer (BC) management. Recent evidence demonstrated that immune and molecular profiling of BC may also play an important role. Despite evidence of differences between invasive ductal (IDC) and lobular (ILC) BC, they are infrequently accounted for when making treatment decisions for individual patients. The purpose of this study was to investigate the relevance of the tumor immune response in the major histological subtypes of BC. We also assessed the relationship between immune responses and molecular subtypes and their prognostic potential.

Methods

Immunostains were done for HLA-I, HLA-E, HLA-G, Treg, NK-cells and CTL for the composition of the immune profiles and Ki67, EGFR, CK5/6, ER, PR and HER2 for molecular profiles in 714 breast cancer patients who underwent primary surgery.

Results

No significant association was found between IDC (90.6%) and ILC (9.4%) and tumor immune subtypes (p=0.4) and molecular subtypes (p=0.4). However, for relapse free period (RFP) tumor immune subtyping was prognostic (p=0.002) in IDC, but not ILC. Contrary to ILC, IDC patients frequently expressed higher cleaved Caspase-3 and Ki67, which was prognostic. Intermediate immune susceptible IDC expressing high cleaved Caspase-3 or Ki67, showed worse RFP than low expression hereof (Caspase-3:p=0.004; Ki67:p=0.002), this was not seen for ILC or in high or low immune susceptible tumor types for neither IDC nor ILC.

Conclusion

Tumor immune characteristics and host immune responses are prognostic in IDC, but not ILC. To add, tumor immune profiles were only prognostic in Luminal A tumors.

INTRODUCTION

Nowadays, breast cancer is the most commonly diagnosed cancer type and the leading cause of cancer related death in the female population in the West [1]. Invasive ductal carcinoma (IDC) is by far the most common type of breast cancer. The second largest group comprises invasive lobular carcinoma (ILC), and reports indicate that 10 to 15 per cent of breast tumors are ILC [2]. Investigations into the differences between IDC and ILC have consistently shown that lobular carcinomas have a particular single-file growth pattern, tend to be larger, more often ER- and progesterone receptor (PR)-positive, and less aggressive than their ductal counterparts [2;3]. Nevertheless, these two types of breast cancer are treated similarly with regard to systemic adjuvant therapy, which is based on tumor size, histological grade, hormone receptor status and human epidermal growth factor receptor 2 (HER2) status.

Gene expression studies have identified several distinct breast cancer subtypes with marked differences in patient prognosis [4-6].This molecular classification proposed four different classes of breast tumors: Luminal A and B, basal-like and tumors overexpressing HER2. Luminal B tumors differ from Luminal A by a lower quantative content of hormone receptors. Basal-like tumors are triple negative tumors and HER2 overexpressing tumors cluster near these basal-like tumors [4-6]. Studies have shown that basal-like and HER2 overexpressing tumors have a more aggressive character, resulting in an unfavourable patient outcome compared to the Luminal tumor types.

With respect to over- and undertreatment, no optimal risk stratification exists for the allocation of the (individual) breast cancer patient to the most appropriate therapeutic regimen. It is likely that the different gene expression profiles explain the observed survival differences seen in the breast cancer population, even after controlling for tumor stage [7]. However, there is also strong evidence that the breast cancer host's adaptive immune system plays a crucial role in the control of tumor growth and progression [8]. On the other hand, breast tumor cells are able to adapt in order to escape the immune system and thus acquire characteristics to evade immunological recognition [9]. Several studies have attempted to elucidate this highly immunogenic disease by pointing out the great variety of immune reactions found in breast cancer.

Cytotoxic T-lymphocytes (CTL) are capable of recognizing tumor-associated antigens presented by classical human leukocytes antigen (HLA) class I on the tumor surface [10]. In order to avoid immune recognition from CTL, cancer cells may lose expression of this classical HLA class I [10]. However, this makes them more prone to natural killer (NK) cell recognition [11]. Non classical HLA class I molecules (HLA-E and HLA-G) play a crucial role in immune surveillance by NK-cells. Expression of these molecules on the cell surface

causes an inhibitory effect on NK-cell attack [11-13]. Another tumor escape mechanism of immunosurveillance is attraction and induction of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment [14]. Together, these studies suggested that complex interactions take place between breast tumor cells and cells of the immune system. We recently reported on these complex interactions in a study on immune subtypes of breast cancer, representing adaptive immune escape variants based on tumor-associated antigens (classical HLA class I and non-classical HLA-E and HLA-G) and tumor-infiltrating lymphocytes (CTL, Treg and NK cells) [15]. In this study a clear association was observed between patient outcome and three tumor immune subtypes (low-, intermediate and high immune subtypes)(Appendix 1).

Our research group also demonstrated that the level of tumor cell proliferative and apoptotic signalling are important predictors in determining tumor development and thus predicting clinical outcome [16], and could thus very well add to the value of immunosubtypes in breast cancer. Assuming that healthy tissue signifies a fine proliferative-apoptotic balance, we propose that tumor growth may be more accurately determined by the outcome of the balance between tumor cell proliferation (Ki67) on one side and apoptosis (cleaved caspase-3) on the other.

With the increasing ability of earlier diagnosis and subsequently the low relapse rate in early breast cancer patients, in combination with the increasingly demanding nature of the contemporary patient population, the bar is raised for clinicians regarding optimal treatment [17]. Therefore, individualized estimation of the true therapeutic benefit is of crucial importance, in order to avoid over- and under-treatment.

Although the two major histological subtypes are frequently treated as similar entities, there are obvious differences in tumor-biological and prognostic characteristics.

The purpose of this study was to investigate the relevance of the host immune response, the apoptotic-proliferative interaction and molecular tumor types in the two major histological subtypes of breast cancer and in different molecular tumor types.

MATERIAL AND METHODS

Patients and tumors

Non-metastatic breast cancer patients who underwent primary surgical treatment at the Leiden University Medical Center (LUMC) between 1985 and 1996, with or without adjuvant systemic treatment were included in the present cohort. Only patients with ILC or IDC were included in the study. Patients with bilateral tumors or an earlier history of cancer (other than basal cell carcinoma or cervical carcinoma *in situ*) were excluded. Data on age, histological type, tumor grade, TNM stage, ER, PR and HER2 were assembled. In

addition, we collected information concerning local and systemic therapy and follow-up until loco-regional and/or distant recurrence and/or death. All tumors were graded according to current pathological standards by a single breast cancer pathologist (VS). Approval for the study was obtained with the LUMC Medical Ethics Committee. All samples were non-identifiable and coded according to the national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Formalin fixed paraffin-embedded tumor blocks of the primary tumor were collected at the pathology department. H&E stained sections with clear histopathological tumor representation were used for assembling of tumor tissue microarray (TMA) paraffin blocks. From each donor breast tumor tissue block, three 0.6 mm^2 tissue cores were punched from tumor areas and transferred into a recipient paraffin block using a custom-made precision instrument. Sections of 4 µm were cut from FFPE tumor TMA material. Tissue sections were deparaffinised and rehydrated. Immunohistochemical staining was performed according to previously described standard protocols [18]. As previously described, sections were incubated overnight with anti-Ki67 (mouse anti human, M7240 Clone MIB-1: Dako, NL) , anti-cleaved Caspase-3 (Rabbit anti human, Anti-Asp175 #9661: Cell Signaling, USA) [16], anti-CD8 (mouse anti human, ab 17147, clone 144B: AbCam, UK), anti-PEN5 (mouse anti human, IM2354, clone 5H10.21.5: Beckman Coulter, NL), mouse monoclonal anti HCA2 and HC10 directed against Classical HLA class I (anti HLA-A and anti HLA-B/C, respectively) and non-classical HLA class I molecules using mouse monoclonal antibodies against HLA-E (ab2216, clone MEM-E/02: AbCam, UK) and HLA-G, ultimately Treg infiltration was determined using anti-FoxP3 antibody (ab 20034, clone 236A/E7: AbCam, UK) with the predetermined optimal dilutions [15;18;19]. For the molecular profiles additional staining was performed for EGFR (NCL-EGFR, Novocastra, UK) and CK5/6 (Clone D5/16 B4, Dako, NL). Immunohistochemical staining and quantification of ER, PGR and HER2 were performed previously. For each staining, all slides were stained simultaneously to avoid inter-assay variation. Negative controls were tissue sections that underwent the whole immunohistochemical staining with omission of the primary antibody.

Evaluation of the immunostaining

Expression of all markers were previously categorized in loss *versus* expression for classical HLA class I; no expression *versus* expression for HLA-E and HLA-G; infiltration absent *versus* infiltration present for Treg cells; presence *versus* absence for PEN5 [15;18;19].The absolute number of infiltrating CD8-positive cells was microscopically assessed per mm^2 and classified into two groups based on two thirds of patients with the lowest number

of CD8 infiltration/mm^2 *versus* the one third of patients with the highest number of CD8 infiltration/mm^2 [15]. For cleaved Caspase-3 staining the mean expression grade of positively stained cells in the TMA was defined as absent, low, intermediate and high scores. Cut-offs for low versus high expression of Ki67, EGFR and CK5/6 were based on the median expression level [16].

Tumor immune subtypes

Tumor immune subtypes, representing tumor adaptive immune escape variants were constructed with data from all known immunological variables of this patient cohort: classical HLA-I (HCA2 and HC10) and non-classical HLA-E and HLA-G expression and Treg (FoxP3), CTL (CD8) and NK cell (PEN5) infiltration in the tumor material [15]. As described by de Kruijf *et al.* , initially seven tumor immune subtypes were defined in ascending order from high immune susceptibility to low immune susceptibility. However, to facilitate clinical applicability the seven immune subtypes were brought back to a more simplified tumor immune subtype variable: high immune susceptibility, intermediate immune susceptibility and low immune susceptibility [15]. Only latter subdivision was used in this experimental design.

Molecular Subtypes

The IHC molecular profiles were previously developed by Carey *et al.* and validated for inter-assay agreement using a gene expression assay [7]). The IHC profile comprised of the markers ER, PGR, HER2, Ki67, EGFR and CK5/6. The Luminal A profile was defined as: ER+ and/or PGR+, HER2- and Ki67-; Luminal B: ER+ and/or PGR+ and HER2+ and/or Ki67+; ERBB2: ER-, PGR- and HER2+; Basal-like: ER-, PGR-, HER2- and EGFR+ and/or CK5/6+ and lastly the unclassified type: ER-, PGR-, HER2-, EGFR- and CK5/6-.

Statistical Analysis

Missing data were imputed (multiple imputation) using a model with IDC/ILC, grade, stage, age, follow-up and recurrence status, tumour immune subtypes, Ki67, Caspase3, molecular subtypes, ER, PR and HER2. With respect to multiple imputation, we generated 25 iterations and combined the estimates and standard errors using Rubin's Rules (micombine in STATA). Prior to running the model, checks were performed to test whether the data was missing at random. Multiple imputation by chained equations was used which assumes a multivariate distribution exists without specifying its form. In STATA the ICE module was used to perform the multiple imputation. Univariable and multivariable binary logistic regression analyses were used to identify differences between IDC and ILC. All variables with a $p \leq 0.1$ in univariable analyses were entered in the multivariable model. Relapse Free Period (RFP) was calculated using multivariable Cox proportional hazard models with any recurrence (locoregional recurrence and/or

distant recurrence, whichever came first) as event, with results stratified for IDC and ILC. Additional analyses were performed and stratified by age, tumor grade, tumor stage and nodal status. With respect to molecular subtypes, regression analyses were performed to assess proportional differences between molecular subtypes and immunological subtypes, Caspase3 and Ki67. In addition, Cox proportional hazard models were used to assess RFP in relation to immunological subtype, and stratified by molecular subtype. STATA/SE 12.0 version was used for all analyses.

RESULTS

Patient and tumor characteristics

Tumor material was available for 87% (704/822) of the patients. Median follow-up was 10 years (range= 0.02-22years), and median age in this cohort was 58 years (range 23-96 years). Clinicopathological and treatment characteristics for the original and imputated cohorts are shown in table 1.

IDC and ILC: differences in associations with clinicopathological parameters

No statistically significant difference was seen between IDC and ILC with regard to the association with tumor immune subtypes (p=0.4) and molecular subtypes (p=0.4). For the classical prognostic variables tumor grade (p<0.001) and pathological tumor stage (p=0.0002), a significant difference was seen between lobular and ductal breast tumor histology. ILC had significantly more grade II tumors and a higher pathological tumor stage. Both remained independent prognostic factors in the multivariate correction (grade: p<0.001, hazard ratio (HR): 12.6, 95% confidence interval (CI): 3.5-44.8); pathological tumor stage: p<0.0001, (HR pT2: 2.3 (95%CI: 1.1-4.8), HR pT3: 9.1 (95%CI: 3.1-26.4), HR pT4: 10.3 (95%CI: 3.0-35.5)). Also, compared to IDC, ILC showed a significantly lower expression pattern for both cleaved Caspase-3 (p=0.0004, HR low: 0.2 (95%CI: 0.1-0.6), HR intermediate: 0.4 (95%CI: 0.1-0.9), HR high: 0.1 (95%CI: 0.01-0.4)) and Ki67 (p=0.03, HR: 0.4, 95%CI: 0.2-0.9) following multivariable analyses.

Interaction with breast cancer histology

An interaction test for RFP and histological subtype was performed to test for differences in effect between IDC and ILC. Results showed a significant effect modification for RFP for immune subtype (p<0.001). In addition, similar results were observed with regard to active Caspase-3 (p<0.001) and molecular subtype (p=0.0005). With regard to Ki67, no effect modification was observed (p=0.09).These findings indicate a possible influence of breast cancer histology on the prognostic value of immune subtype, active Caspase-3 and molecular subtype.

Table 1: Baseline characteristics and distributions in the original and imputated datasets

		Original dataset		Multiple imputations
		N	%	%
Age	<45	137	19.2	19.2
	45-54	175	24.5	24.5
	55-64	157	22.0	22.0
	65+	245	34.3	34.3
Year	1985-1988	251	35.1	35.1
	1989-1992	232	32.5	32.5
	1993-1996	231	32.4	32.4
ER	Negative	288	40.4	42.7
	Positive	393	55.0	57.3
	Missing	33	4.6	
PR	Negative	316	44.3	48.0
	Positive	351	49.1	52.0
	Missing	47	6.6	
HER-2	No overexpression	520	72.8	83.2
	Overexpression	59	8.3	16.8
	Missing	135	18.9	
Grade	Grade I	116	16.3	16.8
	Grade II	342	47.9	48.7
	Grade III	244	34.2	34.5
	Missing	12	1.7	
pT stage	pT1	289	40.5	41.4
	pT2	328	45.9	47.2
	pT3	44	6.2	6.5
	pT4	33	4.6	4.9
	Unknown	20	2.8	
pN stage	Negative	381	53.4	54.9
	Positive	313	43.8	45.1
	Unknown	20	2.8	
Histological subtype	IDC	638	89.4	90.6
	ILC	66	9.2	9.4
	Missing	10	1.4	
Surgery	Mastectomy	416	58.3	58.3
	BCS	298	41.7	41.7

Abbreviations: pT stage: pathological Tumor stage, pN stage: pathological Nodal stage, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma; BCS: breast conserving surgery

Relapse-free period in relation to IDC and ILC

Immunological profile was found to be prognostic for RFP in patients with IDC but not ILC, revealing a hazard ratio (HR) of 3.16 for low immune susceptibility compared to high immune susceptible tumor types for IDC only (p=0.002) (table 2). With regard to ILC a statistically significant association was only found in relation to immune subtype when stratified by tumor grade (grade I&II: p<0.001 and grade III: p=0.01 (data not shown).

For both high expression of apoptotic Caspase-3 (p=0.02, HR1.6, 95%CI: 1.1-2.4) and high expression of proliferative Ki67 (p=0.03, HR1.33, 95%CI: 1.02-1.74) a significantly worse association was found with RFP in IDC, but not for ILC (table 2). When stratified by pathological tumor stage, a significant association with the RFP was only found for stage I and II tumors (high Ki67 HR: 1.37, 955CI 1.01-1.84, p=0.04 and high caspase-3 HR: 1.85, 95%CI: 1.21-2.81, p= 0.0004) in IDC. With regard to IDC stage III and IV and ILC, no significant association was observed for Ki67 and caspase-3 (data not shown).

Table 2: Association between breast cancer histological subtype and active caspase-3, Ki67 and immunological subtypes in relation to relapse-free period

	Ductal breast cancer		Lobular breast cancer	
	HR* (95%CI)	**p-value**	**HR* (95%CI)**	**p-value**
All patients				
Immune subtypes				
High	1 (ref)	**0.002**	1 (ref)	0.3
Intermediate	1.95 (1.09-3.48)		2.10 (0.51-8.73)	
Low	3.16 (1.59-6.25)		3.24 (0.73-14.38)	
Active caspase-3				
Negative	1 (ref)	**0.02**	1 (ref)	0.2
Low	1.04 (0.72-1.52)		1.4 (0.4-4.7)	
Intermediate	1.55 (1.07-2.25)		3.8 (0.8-17.3)	
High	1.58 (1.06-2.36)		4.2 (0.7-24.2)	
Ki67				
Low	1 (ref)	**0.03**	1 (ref)	0.7
High	1.33 (1.02-1.74)		0.83 (0.29-2.37)	

* Adjusted for age, pT and pN

(Statistical interaction tests for histological subtype (IDC-ILC) and immune subtypes: p<0.001; histological subtype (IDC-ILC) and active caspase-3: p<0.001; histological subtype (IDC-ILC) and Ki67: p=0.09; histological subtype (IDC-ILC) and molecular subtypes: p=0.0005)

When Caspase-3 and Ki67 were combined, a statistically significant association was observed in relation to RFP for IDC (p=0.003), but not for ILC (p=0.07) (data not shown).

The highest HR was seen for high caspase-3 expression combined with a high proliferative Ki67 rate (HR2.0, 95%CI: 1.2-3.3, p=0.003). When stratified by immune subtype, intermediate tumor immune phenotypes were significantly associated with Caspase-3 (p=0.004) and Ki67 (p=0.002) expression regarding RFP. With increasing expression rate, both factors showed higher hazard ratios (Caspase-3 high: HR: 2.0, 95%CI: 0.9-4.2 and Ki67 high: HR: 2.2, 95%CI: 1.3-3.6). This was not observed in high or low tumor immune subtypes (data not shown).

Table 3: Associations between molecular subtypes and immune subtypes(**A**), active caspase-3(**B**) and Ki67(**C**)

A. Molecular subtypes (p=0.6)	**Immune High**	**Immune Intermediate**	**Immune Low**
Unclassified	14.0	74.5	11.4
Luminal A	17.0	60.9	22.1
Luminal B	19.7	61.9	18.4
HER2	16.0	58.4	25.6
Basal	19.5	58.1	22.5

B. Molecular subtypes (p<0.001)	**Caspase-3 Negative**	**Caspase-3 Low**	**Caspase-3 Intermediate**	**Caspase-3 High**
Unclassified	36.0	34.2	20.4	9.4
Luminal A	37.8	35.5	19.0	8.7
Luminal B	25.1	34.2	21.9	18.9
HER2	34.2	19.6	19.9	26.4
Basal	18.9	25.2	24.7	31.2

C. Molecular subtypes (p<0.001)	**Ki67 low**	**Ki67 high**
Unclassified	63.8	36.2
Luminal A	92.6	7.4
Luminal B	8.9	91.1
HER2	49.4	50.6
Basal	34.4	65.6

Molecular subtypes: immune profiles and prognosis

There were no proportional differences between molecular subtypes and tumor immune subtypes (p=0.6) (Table 3A). Luminal A tumors frequently did not express Caspase-3, while high expression was more prominent in Basal-like tumors (p<0.001) (Table 3B). Basal-like tumors also expressed higher levels of Ki67 (p<0.001) (Table 3C). As expected, luminal A tumors expressed low levels of Ki67, while luminal B tumors expressed high

Table 4: Associations of molecular and immunological subtypes with Relapse Free Period

		All Histological BC types	
Molecular subtypes	**Immune subtypes**	**HR* (95%CI)**	**p-value**
Luminal A			
	High	1 (ref)	**0.006**
	Intermediate	1.8 (0.8-4.4)	
	Low	3.9 (1.5-10.1)	
Luminal B			
	High	1 (ref)	0.4
	Intermediate	1.8 (0.8-4.2)	
	Low	2.0 (0.7-5.9)	
Basal-like			
	High	1 (ref)	0.1
	Intermediate	2.3 (0.7-7.7)	
	Low	3.8 (1.2-12.5)	

* Adjusted for age, pT and pN; HER2 excluded due to too few numbers

Ki67 levels (Table 3C). Needless to say, immune profiles were strong prognostic indicators in Luminal A tumors, but not in Luminal B, HER2, or Basal-like tumors (Table 4). Luminal A tumors with low immune susceptibility showed a worse RFP than patients with high immune susceptible tumors (high vs. low: HR 3.9, 95%CI:1.5-10.1, p=0.006).

DISCUSSION

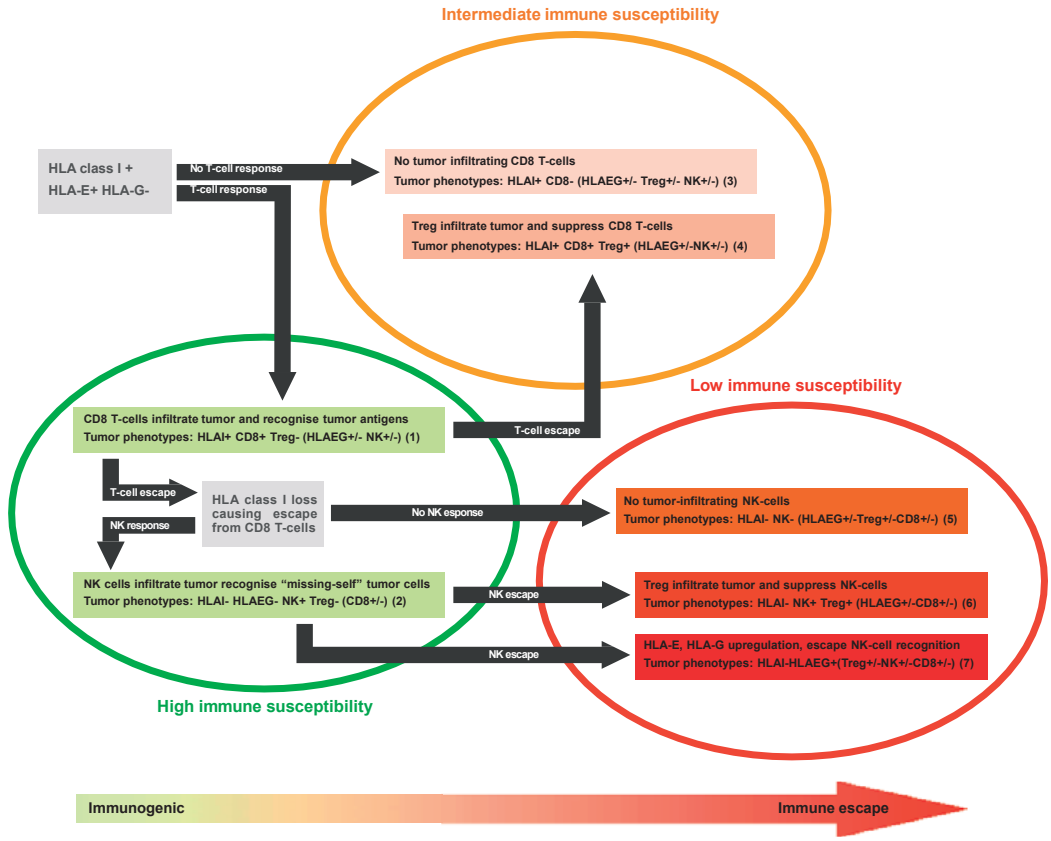
Previously, our study group reported that breast cancer patients with particular immunological profiles were more susceptible to unfavourable outcomes, demonstrating that patients with low immune susceptible tumors had a poorer prognosis when compared with intermediate or high susceptible tumors [15]. Multiple research groups demonstrated that molecular profiling of breast cancer also has important prognostic value [4;5]. The current study focused on distinguishing the major histological subtypes to assess whether tumor immune and molecular profiles were of prognostic value. Our results show that tumor immune profiles are prognostic indicators in different histological subtypes of breast tumors.

IDC and ILC, by far, constitute the largest group of breast tumors, comprising up to 95% of all breast cancers. Although current treatment regimens do not distinguish between these histological subtypes, IDC and ILC are considered to be distinctive entities, and differentiating between these two subtypes may play a role in prognosis and the optimisation of breast cancer treatment in addition to tumor size, histological grade, lymph node, ER/PR and HER2 status. The role of the immune response in cancer prognosis

has been speculated on previously. Several studies have demonstrated a correlation between single immune markers and patient outcome. CD8+ lymphocytes, one of the most studied immune markers worldwide revealed that in various types of cancer the presence hereof results in advantageous outcomes [20]. De Kruijf *et al.* demonstrated that the presence of classical HLA class I and high amounts of Treg infiltration affect prognosis in chemotherapy-treated breast cancer patients only [18]. In all probability, chemotherapy may selectively eliminate Treg, thus enabling CTLs to kill tumor cells that have retained HLA-I expression [18]. The same group demonstrated that presence of non-classical HLA subtypes E and G were associated with a worse relapse-free period [19]. This highly prognostic relation in breast cancer was also seen when the immune markers were combined into immunological subtypes [15]. However, in none of the previous studies the distinction was made between IDC and ILC. Differences in histological subtype may evoke diverse responses on breast cancer cells, thereby rendering one subtype more susceptible to the host immune response than another. In IDC patients, our analyses showed that low immune susceptibility as well as high Caspase-3 and Ki67 expression, were associated with a worse RFP, while this could not be demonstrated for ILC. These results also suggest that neither the apoptotic or proliferative marker, nor immune profiling applies to ILC, again suggesting that these tumors differ biologically from IDC.

With regard to molecular subtype, no correlation was observed between tumor immune subtype and molecular subtype. Based on previous studies, we know that tumors overexpressing HER2 and basal-like tumors generally present with more aggressive clinical characteristics than Luminal A and Luminal B tumors [4;5]. Our results confirm that tumor aggressiveness, as established by molecular subtypes of breast cancer, is not dependent on a tumor's immunological profile. In addition, immunological profiling was found to be prognostic only for Luminal A tumors. Luminal A tumors make up the largest group of IDC. Therefore it is not surprising that these results show a similar prognostic association within the immune profiles. Jung *et al.* proposed that ILC is frequently strongly ER-positive, HER2-negative and presents with low Ki67 expression, making it more likely to be characterized as a Luminal A molecular subtype [21]. This finding may lead to the assumption that outcomes for molecular and histological subtypes are similar, but this was not confirmed in our analyses. This implies that a simple extrapolation cannot be made and that histological subtypes are presumably far more complex.

In this report we investigated the relationship of the clinical outcome of breast cancer patients with immunological and histological profiles. Our results show that tumor immune biology differs greatly between IDC and ILC patients, confirming that ILC and IDC are completely different entities. Further studies are needed to validate these differences between IDC and ILC.



Appendix 1: Tumor immune subtypes: showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape and low immune susceptibility (red), concerning combinations of CTL infiltration, NK-cell infiltration, Treg infiltration, classical HLA class I tumor expression, and HLA-EG tumor expression (de Kruijf *et al.*, BCRT 2013).

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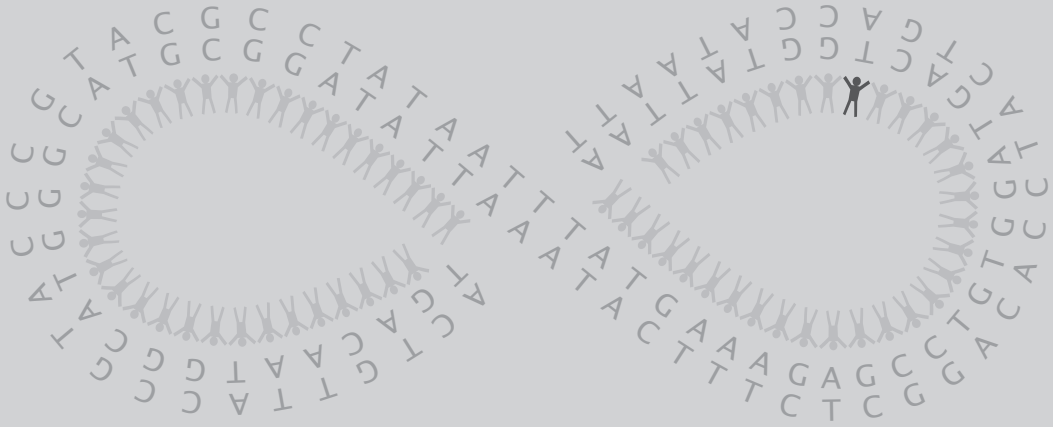

Chapter 5

The prognostic and predictive value of Tregs and tumor immune subtypes in postmenopausal, hormone receptor-positive breast cancer patients treated with adjuvant endocrine therapy: A Dutch TEAM Study Analysis

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ABSTRACT

Purpose

Evidence exists for an immunomodulatory effect of endocrine therapy in hormone receptor-positive (HR+ve) breast cancer (BC). Therefore, the aim of this study was to define the prognostic and predictive value of tumor immune markers and the tumor immune profile in HR+ve BC, treated with different endocrine treatment regimens.

Methods

2596 Dutch TEAM patients were treated with 5 years of adjuvant hormonal treatment, randomly assigned to different regimens: 5 years exemestane or sequential treatment (2.5 years tamoxifen-2.5 years of exemestane). Immunohistochemistry was performed for HLA class I, HLA-E, HLA-G, and FoxP3. Tumor immune subtypes (IS) (low, intermediate & high immune susceptible) were determined by the effect size of mono-immune markers on relapse rate.

Results

Patients on sequential treatment with high level of tumor-infiltrating FoxP3+ cells had significant (p=0.019, HR: 0.729, 95%CI: 0.560-0.949) better OS. Significant interaction for endocrine treatment and FoxP3+ presence was seen (OS p<0.001). Tumor IS were only of prognostic value for the sequentially endocrine treated patients (RFP: p=0.035, HR intermediate IS: 1.420, 95%CI: 0.878-2.297; HR low IS: 1.657, 95%CI: 1.131-2.428; BCSS: p=0.002, HR intermediate IS: 2.486, 95%CI: 1.375-4.495; HR low IS: 2.422, 95%CI: 1.439-4.076) and OS: p=0.005, HR intermediate IS: 1.509, 95%CI: 0.950-2.395; HR low IS: 1.848, 95%CI: 1.277-2.675).

Conclusion

Tregs and the tumor IS presented in this study harbour prognostic value for sequentially endocrine treated HR+ve postmenopausal BC patients, but not for solely exemestane treated patients. Therefore, these markers could be used as a clinical risk stratification tool to guide adjuvant treatment in this BC population.

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed female cancer in the developed world and also leading cause of cancer death, responsible for 14% of cancer-related deaths in women of the West [1]. Nowadays, BC treatment consists of a combination of locoregional treatment (i.e. surgery and radiotherapy) and systemic therapy (i.e. chemotherapy and hormonal therapy), to concur present and less evident metastasis. In the USA, an increased tendency of adjuvant treatment allocation using genomic expression assays such as Onco*type* DX (genomic health, redwood city, CA, USA) and Mammaprint (Agendia, Amsterdam, the Netherlands), providing additional information about the risk of relapse and benefit of adjuvant chemotherapy, is seen [2-4]. However, in the Netherlands, decisions regarding the use of adjuvant systemic therapy in primary BC patients are still mainly based on classical prognostic factors, like lymph node status, tumor-size, -grade, hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) expression [5]. However, currently these do not provide optimal risk-stratification, resulting in over- and undertreatment of certain patients. There is evidence that a host's cellular immune response plays a pivotal role in controlling tumor progression through a number of immunological mechanisms, involving classical human leukocyte antigen (HLA) class I and non-classical HLA-E and HLA-G expression by the tumor, and presence of tumor infiltrating cytotoxic T cells (CTL), Natural Killer (NK) cells and regulatory T cells (Tregs) [6-11], suggesting that complex interactions take place between breast tumor cells and immune cells [12]. Valuable prognostic interactions reported are those between classical HLA class I and Tregs, where loss of HLA class I in combination with presence of Treg in the tumor microenvironment resulted in a worse patient's outcome, and also the interaction between classical HLA class I, HLA-E, and HLA-G tumor expression, where HLA-E and HLA-G expression resulted in worse patient outcome in the co-occurrence of loss of classical HLA class I on the tumor surface [8;9;12]. Together, this emphasizes the importance of research on combinations of markers of immune surveillance together with markers of tumor immune escape.

Our group previously constructed breast tumor immune subtypes (IS) by combining markers of immune surveillance together with markers of tumor immune escape, based on a biological rationale [13]. Data revealed strong associations with patient outcome whereby tumors defined as highly susceptible to immune attack showed favorable clinical outcome compared to patients with tumors harboring a low immune susceptibility profile, independent of known clinicopathological parameters [13]. In the current study we used another approach to define tumor IS. Tumor immune mono-markers in Dutch postmenopausal hormone-sensitive BC patients from the Tamoxifen and Exemestane Adjuvant Multicenter (TEAM) trial were correlated to clinical outcome. Subsequently,

we designed tumor immune subtypes based on statistical effect sizes of the immune monomarkers on relapse rate.

It has already been shown that tumor-infiltrating lymphocytes (TILs) act as an independent predictor of response to chemotherapy treatment [14-16]. Elaborating on this result, evidence also exists for an immunomodulatory effect of tamoxifen; it is thought that tamoxifen induces a shift from cellular (T-helper 1) to humoral (T-helper 2) immunity [17]. Given the fact that T-helper 1 immunity is essential for anti-tumor immune response, a tamoxifen-induced shift away from cellular immunity may represent a significant step in tumor development. This would hamper the cytotoxic effect of tamoxifen and possibly explain the differential effect of aromatase inhibitors versus tamoxifen on clinical outcome [17-19].

The aim of our current study was therefore to investigate the difference in prognostic value of tumor IS in relation with type of hormonal treatment received in HR+ve, postmenopausal BC patients.

PATIENTS AND METHODS

Patients and tumors

Eligibility criteria for the TEAM study have been previously described [20]. In brief, patients were postmenopausal and had HR+ve early BC diagnosed between 2001 and 2006. Patients with bilateral tumors or prior history of cancer were excluded. Patients were randomly assigned in a 1:1 ratio to either exemestane, 25mg daily for five years, or sequential therapy consisting of tamoxifen 20mg daily for 2.5 years followed by exemestane 25mg daily for another 2.5 years [20].

Medical-ethical approval was obtained and the study was conducted in accordance with the Declaration of Helsinki. All TEAM patients gave informed consent prior to enrolment in the study. Surgically resected formalin-fixed paraffin-embedded (FFPE) tumor samples of the Dutch TEAM patients (n=2596) were used. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Data was centrally collected at the Datacenter of the Department of Surgery of the Leiden University Medical Center. For all patients the following data was known: age at diagnosis, histological tumor grade, HR status, tumor size and nodal stage, type(s) of local and systemic treatment, date and type of disease recurrence, death and follow-up data. Reporting of the biomarkers was done according to the REMARK criteria [21].

Immunohistochemistry

Immunohistochemical staining was performed on 4µm FFPE Tissue Micro Array sections consisting of breast cancer tissue of the Dutch TEAM patients (three 0.6 mm^2 tumor tissue punches per patient) [22]. The tissue sections were stained according to the previously described protocol [9]. Sections were incubated at room temperature over night with mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLAB/C, respectively) [9;23] for the detection of classical HLA class I on the tumor cell surface. Non-classical HLA class I staining was performed using mouse monoclonal antibodies against HLA-E (MEM-E/02 Clone (sc-51621, Santa Cruz biotechnology, Dallas, Texas)) and HLA-G (4H84 Clone (sc-21799, Santa Cruz Biotechnology, Dallas, Texas)) [8]. Mouse monoclonal antibodies against FoxP3 (clone 236A/E7 (ab20034, Abcam, Cambridge, United Kingdom)) were used to identify Tregs [9]. All slides were stained simultaneously to avoid inter-assay variation.

Evaluation of immunostaining

Microscopic quantification of positive tumor cells for HCA2, HC10, HLA-E and HLA-G was performed in a blinded manner by two independent observers (C.C.E., A.S. and A.v.V). The scores of the three tissue cores were averaged. For HCA2 and HC10, the percentage of tumor cells with membranous staining was assessed. Classical HLA class I expression status was determined according to the standard set by the International HLA and Immunogenetics Workshop [24]. According to this standard, HCA2 and HC10 staining were scored in two categories: score 1 (0-5% of tumor cells positively stained) or score 2 (5-100% of tumor cells positively stained). Three groups were defined for classical HLA class I expression: HLA class I loss (both HCA2 and HC10 scored 0-5%); HLA class I downregulation (either HCA2 or HC10 scored 0-5%); and HLA class I expression (both HCA2 and HC10 scored 5-100%) [9]. For non-classical HLA class I markers, both HLA-E and HLA-G were scored based on the percentage of tumor cells with membranous staining and re-categorized in a binary manner. Any specific staining of tumor cells was considered positive and no staining was considered negative for HLA-G. HLA-E expression was divided into quartiles, of which the first quartile was categorized as low HLA-E expression and subsequent quartiles (> first quartile) as high. FoxP3+ nuclear presence per mm^2 in tumor epithelium and surrounding stroma tissue was identified with the use of a Panoramic Midi scanner (3DHistech, Hungary) by means of an automated positive cell count analysis using AxioVision 4.6 (Carl Zeiss Vision, Jena, Germany). FoxP3+ presence was scored by two categories: low (≤49 positive cells) and high (>49 positive cells) Treg infiltration per mm^2, based on the median value.

Statistical analysis

Statistical analyses were performed using statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics). Patients of whom tumor material was lost during staining procedure were excluded from analyses. Cohen's kappa coefficient was used to assess inter-observer agreement in quantification of HCA2, HC10, HLA-E and HLA-G. As BC relapse strongly influences survival rates of BC patients, we designed tumor IS based on the regression coefficient of mono-markers in the Cox-regression using Relapse Free Period (RFP) as clinical endpoint for all tumor samples. The regression coefficient value, indicating either negative or positive clinical effect, served as a penalty or bonus (in case of a negative or positive slope, respectively). All regression coefficients (for HLA-I, HLA-E, HLA-G and FoxP3+) were added up to construct the final score per patient. Ultimately, three groups: low, intermediate and high immune susceptible tumor types were constructed based on tertile (≤33%, >33-≤67% and >67%) cut-off points of the final score.

The χ^2 test was used to evaluate associations between the tumor immune monomarkers, and also between clinicopathological parameters and tumor immune monomarkers and tumor IS. The clinical endpoints were RFP, defined as time from date of randomization in the TEAM-trial until any recurrence (loco-regional recurrence and/or a distant recurrence, whichever came first), Breast Cancer Specific Survival (BCSS), defined as time from date of randomization until death due to BC, and Overall Survival (OS), defined as time from randomization until death by any reason. The Kaplan–Meier method was used for survival plotting and log-rank test for RFP, BCSS and OS curve comparison. Cox proportional hazard analysis was used for univariate analysis and was additionally adjusted for clinically relevant confounders (age, pathological tumor and nodal stage, tumor grade, histology, and treatment). All analyses were stratified for hormonal regimen (exemestane or sequential regimen). Interaction between endocrine treatment and tumor IS was tested in a multivariable model.

RESULTS

Patient and tumor characteristics

The Dutch TEAM cohort consists of 2596 postmenopausal non-metastasized BC patients with a median age of 65 years (range: 38-91y). Median follow-up of patients was 5.9 years. Clinicopathological and treatment characteristics in relation with tumor IS are shown in Table 1. Only for radiotherapy a significant difference (chi-square test, p=0.045) was seen for between tumor IS, showing less radiotherapy treatment for intermediate tumor IS compared to low and high tumor IS. Substantial agreement ($K \geq 0.6$) was observed for quantification of all immunohistochemical stainings.

Table 1: patient and tumor characteristics

	High immune subtype		Intermediate immune subtype		Low immune subtype		*p*-value
	N=501	%	N=318	%	N=817	%	
Age							
<65	259	51.7	164	51.6	425	52.0	0.988
≥65	242	48.3	154	48.4	392	48.0	
Missing	0		0		0		
pT stage							
T1	227	45.4	126	39.6	385	47.2	0.218
T2	244	48.8	169	53.1	387	47.4	
T3-4	29	5.8	23	7.2	44	5.4	
Missing	1		0		1		
pN stage							
N0	143	28.5	107	33.6	277	33.9	0.373
N1	319	63.7	192	60.4	490	60.0	
N2-3	39	7.8	19	6.0	49	6.1	
Missing	0		0		1		
Grade							
I	66	13.9	55	18.5	98	12.6	0.100
II	222	46.8	134	45.1	348	44.8	
III	186	39.3	108	36.4	330	42.6	
Missing	27		21		41		
Histology							
Ductal	391	78.5	249	78.6	664	81.8	0.495
Lobular	65	13.1	40	12.6	79	9.7	
Mixed	18	3.6	14	4.4	37	4.6	
Other	24	4.8	14	4.4	32	3.9	
Missing	3		1		5		
Operation							
Mastectomy	263	52.5	183	57.5	434	53.1	0.318
BCS	238	47.5	135	42.5	383	46.9	
Missing	0		0		0		
Radiotherapy							
Yes	318	63.5	174	54.9	500	61.2	**0.045**
No	183	36.5	143	45.1	317	38.8	
Missing	0		1		0		
Chemotherapy							
Yes	129	25.7	102	32.2	247	30.2	0.097
No	372	74.3	215	67.8	570	69.8	
Missing	0		1		0		
Endocrine therapy							
EXE	257	51.3	154	48.4	410	50.2	0.726
TAM→EXE	244	48.7	164	51.6	407	49.8	
Missing	0		0		0		

Abbreviations: pT: pathological tumor pN: pathological nodal BCS: breast conserving surgery EXE: exemestane TAM: tamoxifen

Classical HLA-I expression and association with prognosis

Microscopic quantification for classical HLA-I was successful in 73% (1891/2596) of tumors (79% (2042/2596) for HCA2 and 80% (2083/2596) for HC-10). Classical HLA-I loss was found in 16% (298/1891), down-regulation in 27% (513/1891) and expression in 57% (1080/1891)(Supplementary Table 1A). In the analyses stratified for endocrine treatment, no significant difference in outcome was seen for HLA-I expression in RFP, BCSS or OS (Supplementary Table 2A).

HLA-E and HLA-G expression and association with prognosis

Successful staining for HLA-E was obtained in 74% of tumors, and in 79% for HLA-G. Low HLA-E was found in 26% (495/1914) and high expression in 74% (1419/1914) of the patients, whereas absence of HLA-G was found in 76% (1558/2042) and expression in 24% (484/2042) of the patients (Supplementary Table 1B). Neither of the two immune markers showed significant association with clinical outcome when stratified for endocrine treatment received (Supplementary Table 2B and 2C).

Presence of FoxP3+ cells and association with prognosis

Automated positive cell count was successful in 93% (2426/2596) of tumors for FoxP3+ cells. Low (≤ median value of 49 cells) number of positive cells was seen in 51% (1241/2426) and high number (>median of 49 positive cells) in 49% (1185/2426) of the patients (Supplementary Table 1A). Patients on sequential hormonal therapy showed a significant (univariate: p=0.026, multivariate: p=0.019, HR: 0.729, 95%CI: 0.560-0.949) preferential outcome for high FoxP3+ presence in OS, but not for RFP or BCSS. No association with clinical outcome was seen for patients in the exemestane only treated arm (univariate OS: p=0.138, HR: 0.821, 95%CI: 0.633-1.065) (Supplementary Table 2D). The multivariable interaction model showed a significant predictive effect for endocrine treatment and FoxP3+ presence (p-value OS: <0.001) in OS.

Tumor immune subtypes and association with prognosis

In view of recent evidence stating that the interaction between tumor cells and cells of the immune system is multifaceted and complex [13], we hypothesized that combined analyses of immune markers may better reflect a patients' outcome by taking into account the interaction between tumor cells and cells of the immune system. First, when the four mono-markers were tested in relation to one another in the chi-square test, results showed a significant association between all four mono-markers (chi-square test, p-values: all <0.001, data not shown). No difference in distribution was observed for the defined risk groups in the two hormonal treatment arms (p=0.726). Based on the tumor IS model described in the material and methods section, which is based on the regression coefficient of the mono-markers in the RFP, high tumor immune susceptibility was char-

acterized by either classical HLA-I expression with HLA-EG presence or absence (HLA-EG absence: both or either HLA-E or HLA-G not expressed; HLA-EG positive: both HLA-E and HLA-G positive) on the tumor surface, known for its activation of Natural Killer (NK) cells [8], or classical HLA-I loss or down-regulation combined with mostly HLA-EG absence. Treg presence was equally distributed in the high IS tumor subtypes. Great variability in Treg presence was also seen in the low and intermediate tumor IS (Supplementary Table 3). The tumor IS showed significant preference for the high immune susceptible tumor types for clinical outcome (RFP: p=0.002, HR intermediate (versus high) tumor IS: 1.539, 95%CI: 1.088-2.178; HR low (versus high) tumor IS: 1.634, 95%CI: 1.235-2.163**;** BCSS: p<0.001, HR intermediate (versus high) tumor IS: 2.119, 95%CI: 1.368-3.283; HR low (versus high) tumor IS: 2.103, 95%CI: 1.456-3.038); OS: p=0.002, HR intermediate (versus high) tumor IS: 1.471, 95%CI: 1.065-2.032; HR low (versus high) tumor IS: 1.602, 95%CI: 1.235-2.077, Figure 1).

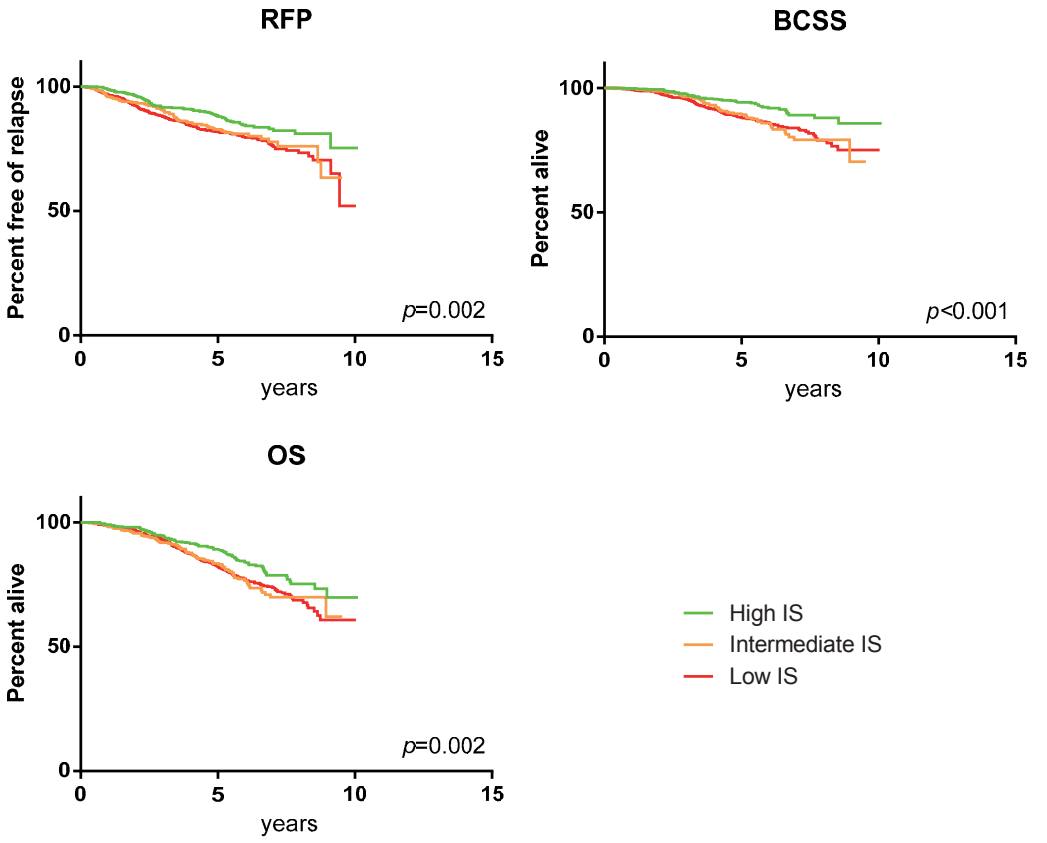

Figure 1: Tumor immune subtypes (high, intermediate and low tumor immune subtypes (IS)) in relation with clinical outcome parameters: Relapse Free Period (RFP); Breast Cancer Specific Survival (BCSS); and Overall Survival (OS), shown with corresponding adjusted (age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy, radiotherapy and endocrine therapy) p-values.

Immune subtypes and adjuvant endocrine treatment

Significant differences were seenfor RFP, BCSS, and OS in the sequentially endocrine treated patient group when stratified for adjuvant hormonal treatment. Again, all outcomes are in favor of high tumor immune susceptibility (RFP: sequential treatment: *p*=0.035, HR intermediate IS (versus high): 1.420, 95%CI: 0.878-2.297; HR low IS (versus high): 1.657, 95%CI: 1.131-2.428; BCSS:sequential treatment: *p*=0.002, HR intermediate IS (versus high): 2.486, 95%CI: 1.375-4.495; HR low IS (versus high): 2.422, 95%CI: 1.439-4.076; and OS:sequential treatment: *p*=0.005, HR intermediate IS (versus high): 1.509, 95%CI: 0.950-2.395; HR low IS (versus high): 1.848, 95%CI: 1.277-2.675 ,Table 2 and Figure 2). No prognostic value was seen for the solely exemestane treated patients. A statistical trend was seen for the interaction between endocrine treatment and tumor IS in the multivariable interaction model (*p*-value RFP: 0.15, BCSS: 0. 19 and OS: 0.17).

Table 2:

Out-come	Hormone therapy	Immune subtype	N	Univariate			Multivariate*			Interac-tion *p*
				HR	95%CI	*p*	HR	95%CI	*p*	
RFP	EXE	High	257	1.00		0.113	-	-	-	0.15
		Intermediate	154	1.556	0.958-2.526					
		Low	410	1.464	0.988-2.171					
RFP	TAM→EXE	High	244	1.00		0.086	1.00		**0.035**	
		Intermediate	164	1.343	0.850-2.122		1.420	0.878-2.297		
		Low	407	1.520	1.049-2.203		1.657	1.131-2.428		
BCSS	EXE	High	257	1.00		0.261	-	-	-	0.19
		Intermediate	154	1.482	0.812-2.708					
		Low	410	1.465	0.907-2.367					
BCSS	TAM→EXE	High	244	1.00		**0.002**	1.00		**0.001**	
		Intermediate	164	2.486	1.375-4.495		2.848	1.509-5.375		
		Low	407	2.422	1.439-4.076		2.869	1.651-4.984		
OS	EXE	High	257	1.00		0.204	-	-	-	0.17
		Intermediate	154	1.428	0.925-2.205					
		Low	410	1.311	0.924-1.858					
OS	TAM→EXE	High	244	1.00		**0.024**	1.00		**0.005**	
		Intermediate	164	1.531	0.993-2.362		1.509	0.950-2.395		
		Low	407	1.636	1.144-2.341		1.848	1.277-2.675		

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

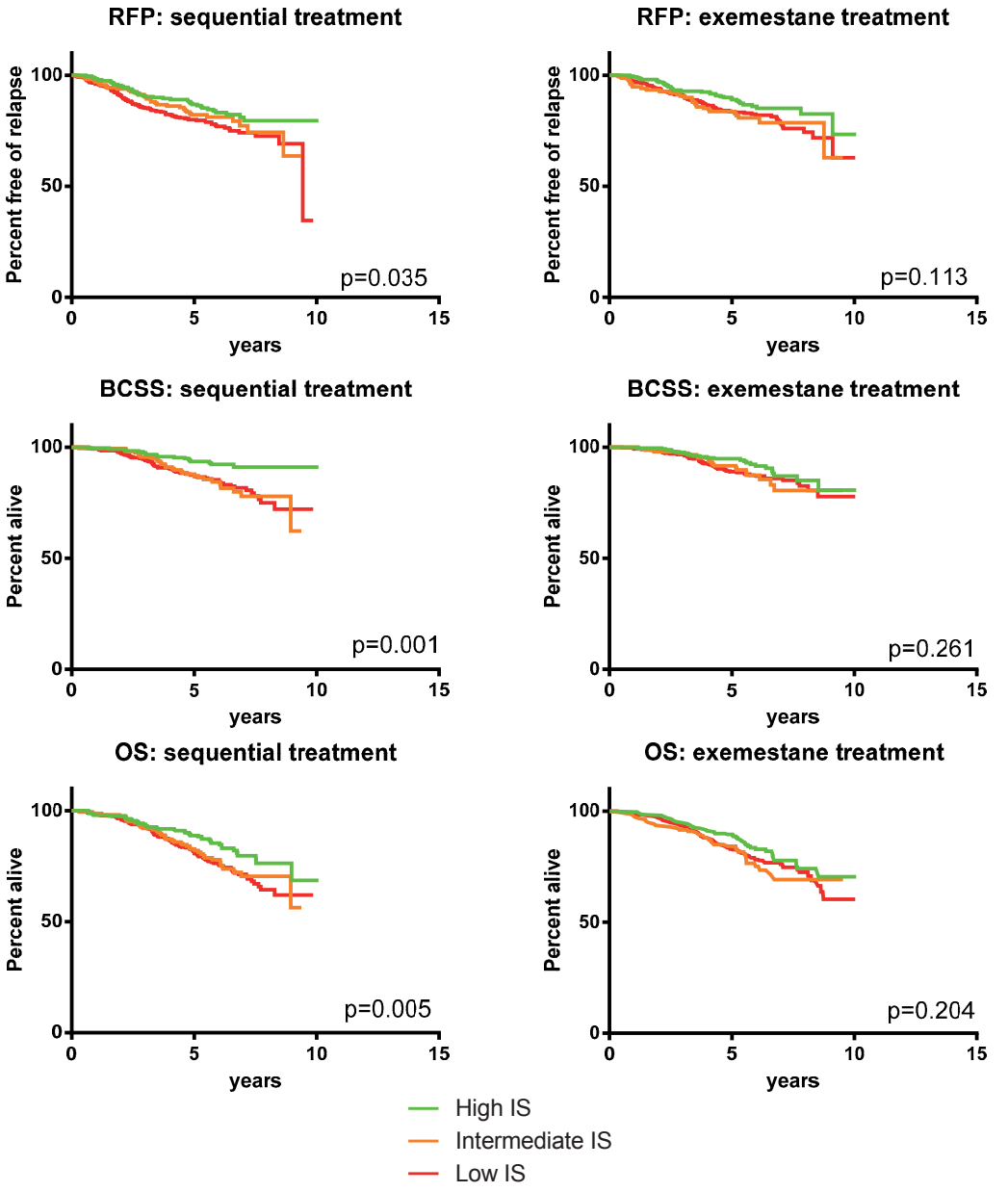

Figure 2: Tumor immune subtypes (high, intermediate and low tumor immune subtypes (IS)) stratified for endocrine therapy in relation with clinical outcome parameters: Relapse Free Period (RFP); Breast Cancer Specific Survival (BCSS); and Overall Survival (OS), shown with corresponding p-values (as seen in Table 3).

DISCUSSION

Evidence is building for an increasingly important role of tumor-immune interaction with regard to clinical outcome of cancer patients [25]. To our knowledge, this is the first study reporting on the effect of endocrine treatment on the prognostic value of Treg cells and tumor IS in a HR+ve BC cohort.

Our data suggest a positive effect of Treg presence on overall survival outcome in the sequentially endocrine treated patient group, which is further supported by a highly significant interaction term for endocrine treatment and Treg presence. This could possibly be explained by recent data indicating that Tregs harbour a dual role in cancer, suppressing anti-tumor immune response (inducible Treg) and suppressing inflammation which is known to promote carcinogenesis (natural Treg) [26;27]. These same studies suggest that the clinical and prognostic significance of Tregs in cancer depends on its environmental factors. Our investigated patient population harbours a number of pro-inflammatory risk factors, namely, a post-menopausal status which is known to be associated with systemic inflammation, and HR+ve breast tumors [28]. Assuming that HR+ve tumors attract higher estrogen levels in and around the tumor due to an increased tendency of estrogen binding, we hypothesize that this estrogen rich environment leads to higher Adenosine Deaminase Gene expression, which in turn is responsible for the degradation of Adenosine (ADO), a potent anti-inflammatory agent [29;30]. This presumed high inflammatory state in our patient population would assume a preference for natural Tregs, explaining the positive effect of high FoxP3+ presence in the tumors and the loss of prognostic significance in solely exemestane treated patients, as aromatase inhibition leads to lower estrogen levels, which will diminish ADO degradation.

For BC patients treated with sequential endocrine therapy, the tumor IS bare a strong independent significant prognostic value for BC specific survival and also, although to a lesser degree, for relapse rate and overall survival, while this association was not seen for patients treated solely with aromatase inhibition for five consecutive years. These data might imply that the immune profile of the breast tumor in sequentially endocrine treated breast cancer patients could predict BC death and overall death in HR+ve breast disease, and thus additional adjuvant therapy, such as chemotherapy and radiotherapy, could be optimally allocated based on this prognostic indicator. Since no prognostic effect was noted for the tumor IS in the solely exemestane treated patient population, the question remains whether there would be any benefit of additional adjuvant treatment for these patients, suggesting that currently we might have obtained the best attainable clinical outcome with five consecutive years of exemestane treatment, even for the low tumor immune susceptible HR+ve patient population. However, the multivariable interaction term for endocrine treatment and breast tumor immune subtypes hinted to a possible statistical trend for clinical outcome. The lack of significance in this test could be explained by the limited power of the statistical interaction test and also due to the low number of clinical events in our cohort.

In this study it was hypothesized that high immune susceptible tumor types, due to a tamoxifen induced shift from Th1 to Th2 immunity, would have the highest likelihood of showing regression of clinical outcome to mean relapse and survival rates of the overall cohort. Based on the data presented in this manuscript, the difference in prognostic

value of tumor immune subtyping between the two endocrine treatment arms cannot be explained by the previously described tamoxifen driven shift from Th1 to Th2 immunity [17]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift, by means of other immune interactions not requiring Th1 activation. A possible explanation for the loss of prognostic value of the tumor IS in the exemestane-treated patient arm of this cohort could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published by Chan *et al.*, showing a significant increase in the CD8+/Treg ratio in ER+ve patients, responding well to aromatase inhibiting therapy, herewith reflecting the dynamic process in which the hosts immune response to tumor antigens changed in consequence of estrogen depletion caused by the aromatase inhibitor [31]. Similarly, Generali *et al.* observed that FoxP3+ cell counts decreased significantly after letrozole treatment [32]. Therefore, one could hypothesize that in this specific HR+ve, postmenopausal BC cohort, exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor IS in the solely exemestane treated adjuvant treatment arm. If this would be true, one could speculate on the great importance of Treg for inhibition of tumor development in a post-menopausal, HR+ve tumor environment. Thereby proposing that under these conditions, HLA-I, HLA-E and HLA-G seem to merely have a supportive role in relation to Treg cells.

This is the first study that assessed the relation between adjuvant endocrine therapy and the prognostic value of tumor immune markers and tumor IS of postmenopausal HR+ve, early BC patients. Of course, the external validity of our results should be investigated in other large studies with tumor material available of HR+ve BC patients treated with different hormonal regimens, such as, for example the ATAC, BIG, or IES study. [18;33;34] The major strength of this study is the use of data from the TEAM-trial, as this provides well-registered data in a large number of patients. This study, however, also has its limitations. First, one could stress the shortcomings of FoxP3 staining, without co-staining of CD25 and CD4, for the detection of Tregs. Herewith, the margin of error for mistakenly scoring FoxP3+ breast tumor cells is increased [35]. However, based on careful review of the histology of the breast cancer tissue and given the fact that the majority of FoxP3+ cells were seen in the stromal region of the tumor tissue, we can state with reasonable certainty that the majority of positive cells were true Treg cells. Second, there were no standard tumor IS categories available from previous literature. Therefore, we categorized patients by tumor IS based on the regression coefficient of the mono-markers in the Cox-regression using RFP. One could criticize that this is an over-fitted model for RFP, but our results also showed significant association with the

other clinical outcome parameters BCSS and OS. Furthermore, our results did not show a difference in the distribution of the tumor IS for the two hormonal treatment arms, nevertheless, results showed a clear significant difference in the prognostic value of the IS based on the hormonal treatment received. Third, patients on sequential hormonal therapy received exemestane after the first 2.5 years of tamoxifen treatment. It would be desirable to compare two endocrine treatment regimens, consisting of solely exemestane and solely tamoxifen given for five consecutive years, eliminating the potential immune modulating effects of endocrine drugs with a different mode of action. Lastly, the immune contribution on clinical outcome described in this manuscript are all based on surgically derived tumor material, assuming that metastasizing cells harbour the same immunogenic characteristics. It should not be ignored that this approach disregards the possible interplay of systemic immune cells which undoubtedly also play a major role in anti-tumor immunity.

In conclusion, when taking into account the difference in associations of the tumor immune markers and tumor IS per endocrine treatment arm, these data partially support the hypothesis of previous manuscripts stating that endocrine treatment harbours an immune modulating effect [17;31]. Nonetheless, this study merely showed a statistical trend for interaction between tumor IS and type of endocrine treatment, and a strong interaction for FoxP3+ cells present in the tumor and endocrine treatment, implying that Based on the data presented in this manuscript, the difference in prognostic value of tumor immune subtyping between the two endocrine treatment arms cannot be explained by the previously described tamoxifen driven shift from Th1 to Th2 immunity [17]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift, by means of other immune interactions not requiring Th1 activation. A possible explanation for the loss of prognostic value of the tumor IS in the exemestane-treated patient arm of this cohort could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published by Chan *et al.*, showing a significant increase in the CD8+/Treg ratio in ER+ve patients, responding well to aromatase inhibiting therapy, herewith reflecting the dynamic process in which the hosts immune response to tumor antigens changed in consequence of estrogen depletion caused by the aromatase inhibitor [31]. Similarly, Generali *et al.* observed that FoxP3+ cell counts decreased significantly after letrozole treatment [32]. Therefore, one could hypothesize that in this specific HR+ve, postmenopausal BC cohort, exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor IS in the solely exemestane treated adjuvant treatment arm. If this would be true, one could speculate on the great

importance of Treg for inhibition of tumor development in a post-menopausal, HR+ve tumor environment. Thereby proposing that, despite the call for strong immune cell interplay recognition in tumor development, under these specific conditions, HLA-I, HLA-E and HLA-G seem to merely have a supportive role in relation to Treg cells.

To the best of our knowledge, this is the first study showing different associations in the prognostic value of tumor infiltrating Tregs and tumor IS with adjuvant endocrine treatment, and thus could be used as a clinical risk stratification tool in sequentially endocrine-treated HR+ve, postmenopausal BC patients. Therewithal, the results of this study add to previous studies on tumor-immune interactions in BC [6;13;17;36;37]. More research is needed to further elucidate this clinically relevant matter.

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SUPPLEMENTARY TABLES

Supplementary Table 1A: Clinicopathologic characteristics of the Dutch TEAM cohort for the expression of classical HLA class I and FoxP3+.

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	%	N	%	N	%	N	%		N	%	N	%	
	2596	100	298	100	513	100	1080	100		1241	100	1185	100	
Age in years														
< 40	1	0.0	0	0.0	0	0.0	1	0.1	0.534	1	0.1	0	0.0	0.484
40-50	57	2.2	11	3.7	11	2.1	19	1.8		31	2.5	22	1.9	
50-60	824	31.7	94	31.5	160	31.2	332	30.7		387	31.2	385	32.5	
≥60	1714	66.0	193	64.8	342	66.7	728	67.4		822	66.2	778	65.7	
Missing	0		0		0		0			0		0		
pT stage														
T1	1173	45.3	114	38.3	239	46.7	494	45.8	0.152	529	42.6	557	47.1	0.095
T2	1247	48.1	165	55.4	240	46.9	521	48.3		625	50.5	555	46.9	
T3/4	171	6.6	19	6.4	33	6.4	64	5.9		84	6.8	71	6.0	
Missing	5		0		1		1			3		2		
pN stage														
N0	785	30.2	78	26.2	157	30.6	352	32.6	0.431	369	29.7	383	32.3	0.467
N1	1641	63.2	201	67.4	325	63.4	655	60.6		793	63.9	720	60.8	
N2/3	168	6.6	19	6.4	31	6.0	72	6.7		78	6.4	81	6.8	
Missing	2		0		0		1			1		1		
Grade														
1	398	16.4	52	18.3	91	18.7	117	11.6	**0.001**	215	18.5	146	13.1	**<0.001**
2	1148	47.3	137	48.2	218	44.8	480	47.5		580	50.0	488	43.8	
3	883	36.4	95	33.5	178	36.6	414	40.9		365	31.5	479	43.0	
Missing	167		14		26		69			81		72		

Supplementary Table 1A: (continued)

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	%	N	%	N	%	N	%		N	%	N	%	
	2596	100	298	100	513	100	1080	100		1241	100	1185	100	
Histology														
Ductal	1936	75.2	229	78.2	388	75.9	861	80.2	0.172	892	72.8	929	78.8	**<0.001**
Lobular	409	15.9	44	15.0	72	14.1	122	11.4		227	18.5	146	12.4	
Mixed	123	4.8	7	2.4	29	5.7	46	4.3		52	4.2	60	5.1	
Other	106	4.1	13	4.4	22	4.3	45	4.2		55	4.5	44	3.7	
Missing	22		5		2		6			15		6		
ER status														
Positive	2543	98.0	295	99.0	503	98.1	1054	97.6	0.315	1223	98.5	1153	97.4	**0.041**
Negative	52	2.0	3	1.0	10	1.9	26	2.4		18	1.5	31	2.6	
Missing	1		0		0		0			0		1		
PGR status														
Positive	1884	76.9	204	72.6	379	78.3	785	76.9	0.188	907	77.2	859	77.0	0.900
Negative	567	23.1	77	27.4	105	21.7	236	23.1		268	22.8	257	23.0	
Missing	145		17		29		59			66		69		
HER-2 status														
Overexpression	5	6.3	0	0	2	9.5	3	7.3	0.701	1	2.9	4	9.1	0.272
No overexpression	75	93.7	7	100	19	90.5	38	92.7		33	97.1	40	90.9	
Missing	2516		291		492		1039			1207		1141		
Hormone Therapy														
TAM-EXE	1298	50.0	145	48.7	244	47.6	552	51.1	0.382	621	50.0	593	50.0	0.968
EXE	1298	50.0	153	51.3	269	52.4	528	48.9		620	50.0	592	50.0	
Missing	0		0		0		0			0		0		

Supplementary Table 1A: (continued)

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**		**N**	**%**	**N**	**%**	
	2596	**100**	**298**	**100**	**513**	**100**	**1080**	**100**		**1241**	**100**	**1185**	**100**	
Surgery type														
Mastectomy	1426	55.0	175	58.7	293	57.1	557	51.6	**0.027**	722	58.2	604	51.0	**<0.001**
Local Excision	1169	45.0	123	41.3	220	42.9	523	48.4		519	41.8	580	49.0	
Missing	1	0	0		0		0			0		1		
Axillary dissection														
Yes	1996	76.9	236	79.2	395	77.0	814	75.6	0.363	965	77.8	886	74.8	0.083
No	600	23.1	62	20.8	118	23.0	266	24.6		276	22.2	299	25.2	
Missing	0		0		0		0			0		0		
Chemotherapy														
Yes	771	29.7	97	32.6	154	30.0	300	27.8	0.246	364	29.4	357	30.1	0.678
No	1824	70.3	201	67.4	359	70.0	779	72.2		876	70.6	828	69.9	
Missing	1		0		0		1			1		0		
Radiotherapy														
Yes	1590	61.3	170	57.0	290	56.6	681	63.1	**0.021**	719	58.1	765	64.6	**0.001**
No	1002	38.7	128	43.0	222	43.4	398	36.9		519	41.9	420	35.4	
Missing	4		0		1		1			3		0		

Abbreviations: pT: pathological tumor pN: pathological nodal ER: estrogen receptor PGR: progesterone receptor HER2: human epidermal growth factor receptor 2 TAM: tamoxifen EXE: exemestane

Supplementary Table 1B: Clinicopathologic characteristics of the Dutch TEAM cohort for the expression of HLA-E and HLA-G.

Total	Total population		HLA-E low		HLA-E high		*p*	HLA-G absence		HLA-G expression		*p*
	N	%	N	%	N	%		N	%	N	%	
	2596	100	495	100	1419	100		1558	100	484	100	
Age in years												
< 40	1	0.0	0	0.0	1	0.1	0.087	1	0.1	0	0.0	0.643
40-50	57	2.2	16	3.2	24	1.7		35	2.2	9	1.9	
50-60	824	31.7	142	28.7	462	32.6		480	30.8	162	33.5	
≥60	1714	66.0	337	68.1	932	65.7		1042	66.9	313	64.7	
Missing	0		0		0			0		0		
pT stage												
T1	1173	45.3	212	43.0	646	45.6	0.176	708	45.5	205	42.4	0.487
T2	1247	48.1	243	49.3	693	48.9		750	48.2	247	51.1	
T3/4	171	6.6	38	7.7	78	5.5		97	6.2	31	6.4	
Missing	5		2		2			3		1		
pN stage												
N0	785	30.2	131	26.5	484	34.1	**0.010**	475	30.5	171	35.4	0.160
N1	1641	63.2	324	65.5	849	59.8		986	63.3	281	58.2	
N2/3	168	6.6	40	8.1	85	6.0		96	6.2	31	6.4	
Missing	2		0		0			1		1		
Grade												
1	398	16.4	95	20.5	160	11.9	**<0.001**	236	16.1	51	11.2	**<0.001**
2	1148	47.3	236	50.9	598	44.5		704	48.0	189	41.4	
3	883	36.4	133	28.7	585	43.6		526	35.9	217	47.5	
Missing	167		31		76			92		27		
Histology												
Ductal	1936	75.2	345	70.3	1156	82.0	**<0.001**	1194	77.3	394	81.7	**0.002**
Lobular	409	15.9	99	20.2	144	10.2		217	14.0	41	8.5	
mixed	123	4.8	25	5.1	57	4.0		76	4.9	18	3.7	
Other	106	4.1	22	4.5	53	3.8		58	3.8	29	6.0	
Missing	22		4		9			13		2		
ER status												
Positive	2543	98.0	488	98.6	1385	97.7	0.222	1527	98.0	472	97.7	0.697
Negative	52	2.0	7	1.4	33	2.3		31	2.0	11	2.3	
Missing	1		0		1			0		1		
PGR status												
Positive	1884	76.9	376	80.0	1011	75.8	0.066	1135	77.5	348	75.7	0.404
Negative	567	23.1	94	20.0	322	24.2		329	22.5	112	24.3	
Missing	145		25		86			94		24		

Supplementary Table 1B: (continued)

Total	Total population		HLA-E low		HLA-E high		*p*	HLA-G absence		HLA-G expression		*p*
	N	%	N	%	N	%		N	%	N	%	
	2596	100	495	100	1419	100		1558	100	484	100	
HER-2 status												
Overexpression	5	6.3	0	0.0	5	9.1	0.226	5	8.8	0	0.0	0.220
No overexpression	75	93.7	15	100	50	90.9		52	91.2	16	100.0	
Missing	2516		480		1364			1501		468		
Hormone Therapy												
TAM-EXE	1298	50.0	241	48.7	719	50.7	0.447	762	48.9	238	49.2	0.919
EXE	1298	50.0	254	51.3	700	49.3		796	51.1	264	50.8	
Missing	0		0		0			0		0		
Surgery type												
Mastectomy	1426	55.0	272	54.9	753	53.1	0.478	845	54.2	257	53.2	0.692
Local Excision	1169	45.0	223	45.1	665	46.9		713	45.8	226	46.8	
Missing	1	0	0		1			0		1		
Axillary dissection												
yes	1996	76.9	380	76.8	1060	74.7	0.359	1190	76.3	369	76.2	0.949
no	600	23.1	115	23.2	359	25.3		368	23.6	115	23.8	
Missing	0		0		0			0		0		
Chemotherapy												
yes	771	29.7	129	26.1	441	31.1	**0.035**	454	29.1	148	30.6	0.527
no	1824	70.3	366	73.9	977	68.9		1104	70.9	335	69.4	
Missing	1		0		1			0		1		
Radiotherapy												
yes	1590	61.3	302	61.0	871	61.5	0.857	950	61.0	292	60.5	0.838
no	1002	38.7	193	39.0	546	38.5		608	39.0	191	39.5	
Missing	4		0		2			0		1		

Abbreviations: pT: pathological tumor pN: pathological nodal ER: estrogen receptor PGR: progesterone receptor HER2: human epidermal growth factor receptor 2 TAM: tamoxifen EXE: exemestane

Supplementary Table 2A: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-I expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-I**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Loss	153	1.00		0.795	-	-	-
		Downregulation	269	1.180	0.715-1.947				
		Expression	528	1.153	0.731-1.817				
RFP	TAM→EXE	Loss	145	1.00		0.265	-	-	-
		Downregulation	244	1.512	0.919-2.485				
		Expression	552	1.353	0.857-2.137				
BCSS	EXE	Loss	153	1.00		0.988	-	-	-
		Downregulation	269	0.962	0.527-1.758				
		Expression	528	0.993	0.851-1.696				
BCSS	TAM→EXE	Loss	145	1.00		0.323	-	-	-
		Downregulation	244	1.528	0.838-2.784				
		Expression	552	1.215	0.696-2.120				
OS	EXE	Loss	153	1.00		0.600	-	-	-
		Downregulation	269	1.090	0.705-1.685				
		Expression	528	0.925	0.620-1.379				
OS	TAM→EXE	Loss	145	1.00		0.094	1.00		0.183
		Downregulation	244	1.474	0.940-2.312		1.390	0.880-2.195	
		Expression	552	1.084	0.712-1.649		1.055	0.689-1.615	

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2B: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-E expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-E**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Low	254	1.00		0.793	-	-	-
		High	700	1.049	0.734-1.500				
RFP	TAM→EXE	Low	241	1.00		0.103	-	-	-
		High	719	1.345	0.942-1.919				
BCSS	EXE	Low	254	1.00		0.505	-	-	-
		High	700	1.171	0.736-1.862				
BCSS	TAM→EXE	Low	241	1.00		0.101	-	-	-
		High	719	1.459	0.929-2.291				
OS	EXE	Low	254	1.00		0.621	-	-	-
		High	700	0.921	0.666-1.274				
OS	TAM→EXE	Low	241	1.00		0.869	-	-	-
		High	719	1.027	0.750-1.406				

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2C: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-G expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-G**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Absence	796	1.00		0.821	-	-	-
		Presence	246	0.960	0.676-1.364				
RFP	TAM→EXE	Absence	762	1.00		0.441	-	-	-
		Presence	238	0.876	0.625-1.227				
BCSS	EXE	Absence	796	1.00		0.958	-	-	-
		Presence	246	0.988	0.641-1.526				
BCSS	TAM→EXE	Absence	762	1.00		0.108	-	-	-
		Presence	238	0.698	0.451-1.082				
OS	EXE	Absence	796	1.00		0.165	-	-	-
		Presence	246	0.786	0.560-1.104				
OS	TAM→EXE	Absence	762	1.00		0.255	-	-	-
		Presence	238	0.830	0.601-1.144				

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2D: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for FoxP3+ expression.

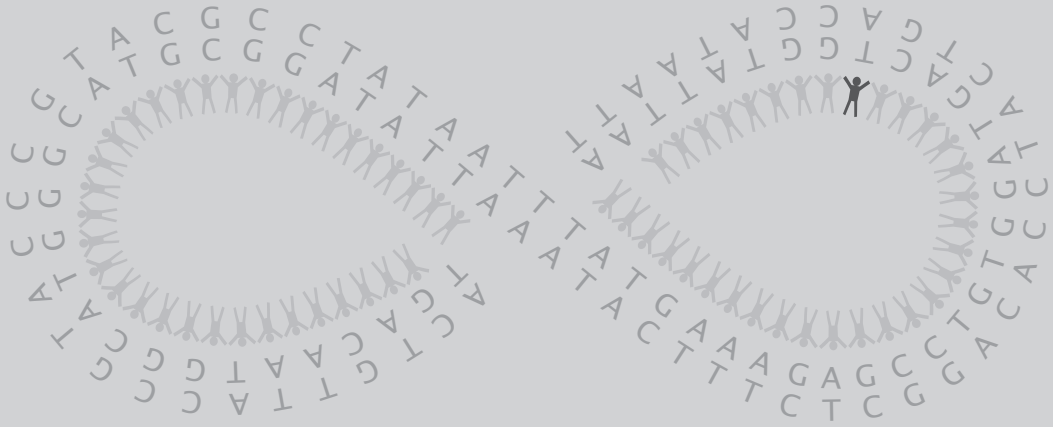
Outcome	Hormone therapy	FoxP3+	N	Univariate HR	95%CI	*p*	Multivariate* HR	95%CI	*p*	Interaction *p*
RFP	EXE	Low	621	1.00		0.230	-	-	-	
		High	592	0.843	0.637-1.114					
RFP	TAM→EXE	Low	620	1.00		0.874	-	-	-	-
		High	593	0.979	0.755-1.270					
BCSS	EXE	Low	621	1.00		0.745	-	-	-	
		High	592	0.944	0.664-1.340					
BCSS	TAM→EXE	Low	620	1.00		0.197	-	-	-	-
		High	593	0.808	0.585-1.117					
OS	EXE	Low	621	1.00		0.138	-	-	-	
		High	592	0.821	0.633-1.065					
OS	TAM→EXE	Low	620	1.00		0.026	1.00		0.019	**<0.001**
		High	593	0.752	0.586-0.966		0.729	0.560-0.949		

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 3: Composition of tumor immune subtypes based on the regression coefficient of the mono-markers in the relapse free period.

Tumor immune subtype category	HLA-I	HLA-G	HLA-E	FoxP3+	N	Regression coefficient range
High	Loss	Negative/Positive	Negative	Low/High	152	(-0.485) – (-0.108) (1st tertile)
	Loss	Positive	Positive	Low/High	6	
	Down-regulation	Positive	Negative	Low/High	14	
	Expression	Negative/Positive	Negative	Low/High	116	
	Expression	Positive	Positive	High	213	
Intermediate	Loss	Negative	Positive	Low/High	100	(-0.086 – (0.011) (2nd tertile)
	Down-regulation	Negative	Negative	Low/High	127	
	Expression	Positive	Positive	Low	91	
Low	Down-regulation	Negative/Positive	Positive	Low/High	288	(0.065) – (0.287) (3rd tertile)
	Expression	Negative	Positive	Low/High	529	



Chapter 6

The clinical prognostic value of molecular intrinsic tumor subtypes in older breast cancer patients: A FOCUS study analysis

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ABSTRACT

Introduction

It was recently proposed that the molecular breast tumor subtypes are differently distributed in the elderly breast cancer patients, and also lack prognostic value. Given the limited number of elderly patients in previous studies, the aim of this study was to determine the prognostic effect of the molecular intrinsic subtypes in a large older breast cancer population.

Material and method

Older breast cancer patients with invasive, non-metastatic breast cancer with tumor material available for immunohistochemical determination of Ki67, EGFR, CK5/6 and HER-2 were included. ER and PR expression was retrieved from the pathology report. Molecular subtypes were: Luminal A, Luminal B, ERBB2, Basal-like and Unclassified. Primary endpoint was Relapse Free Period (RFP), taking into account the competing risk of mortality, and adjusted for the most important patient, tumor and treatment characteristics. Secondary endpoint was Relative Survival (RS).

Results

Overall, 1,362 patients were included. Patients with a Luminal A subtype had the lowest risk of recurrence (11% at 5 yrs). Patients with a Basal (24% at 5yrs) or ERBB2 (34% at 5yrs) molecular breast tumor subtype had the highest risk of recurrence. The ERBB2 subtype had the worst prognosis in terms of RFP (SHR 2.07, 95% CI 1.35-3.20; p=0.001). The worst RS was again observed for the ERBB2 subtype (48% at 10 yrs). In multivariable analyses, the relative excess risk of death for all molecular subtypes was significantly worse compared to the Luminal A subtype.

Conclusion

Molecular intrinsic breast tumor subtypes have significant prognostic value in the elderly population, even after taking competing mortality into account.

INTRODUCTION

Due to a lack of presentation in clinical trials and translational studies, our knowledge on the effects and associations of prognostic and predictive biomarkers in the elderly breast cancer population is limited. Current breast cancer treatment guidelines are based on studies performed in relatively young or fit elderly populations [1-3]. This observation is alarming for the clinical care of older breast cancer patients, given the fact that breast cancer is increasingly becoming a disease affecting the aged population [4]. It has been shown that older breast cancer patients tend to present more frequently with hormone receptor (HR) positive tumors, less human epidermal growth factor (HER-2) receptor overexpression and with lower proliferation rates than their younger counterparts [5;6]. Although the tumor characteristics may seem more favorable, recent studies have shown an inferior breast cancer specific prognosis for older breast cancer patients [7;8]. Current treatment guidelines are based on studies performed in younger breast cancer patients, herewith increasing the chance of suboptimal treatment in the elderly breast cancer patients. Lately therefore, the demand for prognostic and predictive research focusing on the elderly breast cancer population has greatly increased.

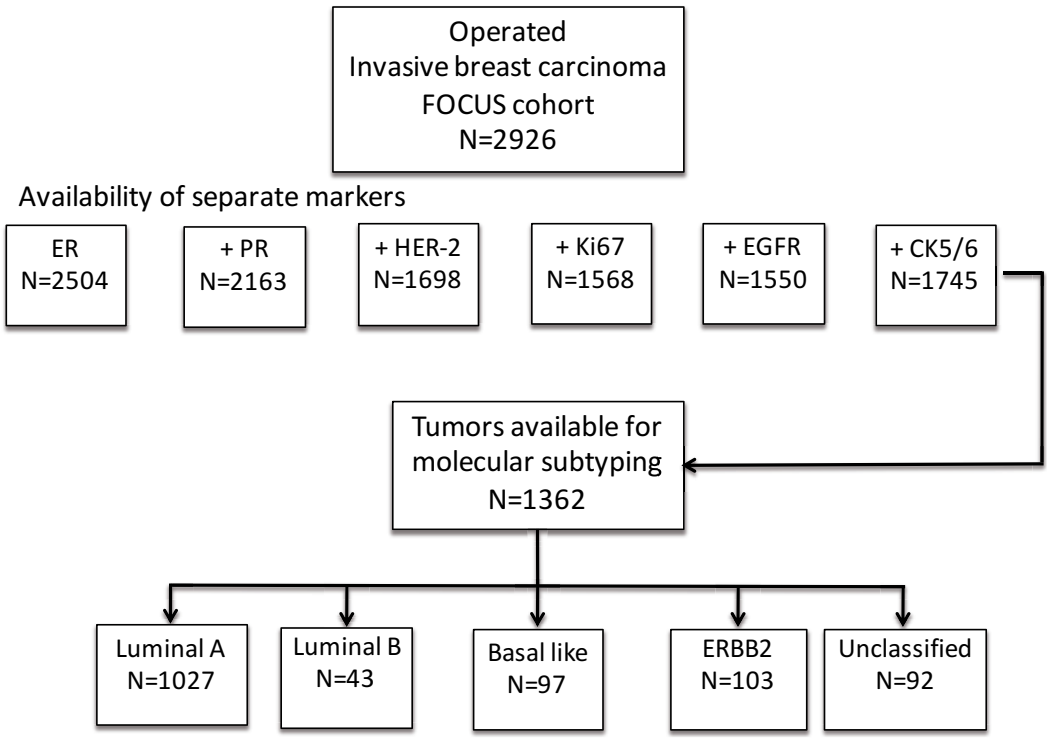
A modern-day genetic array showing promising prognostic results is the intrinsic breast tumor classification, also known as the molecular breast cancer subtypes [9-11]. The intrinsic classification proposes four different classes of breast tumors: Luminal A and B, which are mostly hormone receptor positive and express high amounts of genes related to the luminal epithelial cell layer [9-11]. Compared to Luminal A tumors, Luminal B tumors tend to have more cellular proliferation. Furthermore, the intrinsic subtypes also include two tumor subtypes which do not express hormonal receptors, namely: the Basal like tumors, which are triple negative tumors (estrogen receptor (ER) negative, progesterone receptor (PR) negative and Human Epidermal Growth Factor Receptor-2 (HER-2) negative) combined with expression of genes characteristic of the basal epithelial layer such as cytokeratin (CK) 5 and 6; and the ERBB2 tumor subtype, which clusters near the basal-like subtypes, but expresses high HER-2 on the tumor surface. Previous studies showed that Luminal A tumors have the most indolent character, closely followed by Luminal B. ERBB2 and Basal-like tumors are both characterized by more aggressive phenotypes, resulting in unfavorable patient outcome [10].

Recently, de Kruijf *et al.* showed that the molecular intrinsic breast cancer subtypes have a different distribution in elderly breast cancer compared to their younger counterparts [12]. However, in this study no prognostic effect of the intrinsic breast tumor subtypes was shown within the older breast cancer patients. However, this study only included 189 breast cancer patients above the age of 65. Therefore, the aim of our current study was to determine the prognostic effect of the intrinsic molecular breast cancer subtypes in a large population of elderly breast cancer patients.

MATERIAL AND METHODS

Patients and tumors

For this study, all patients with invasive, non-metastatic breast cancer from the FOCUS cohort (Female breast cancer in the elderly, Optimizing Clinical guidelines USing clinic-pathological and molecular data) who received surgery and had formalin fixed paraffin embedded (FFPE) intra-operative tumor samples available with successful staining of HER-2, Ki67, EGFR and CK5/6 were included (CONSORT diagram). ER and PR status was retrieved from the pathology report of each patient. The FOCUS cohort has been described extensively in previous publications [13]. In brief, the cohort consists of all women aged ≥ 65 years at time of diagnosis, with invasive and *in situ* breast cancer, diagnosed between 1997 and 2004 in the South Western region of The Netherlands. Follow-up on survival status was available until the 1st of January 2013. All tumor samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).



CONSORT diagram

Immunohistochemistry

Tissue sections of 4µm were cut from intra-operatively derived FFPE tumor material of the FOCUS cohort processed into a tissue microarray (TMA). Mouse anti-Ki67 (cline

MIB-1, Dako, NL), anti-epidermal growth factor receptor (EGFR) (NLC-EGFR, Novocastra, UK), anti-CK5/6 (clone D5/16 B4, Dako, NL) and rabbit anti-c-erbB-2 Oncoprotein (A0485, Dako, Denmark) were used for immunohistochemical staining. Immunohistochemical staining was performed according to previously described standard protocol [12]. Briefly, tissue sections were deparafinized and antigen retrieval was performed using a Pre Treatment (PT) module (PT link, DAKO, Denmark). Endogenous peroxidase activity was blocked with hydrogen peroxidase 0.3% in PBS for 30 minutes. Sections were incubated with the primary antibody at room temperature overnight or for 20 minutes (only for c-erbB2 antibody). Subsequently, all TMA slides were incubated with Envision anti-mouse (DAKO, Denmark, Cytomation K4002) or anti-rabbit (DAKO, Denmark, Cytomation K4003) for 30 minutes at room temperature. DAB was used for visualization of positively stained breast tumor tissue on the TMA and counterstained with haematoxylin, dehydrated and finally mounted with pertex. Per staining, all slides were stained simultaneously to avoid inter-assay variation. Negative controls were slides that underwent the entire staining protocol without primary antibody.

Evaluation of immunostaining and molecular subtype determination

Microscopic quantification of positive tumor cells for Ki67, EGFR, CK5/6 and c-erbB-2 protein was performed by two independent observers. Ki67 staining was considered negative if less than 10% of the tumor cells had visible staining and positive if Ki67 was immunohistochemically present in ≥10% of the tumor cells. Cut-offs for low versus high expression of EGFR and CK5/6 were based on the median expression level, which was 0% for both stainings. HER-2 staining was scored as follows: 0 for no staining at all or incomplete or faint/barely perceptible membrane staining in <10% of the invasive tumor cells; 1+ for a faint/barely perceptible partial membrane staining in >10% of the tumor cells; 2+ for weak to moderate complete membrane staining in >10% of the tumor cells; and 3+ for strong to complete membrane staining in >30% of the tumor cells. For all patients, the highest score out of the three punches of the same tumor was used for statistical analysis. If one or more punches were missing, the highest score of the remaining punch(es) was included for analyses. Immunohistochemical HER-2 scores 0, 1+ and 2+ were considered HER-2 negative and a HER-2 3+ score was considered HER-2 positive.

Immunohistochemical profiles have been previously developed and validated by combinations of the following immunohistochemically determined markers: ER, PR, HER-2, Ki67, EGFR, and CK5/6. Based on these papers, we defined the immunohistochemical molecular intrinsic breast tumor subtypes as follows: *Luminal A*: ER+ and/or PR+, HER-2- and Ki67-; *Luminal B*: ER+ and/or PR+, HER-2- and Ki67+; *ERBB2*: HER-2+; *Basal-like*: ER-, PR-, HER-2- and EGFR+ and/or CK5/6+; *Unclassified*: ER-, PR-, HER-2-, EGFR-, and CK5/6- [9;10;12].

Statistical analyses

Statistical analyses were performed using the statistical packages SPSS (version 20.0 for Windows, IBM SPSS statistics) and Stata SE 12.0.

Cohen's kappa coefficient was used to assess the inter-observer agreement in quantification of HER-2, Ki67, EGFR and CK5/6 tumor expression. The χ^2 test was used to evaluate associations between various clinicopathological parameters and molecular intrinsic tumor subtypes.

The primary endpoint examined was Relapse-Free Period (RFP), defined as the time from date of diagnosis until any recurrence (any registered relapse of breast cancer, either locoregional recurrence, distant recurrence or contralateral breast cancer, whichever came first). The Cumulative Incidence Competing Risks method was used for plotting of the cumulative incidence of recurrence, taking into account the competing risk of death [14]. Fine & Gray competing risks regression analyses were used for univariable and multivariable analysis for RFP, taking into account the competing risk of death of any cause [15]. Multivariable analyses were adjusted for patient (age), tumor (TNM stage, grade) and treatment factors (type of breast surgery, axillary surgery, radiotherapy, endocrine therapy and chemotherapy).

The secondary endpoint was relative survival, calculated as the ratio between the observed survival in the cohort and the expected survival as calculated from the age-, sex- and year-matched background population [16]**.** Assuming that other factors influencing mortality risk are the same in the cohort and background population, this means that the excess risk of death, as measured in the cohort, can be attributed to breast cancer. Therefore, the excess mortality can be interpreted as cancer-specific mortality.

Patients with missing data on the determinant of interest due to material handling (which is considered to happen randomly, so we assume these data to be missing at random) were excluded from the statistical analyses. To test for the robustness of results, due to the relatively large proportion of missing values, as a sensitivity analysis we used multiple imputation to impute the six markers ER, PR, HER-2, EGFR, CK5/6 and Ki67. Therefore we used 25 replications of the original dataset, with the following variables as predicting variables: age, morphology, grade T-stage, N-stage, screen-detected, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, and chemotherapy. We also accounted for the outcomes: time to first recurrence, recurrence status, time to death or last follow-up and vital status. We analyzed the primary endpoint (RFP with competing risks regression) using the imputed dataset. We were not able to analyze the secondary endpoint (relative survival) with the imputed dataset, because the statistical package does not support this analysis with imputed data.

RESULTS

Patient and tumor characteristics

Overall, 1,362 patients were included in all analyses.

Median age of patients in the cohort was 75 years (range 65-98 years). The majority of the patients had an early stage tumor of ductal morphology. Molecular tumor subtypes were associated with different tumor stage, showing more early stage tumors in the luminal tumor subtypes, and more stage III tumors in the ERBB2, basal and unclassified subtypes (p=0.014 (Table 1)). In addition, molecular subtypes were significantly associated with tumor grade and morphology. Regarding therapies, patients with an ERBB-2, basal and unclassified subtype received more chemotherapy, whereas the patients with a luminal A or B subtype received more adjuvant endocrine therapy (both therapies p<0.001) (Table 1). The Cohen's kappa coefficient for inter-observer agreement for HER-2, Ki67, EGFR and CK5/6 were all >0.6.

Relapse Free Period for molecular intrinsic breast tumor subtypes

Median follow-up time for the endpoint of relapse free period (RFP) was 5.2 years (range 0-13.4). Patients with a luminal A subtype had the lowest risk of recurrence (11% at 5 years), followed by luminal B and unclassified (both 18%). Patients with a Basal or ERBB2 molecular breast tumor subtype had the highest risk of recurrence (24% and 34% at 5 years, respectively). Cumulative incidences of recurrence are depicted in Figure 1. Table 2 shows the results of the crude and adjusted Fine & Gray competing risks regression analyses, where patients with Luminal A were taken as reference group. Patients with an ERBB2 subtype had, after taking into account the competing risk of mortality and after adjustment for the most important clinical factors, the worst prognosis in terms of RFP (adjusted sub-distribution hazard ratio (SHR) 2.07, 95% confidence interval (CI) 1.35-3.20; p=0.001). Patients with a Basal subtype also had a significantly higher risk of recurrence (adjusted SHR 1.80, 95% CI 1.14-2.85; p=0.012). Patients with a Luminal B or unclassified subtype had no statistically significant different RFP than patients with a Luminal A subtype.

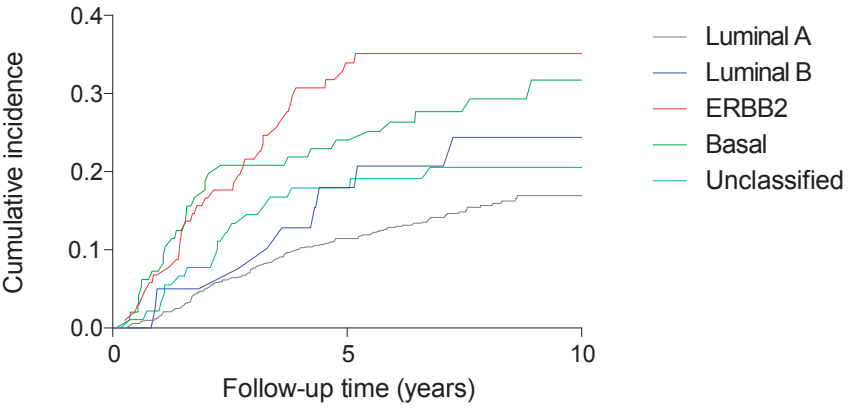
Results of the sensitivity analysis, using multiple imputation of missing values is shown in web-table 1, confirming the higher risk of recurrence for patients with an ERBB-2 and Basal breast cancer subtype.

When separate analyses were performed for loco-regional and distant relapse, no significant association was seen for loco-regional relapse and molecular breast tumor subtypes (web-table 2). Contrarily, the same significant association was seen for the molecular breast tumor subtypes and distant metastases as with RFP (web-table 3). These results could be explained by the greater number of events for distant metastases compared to loco-regional relapse in this patient set. Implying that the significant association seen in our study for RFP and the molecular breast tumor subtypes is largely dependent on the distant metastases.

Table 1: Patient, tumor and treatment characteristics

	Luminal A		Luminal B		ERBB2		Basal		Unclassified		*P**
	N=1027		N=43		N=103		N=97		N=92		
	N	%	N	%	N	%	N	%	N	%	
Age in years (mean, SD)	76.3 (7.2)		75.2 (7.2)		74.9 (6.9)		75.9 (6.8)		76.4 (7.8)		0.392
Number of comorbidities											0.712
0-1	492	47.9	20	46.5	51	49.5	49	50.5	41	44.6	
2-4	443	43.1	20	46.5	39	37.9	39	40.2	46	50.0	
5 or more	92	9.0	3	7.0	13	12.6	9	9.3	5	4.5	
TNM stage											**0.014**
I	355	34.6	14	32.6	21	20.4	25	25.8	27	29.3	
II	528	51.4	28	65.1	66	64.1	57	58.8	45	48.9	
III	124	12.1	0	0.0	14	13.6	14	14.4	15	16.3	
Missing	20	1.9	1	2.3	2	1.9	1	1.0	5	5.4	
Grade											**<0.001**
1	164	16.0	2	4.7	4	3.9	2	2.1	5	5.4	
2	374	36.4	9	20.9	16	15.5	11	11.3	20	21.7	
3	218	21.2	17	39.5	62	60.2	56	57.7	45	48.9	
Missing	271	26.4	15	34.9	21	20.4	28	28.9	22	23.9	
Morphology											**0.002**
Ductal	780	75.9	33	76.7	91	88.3	71	73.2	75	81.5	
Lobular	118	11.5	8	18.6	4	3.9	5	5.2	8	8.7	
Other/missing	129	12.6	2	4.7	8	7.8	21	21.6	9	9.8	
Breast surgery*											0.052
BCS	389	37.6	18	41.9	24	23.3	32	33.0	35	38.0	
Mastectomy	641	62.4	25	58.1	79	76.7	65	67.0	57	62.0	
Axillary surgery*											0.4
No axillary surgery	121	11.8	3	7.0	16	15.5	11	11.3	15	16.3	
Sentinel node	258	25.1	12	27.9	16	15.5	21	21.6	22	23.9	
ALND	648	63.1	28	65.1	71	68.9	65	67.0	55	59.8	
Adjuvant radiotherapy											0.944
No	522	50.9	21	48.8	54	52.4	49	50.5	43	46.7	
Yes	505	49.2	22	51.2	49	47.6	48	49.5	49	53.3	
Adjuvant endocrine therapy											**<0.001**
No	470	45.8	13	30.2	66	64.1	82	84.5	74	80.4	
Yes	557	54.2	30	69.8	37	35.9	15	15.5	18	19.6	
Adjuvant chemotherapy											**<0.001**
No	981	95.5	42	97.7	89	86.4	88	90.7	75	81.5	
Yes	46	4.5	1	2.3	14	13.6	9	9.3	17	18.5	

*calculated by one-way ANOVA test for continuous variables and a Chi-square test for categorical variables. P-values in bold indicate a statistical significant difference between the molecular subtypes at the p-level of 0.05.



Numbers at risk:

Luminal A	1025	579	48
Luminal B	43	22	5
ERBB2	103	33	4
Basal	97	49	11
Unclassified	92	46	6

Figure 1: Cumulative incidence of recurrence by molecular subtype.

Table 2: Relapse free period (Fine & Gray regression)

	N	N of events	Cumulative incidence of recurrence at 5 years (%)	95% CI			
				SHR	lower	upper	*P*
Luminal A	1027	112	11%	1 (reference)			
Luminal B	43	7	18%	1.56	0.81	3.02	0.184
ERBB2	103	34	34%	2.78	1.91	4.04	**<0.001**
Basal	97	23	24%	2.19	1.44	3.31	**<0.001**
Unclassified	92	16	18%	1.49	0.92	2.41	0.106

SHR*	95% CI		*P*
	lower	upper	
1 (reference)			
1.31	0.69	2.48	0.407
2.07	1.35	3.20	**0.001**
1.80	1.14	2.85	**0.012**
1.27	0.75	2.15	0.372

*Adjusted for age, morphology, grade, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

Relative survival for molecular intrinsic breast tumor subtypes

Median follow-up time was 8.6 years (range 0-17.0 years). Relative survival, calculated as the observed survival in the cohort, divided by the expected survival in the age-, year and sex matched general population, was highest for the patients with a Luminal A subtype (88%). All other subtypes had a worse relative survival at 10 years. The worst clinical outcome was again observed for patients with an ERBB2 subtype, showing a relative survival at 10 years of 48%. In multivariable analyses, the relative excess risk (RER) of death for all molecular breast cancer subtypes was significantly worse than patients with a Luminal A subtype (Table 3).

Table 3: Relative survival

	N of observed deaths / N of Expected deaths	Relative survival at 10 years (%)	95% CI				95% CI			
			RER	lower	upper	*P*	RER*	lower	upper	*P*
Luminal A	533/430	88%	1 (reference)				1 (reference)			
Luminal B	26/16	67%	2.59	0.97	6.93	0.058	2.88	1.26	6.57	**0.012**
ERBB2	69/23	48%	5.79	3.53	9.51	**<0.001**	4.28	2.51	7.30	**<0.001**
Basal	59/32	68%	3.34	1.74	6.41	**<0.001**	3.11	1.74	5.55	**<0.001**
Unclassified	59/33	63%	2.84	1.46	5.53	**0.002**	2.22	1.13	4.35	**0.020**

*Adjusted for age, morphology, grade, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

DISCUSSION

In this study it was shown that molecular intrinsic breast tumor subtypes are of significant prognostic value in the older (≥65years) breast cancer population. Our results indicate that the ERBB2 and Basal molecular breast tumor subtypes are associated with worse Relapse Free Period. Moreover, all molecular subtypes hold a poor prognosis in terms of Relative Survival, compared to the Luminal A breast tumor subtype in the elderly breast cancer patients.

These results are in contrast with the results of a recent study performed by de Kruijf *et al.*, in which it was proposed that intrinsic breast tumor subtyping is of limited prognostic value in the older breast cancer population. However, the study from the Kruijf *et al.* only included 189 patients aged 65 years or older, resulting in small molecular subtype subgroups with minimal discriminative capacity. The current study contained 1,362 breast cancer patients aged 65 years or older, resulting in much more statistical power and thus more reliable, clinically translatable outcome.

In addition to the results of our current study, evidence is accumulating about the prognostic role of tumor biology, even in the presence of high competing risk of mortality. For instance, Mook *et al.* showed that usage of the 70-gene prognosis signature is

able to accurately select postmenopausal breast cancer patients, between 55 and 70 years of age, who are at low risk of breast cancer-related death within 5 years of diagnosis [17]. These results may help to more adequately select patients for adjuvant systemic treatment.

In our study, the distribution of the molecular intrinsic breast tumor subtypes in this older breast cancer population showed a higher prevalence of the assumed more indolent Luminal A tumor and a relatively low prevalence of the more aggressive Basal molecular tumor subtype compared to the studies performed in a younger breast cancer population [18]. Noteworthy is the same prevalence, of around 7%, of the ERBB2 intrinsic molecular breast tumor subtype in both the younger and the older breast cancer population. These results imply that the chance of getting a more aggressive molecular tumor subtype decreases with increasing age, which is in accordance with the observation of milder tumor characteristics in the older breast cancer population. However, our results confirm the prognostic value of the more aggressive tumor subtypes (Basal and ERBB2) in the older breast cancer population, which is reflected in a worse relapse free period as well as a worse relative survival. These results imply that older breast cancer patients with aggressive tumor types could potentially benefit from a more aggressive (systemic) treatment, irrespective of their advanced age.

The major strength of our study is that we used the largest consecutive series of older breast cancer patients from a population-based cohort, from which tumor material was available. Therefore, our study is not affected by selection bias. A limitation of the study is that there was no tumor material available for all patients from the original cohort. This was mostly due to logistical reasons and tissue loss during experimental procedures. After statistical imputation of the missing data, our results did not change. Second, by using TMA for immunohistochemical stainings one does not have an overview of the exact number of positively stained cells on the histological slide. Therefore, some degree of underscoring cannot be ruled out. Third, no confirmatory microarray genetic analysis was performed. However, immunohistochemical surrogates, like those used in this study, have been validated with good agreement in previous studies [12].

In conclusion, the molecular intrinsic classification and its impact on clinical outcome have been extensively investigated in breast cancer. So far, molecular breast cancer studies identified breast cancer as a heterogeneous disease, emphasizing the need for different systemic treatment approaches. However, as is the case with most translational studies and clinical trials, these studies mostly include relatively young or fit elderly patients. Our present study is the first study performed in a large unselected population of older breast cancer patients, showing significant prognostic value of the molecular breast tumor subtypes, even after taking the risk of competing mortality into account.

Therefore, the result of this study supports the use of molecular subtyping in the older breast cancer patients, even when dealing with the older, more fragile breast cancer population for prognostication and consequently, therapy allocation.

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SUPPLEMENTARY TABLES

Webtable 1: Relapse free period (Fine & Gray regression) - sensitivity analysis using multiple imputation for missing values

		95% CI				95% CI		
	SHR	**lower**	**upper**	***P***	**SHR***	**lower**	**upper**	***P***
Luminal A	1 (reference)				1 (reference)			
Luminal B	1.35	0.80	2.30	0.259	1.39	0.81	2.39	0.233
ERBB2	2.65	1.81	3.90	**<0.001**	2.33	1.55	3.52	**<0.001**
Basal	1.72	1.21	2.44	**0.003**	1.66	1.15	2.39	**0.006**
Unclassified	1.31	0.91	1.88	0.14	1.34	0.90	1.99	0.142

*Adjusted for age, morphology, grade, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

Web-table 2: Locoregional relapse free period (Fine & Gray regression)

	N of events	**Cumulative incidence of recurrence at 5 years (%)**	**SHR**	**95% CI**		***P***
				lower	**upper**	
Luminal A	23	2%	1 (reference)			
Luminal B	2	5%	1.50	0.35	6.31	0.584
ERBB2	5	5%	1.85	0.78	4.41	0.164
Basal	4	4%	1.92	0.80	4.60	0.143
Unclassified	4	4%	1.36	0.48	3.88	0.562

*Adjusted for age, morphology, grade, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

	95% CI		
SHR*	**lower**	**upper**	***P***
1 (reference)			
1.36	0.32	5.76	0.676
1.42	0.57	3.50	0.451
1.33	0.52	3.37	0.548
1.09	0.38	3.10	0.877

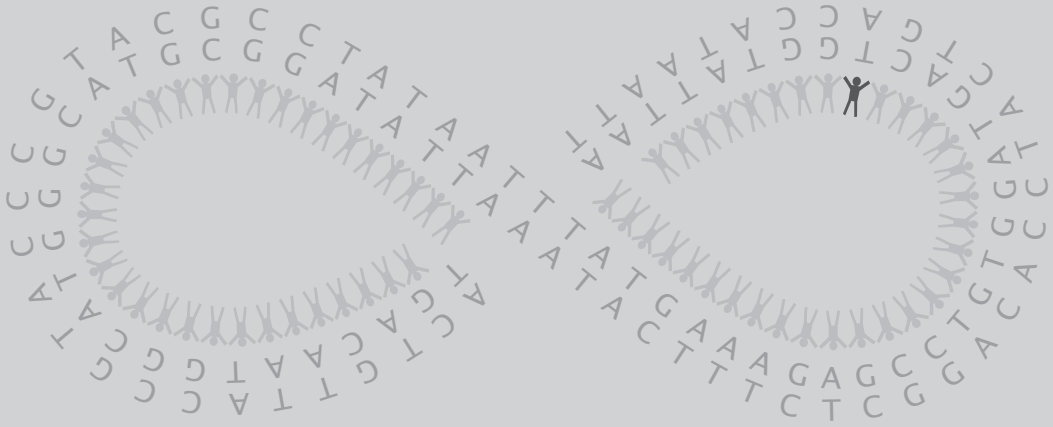
Web-table 3: Distant metastasis free period (Fine & Gray regression)

	N of events	**Cumulative incidence of recurrence at 5 years (%)**		**95% CI**		***P***
				lower	**upper**	
Luminal A	88	9%	1 (reference)			
Luminal B	5	12%	1.53	0.73	3.21	0.263
ERBB2	29	29%	2.95	1.95	4.47	**<0.001**
Basal	19	20%	2.26	1.41	3.60	**0.001**

Unclassified	10	11%	1.32	0.74	2.34	0.346

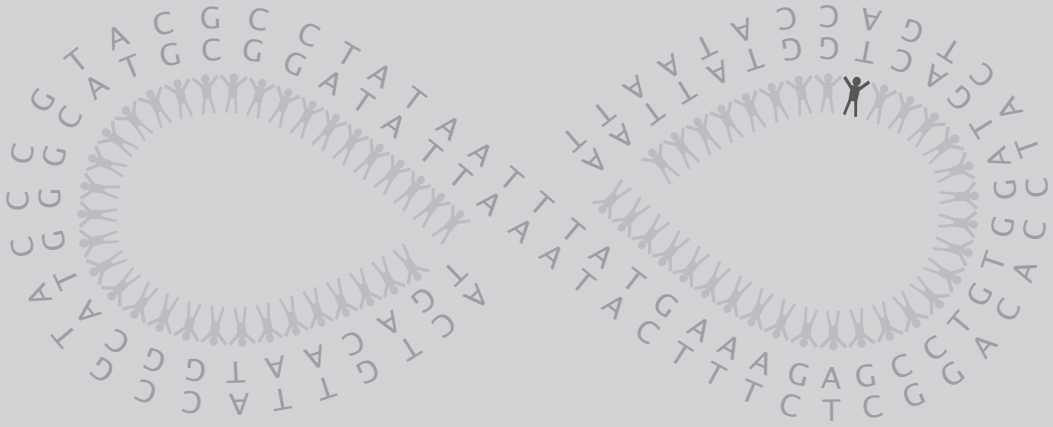
*Adjusted for age, morphology, grade, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

	95% CI		
SHR*	**lower**	**upper**	***P***
1 (reference)			
1.28	0.62	2.64	0.504
2.18	1.33	3.55	**0.002**
1.96	1.16	3.32	**0.012**
1.14	0.61	2.14	0.681



Part II

Predictive biomarkers in breast cancer and targeted treatment



Chapter 7

The influence of Insulin-like Growth Factor-1-Receptor expression and endocrine treatment on clinical outcome of postmenopausal hormone receptor positive breast cancer patients: A Dutch TEAM substudy analysis

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ABSTRACT

Background

Signaling via the Insulin-like Growth Factor type 1 Receptor (IGF1R) plays a crucial role in cancer development. In breast cancer (BC), IGF1R and estrogen receptor expression are correlated. In this current study we explored the hypothesis that postmenopausal hormone receptor positive (HR+ve) BC patients with high IGF1R tumor expression still have estrogen driven IGF1R stimulated tumor growth when treated with tamoxifen, resulting in detrimental clinical outcome compared to patients treated with exemestane. Additionally, we assessed the added value of metformin as this drug may lower IGF1R stimulation.

Methods

Of 2,446 Dutch TEAM patients, randomized to either exemestane for 5 years or sequential treatment (tamoxifen for 2-3 years followed by exemestane for another 3-2 years) tumor tissue microarray sections were immunohistochemically stained for IGF1R. Overall Survival (OS), Breast Cancer specific Survival (BCSS) and Relapse-Free Survival (RFS) were assessed in patient subgroups with low and high IGF1R expression, and in patients with or without metformin use.

Results

High IGF1R tumor expression was significantly associated with exemestane therapy for RFS (Hazard Ratio (HR) 0.74, 95% Confidence Interval (CI) 0.58-0.95, *p=0.02*). In addition, the combination of metformin with exemestane resulted in improved efficacy, yielding a 5-yrs RFS of 95% (HR 0.32, 95% CI 0.10-1.00, *p=0.02*, compared to sequential treatment). No relation was observed in tumors with low IGF1R expression.

Conclusion

This study suggests IGF1R as a potential biomarker of improved clinical outcome in HR+ve BC patients treated with exemestane. Adding metformin to exemestane treatment may add to this effect.

INTRODUCTION

Breast cancer (BC) is still the most frequent cause of cancer related death in women in developed countries, and marks one of the leading health problems worldwide [1-3]. Over the past decades, a substantial reduction in BC related mortality has been observed, mostly due to mayor advances in (neo-)adjuvant systemic treatment [4-8]. Decisions regarding optimal treatment of breast cancer patients are largely based on prognostic and predictive markers. However, the various currently used classical markers do not provide optimal risk stratification, hampering further personalization of therapy.

Estrogen receptor (ER) expression is present in approximately 65-75% of all postmenopausal breast cancers [9]. Anti-estrogens, such as tamoxifen, are known to inhibit cell proliferation and disease progression by competitive blocking of estrogen binding to the ER, whereas aromatase inhibitors (AIs) act by blocking the estrogen biosynthesis via aromatase inhibition in postmenopausal women, thus lowering the already low postmenopausal estrogen levels.

It is known that signaling via the Insulin-like Growth Factor type 1 Receptor (IGF1R) plays a crucial role in the development of many cancers, including BC, by influencing cellular proliferation, cell survival, invasion and metastatic behavior [10;11]. It has been shown that IGF1R expression is correlated with the expression of the ER [12;13]. IGF1R has been shown to be up-regulated in tamoxifen-resistant BC, which retained the tamoxifen antagonism of classical ER genomic function [14]. Subsequently, a study performed by Song *et al.*, has shown that 17β-Estradiol, although to a lesser extent than IGF1, can activate a linear pathway involving the activation of IGF1R, which subsequently leads to a boost of the mitogen-activated protein kinase (MAPK) [15-17]. Patients treated with an AI could lose this additional tumor growth-stimulating pathway due to complete blockage of estrogen production, independent of IGF1 stimulation.

Another drug that may be of interest in relation to the IGF1R is metformin, which has long been known for lowering plasma insulin and insulin growth factor levels by increasing insulin sensitivity [18]. Several observational studies have suggested that metformin may be beneficial in BC treatment [19;20]. It could be postulated that an additional effect of metformin treatment in BC patients with high IGF1R expression could be observed, by means of lowering direct IGF1R stimulation.

Therefore, in the current analyses we explored the hypothesis that postmenopausal hormone-receptor positive (HR+ve) early BC patients with high IGF1R tumor expression treated with tamoxifen still have estrogen driven IGF1R stimulated tumor growth, resulting in detrimental clinical outcome compared to patients treated with the AI exemestane. In addition, the combined effect of endocrine therapy with metformin use on clinical outcome in both IGF1R positive and IGF1R negative HR+ve postmenopausal BC patients was explored.

MATERIAL AND METHODS

Patients and tumors

For this study, intra-operative breast tumor samples of Dutch patients participating in the Tamoxifen and Exemestane Adjuvant Multicenter trial (TEAM) (n=2,764) were used. All patients signed an informed consent form prior to enrolment in the TEAM study. Local ethics approval was received and the study was conducted in accordance with the Declaration of Helsinki.

The TEAM study is a randomized, open-label, phase III trial, conducted in postmenopausal women with early stage ER and/or progesterone receptor-positive BC, who were eligible for adjuvant endocrine treatment. Patients were randomly assigned to receive either exemestane 25 mg once daily for 5 years or tamoxifen 20 mg once daily for 2.5 to 3 years, followed by exemestane 25 mg once daily for 2.5 to 2 years (sequential regimen) [21]. All patients were diagnosed and treated between 2001 and 2006. For this sub-study, patients with bilateral tumors or a history of another cancer within five years prior to inclusion in the TEAM study were excluded, with an exception for patients with basal cell carcinoma of the skin and cervical intraepithelial neoplasia.

For all patients included in this study the following data was retrieved from the central TEAM database at the datacenter of the Leiden University Medical Center: age at diagnosis, histological tumor grade (classified as Grade I, II or III) and tumor type (ductal, lobular or "other"), ER and progesterone receptor status, pathological tumor and nodal stage, adjuvant treatment received, Body Mass Index (BMI), used co-medication, date and type of loco-regional/distant recurrence, and date and cause of death if relevant.

It should be noted that some differences were seen between the Dutch patients and the other patients in the TEAM trial. Most of these can be explained by differences in the number of patients with missing data. However, patients from the Netherlands presented with more advanced tumor stages than patients from other countries, as they had higher T- and N-stages (web-table 1). This probably also explains the difference in survival between the countries (web-table 2).

Immunohistochemistry

All tumor samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). Immunohistochemical staining for IGF1R was performed on 4µm tissue sections from FFPE tumor samples of the Dutch TEAM BC patients processed into a Tissue Micro Array (TMA, containing three 0.6mm^2 tumor tissue punches per patient) [22]. The tissue sections were deparaffinized and rehydrated according to standard protocols [23]. Endogenous peroxidase activity was blocked with hydrogen peroxidase 0.3% in PBS for 20 minutes. Antigen retrieval was performed using a Pre Treatment (PT)

module (PT link, DAKO, Denmark) in low pH buffer. Sections were incubated at room temperature over night with rabbit polyclonal antibody (1:50, diluted in 1% PBSA) directed against IGF-I receptor β (#3027 Cell Signaling, BIOKÉ, Leiden, the Netherlands). The following day all TMA slides were washed in PBS and incubated with Envision anti-rabbit (DAKO Cytomation K4003) for 30 minutes at room temperature. DAB was used for visualization of positively stained breast tumor tissue on the TMA and counterstained with haematoxylin, dehydrated and finally mounted with pertex. All slides were stained simultaneously to avoid inter-assay variation. Placenta tissue served as positive- and negative-control, the latter was obtained by omitting the primary antibody.

Evaluation of immunostaining

Microscopic quantification of positive tumor cells for the IGF1R antibody was performed in a blinded manner by two independent observers (CCE and AS). IGF1R expression was scored: 0 for no staining at all or membrane staining in <10% of the tumor cells; 1+ for a faint/barely perceptible partial membrane staining in >10% of the tumor cells; 2+ for weak to moderate complete membrane staining in >10% of the tumor cells; and 3+ for strong to complete membrane staining in >10% of the tumor cells (Figure1). In accordance with previous studies, the highest score out of the three punches of the same tumor was used for statistical analyses [24]. If one or more punches were missing, the highest score of the remaining punch(es) was included for analyses. The Cohen's Kappa was 0.86, indicating substantial agreement between the two observers.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics). Hypotheses and analysis plan were drafted before the pathological data became available. Patients with missing data regarding IGF1R, due to material handling, were excluded from statistical analyses as it can be assumed that these data were "missing at random". IGF1R scores were dichotomized: scores 0 and 1+ were considered IGF-1R low, and scores 2+ and 3+ were considered IGF1R high [25;26]. The χ^2 test was used to evaluate associations between various clinico-pathological parameters and tumor IGF1R expression. The clinical endpoints examined were Overall Survival (OS), defined as the time from date of randomization in the TEAM-trial until death by any reason; Breast Cancer Specific Survival (BCSS), defined as the time from date of randomization until death due to BC; and Relapse-Free Survival (RFS), defined as the time from date of randomization until loco-regional recurrence, contralateral BC, distant recurrence or BC death (whichever came first).

First, we assessed the relation between the two treatment regimens of the TEAM trial in patients with either high or low IGF1R expression. The Kaplan–Meier method was used to compose survival plots, and the log-rank test was performed for comparison of

OS, BCSS and RFS curves. Cox Proportional Hazard analyses were used to calculate corresponding Hazard Ratio's (HRs), using univariate analyses for OS, BCSS and RFS. Since the TEAM-trial was randomized, no additional adjustments were made for these analyses.

Next, we assessed the relation between the type of adjuvant endocrine treatment with or without metformin use (subgroups: sequential endocrine treatment only, sequential endocrine treatment with metformin, exemestane only, and exemestane with metformin) in patients with either high or low IGF1R tumor expression using Cox Proportional Hazard Models. These analyses were additionally adjusted for clinically relevant confounders (including age at diagnosis, Body Mass Index (BMI), T-stage, N-stage and histological grade).

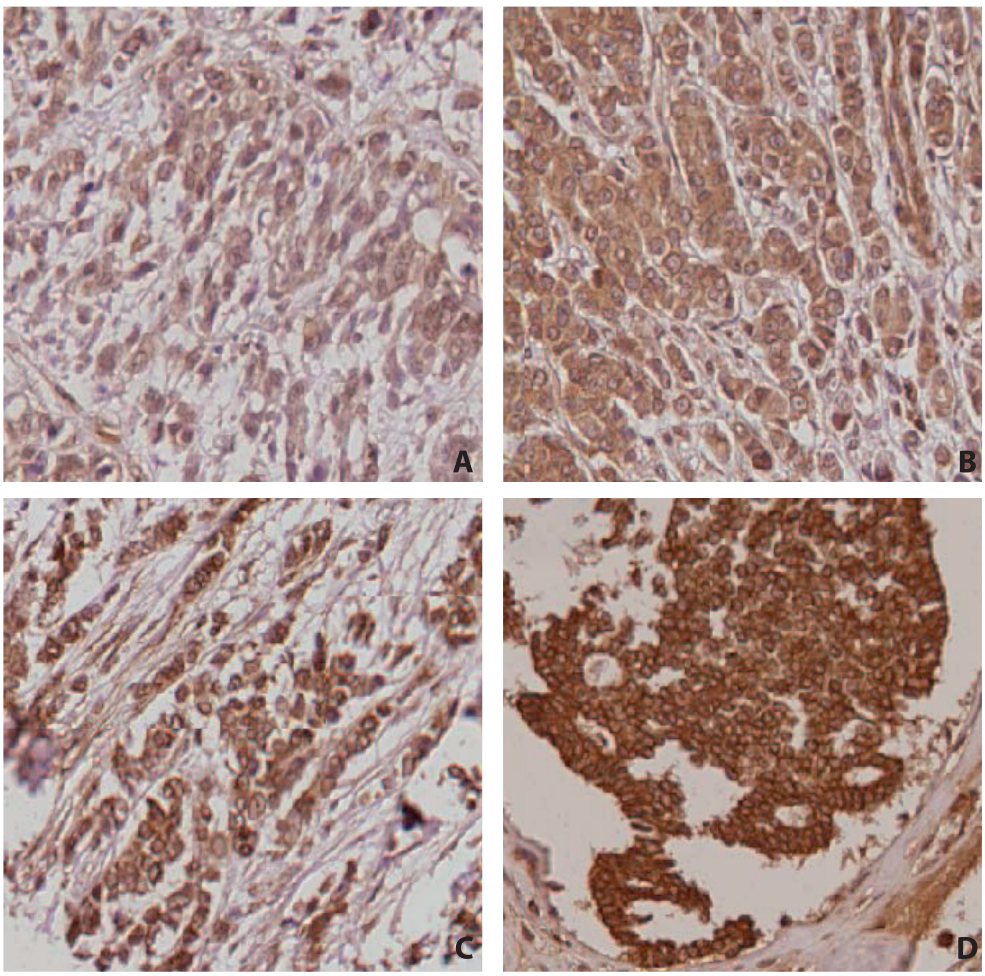

Figure 1: 20x pictures of immunohistochemical IGF-1R staining showing:
A- No staining at all or membrane staining in <10% of the tumor cells (0)
B- Faint/barely perceptible partial membrane staining in >10% of the tumor cells (1+)
C- Weak to moderate complete membrane staining in >10% of the tumor cells (2+)
D- Strong to complete membrane staining in >10% of the tumor cells (3+)

Table 1: patient and tumor characteristics

	IGF1R low (n=830)		IGF1R high (n=1,616)		
	N	(%)	N	(%)	*p-value*
Age					
<55	110	13,3	221	13,7	0,33
55-59	175	21,1	321	19,9	
60-64	145	17,5	315	19,5	
65-69	123	14,8	277	17,1	
70-74	130	15,7	222	13,7	
>=75	147	17,7	260	16,1	
BMI					
<20	23	2,8	39	2,4	0,81
20-24	253	30,5	484	30	
25-29	282	34	559	34,6	
>=30	176	21,2	366	22,6	
Unknown	96	11,6	168	10,4	
T-stage					
T1	359	43,3	733	45,4	0,62
T2	411	49,5	781	48,3	
T3	35	4,2	62	3,8	
T4	22	2,7	38	2,4	
Missing	3	0,4	2	0,1	
N-stage					
N0	239	28,8	517	32	0,16
N+	591	71,2	1.097	67,9	
Unknown	0	0	2	0,1	
Histological grade					
Grade 1	130	15,7	227	14	0,06
Grade 2	368	44,3	717	44,4	
Grade 3	270	32,5	586	36,3	
Unknown	62	7,5	86	5,3	
ER- and/or PR-status					
Negative	3	0,4	3	0,2	0,33
Positive	827	99,6	1.613	99,8	
Most extensive surgery					
No surgery	0	0	1	0,1	0,77
BCS	380	45,8	735	45,5	
Mastectomy	450	54,2	880	54,5	
Radiotherapy					
No	318	38,3	623	38,6	0,92
Yes	511	61,6	990	61,3	

Table 1: (continued)

	IGF1R low (n=830)		IGF1R high (n=1,616)		
	N	**(%)**	**N**	**(%)**	***p-value***
Unknown	1	0,1	3	0,2	
Chemotherapy					
No	592	71,3	1.119	69,2	0,29
Yes	238	28,7	497	30,8	
Randomization					
TAM → EXE	402	48,4	822	50,9	0,26
EXE	428	51,6	794	49,1	
Metformin user					
No	780	94	1.511	93,5	0,65
Yes	50	6	105	6,5	

Abbreviations: BMI: body mass index T-stage: tumor-stage N-stage: nodal stage ER: estrogen receptor PR: progesterone BCS: breast conserving surgery TAM: tamoxifen EXE: exemestane

RESULTS

Patient and tumor characteristics

Of the original Dutch TEAM cohort (n=2,764), 2,446 postmenopausal, early hormone sensitive BC patients were included in the current analyses (116 patients were excluded because of history of malignancy within five years prior to inclusion, and 202 patients were excluded due to missing IGF1R-status, as a result of sample errors. Clinico-pathological and treatment characteristics of the selected patients are shown in Table 1. Median age at diagnosis was 64 years (range 38-91 years). Median follow-up of patients who were alive was 5,4 years (range 0.1-8.7 years). The majority of the BCs had high IGF1R expression (n=1,616, 66.0%). IGF1R expression was not significantly associated with any of the patient, tumor or treatment characteristics.

Stratified analyses for endocrine therapy and metformin use

After stratification of the cohort by IGF1R status, exemestane therapy was significantly associated with improved RFS in patients with high IGF1R tumor expression (HR for exemestane versus sequential therapy: 0.74, 95% Confidence Interval (CI) 0.58-0.95, *p=0.02*) (Table 2 and Figure 2). In this cohort, OS and BCSS were not significantly related with either of the treatment arms, showing a HR of 0.83 (95% CI 0.66-1.04, *p=0.10)* for OS, and a HR of 0.74 (95% CI 0.54-1.01, *p=0.06)* for BCSS. However, it should be noted that both estimates were below one and the p-value for BCSS approached statistical significance. In low IGF-1R expressing tumors, no association between treatment and any of the outcomes was observed.

Regarding metformin use in addition to the endocrine therapy, survival analyses showed no significant association in the patient population with low IGF1R tumor expression (Table 3). In contrast, in patients with high IGF1R expressing tumors, the combination of metformin with the endocrine treatment arm was significantly associated with RFS (HR 1.12, 95% CI 0.57-2.23 for sequential treatment with metformin, HR 0.73 95% CI 0.56-0.94 for exemestane only, and HR 0.32, 95% CI 0.10-1.00 for exemestane with metformin, *p=0.02*, compared to the sequential treatment arm, Table 3). Although BCSS was not significantly associated with the combined therapies, the estimates were similar to the RFS outcomes. Ultimately, significant association was also seen for the OS (multivariable adjusted HR for OS 1.72, 95% CI 0.96-3.08 for sequential treatment with metformin, HR 0.80, 95% CI 0.62-1.03 for exemestane only, and HR 0.67, 95% CI 0.31-1.45 for exemestane with metformin, *p=0.03,* compared to the sequential treatment arm, Table 3). It should be noted that in all analyses the number of events was low for the metformin users.

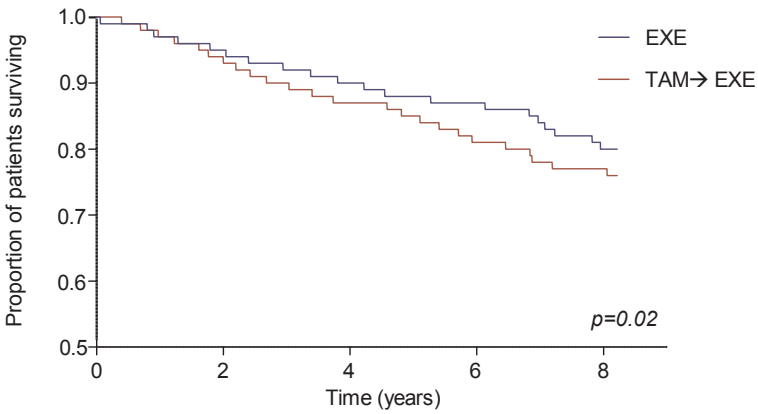

Figure 2: Relapse-free survival of patients with high IGF1-R expression stratified for hormone treatment.

Table 2

			Overall survival				Breast cancer specific survival				Relapse-free survival			
		Patients	**Events**	**HR**	**95% CI**	***p-value***	**Events**	**HR**	**95% CI**	***p-value***	**Events**	**HR**	**95% CI**	***p-value***
IGF1R low	TAM→EXE	402	75	Ref		0,6	52	Ref		0,66	68	Ref		0,73
	EXE	428	84	1,08	(0.81-1.43)		46	1,09	(0.74-1.63)		76	1,06	(0.76-1.47)	
IGF1R high	TAM→EXE	822	142	Ref		0,1	92	Ref		0,06	142	Ref		**0,02**
		794	118	0,83	(0.66-1.04)		67	0,74	(0.54-1.01)		105	0,74	(0.58-0.95)	

OS, BCSS and RFS comparing hormonal treatment, stratified for IGF1R expression on the tumor
Abbreviations: TAM: tamoxifen EXE: exemestane

Table 3

			Overall survival				Breast cancer specific survival				Relapse-free survival			
				Adjusted*				Adjusted*				Adjusted*		
		Patients	**Events**	**HR**	**95% CI**	***p-value***	**Events**	**HR**	**95% CI**	***p-value***	**Events**	**HR**	**95% CI**	***p-value***
IGF1R low	TAM only	376	70	Ref		0,98	44	Ref		0,95	64	Ref		0,94
	TAM & metformin	26	5	0,82	(0.33-2.07)		2	0,73	(0.18-3.08)		4	1,22	(0.48-3.09)	
	EXE only	404	81	0,99	(0.71-1.37)		50	1,04	(0.68-1.62)		72	1,06	(0.75-1.49)	
	EXE & metformin	24	3	1,07	(0.42-2.70)		2	0,8	(0.19-3.39)		4	1,28	(0.50-3.26)	
IGF1R high	TAM only	775	131	Ref		**0,03**	87	Ref	0,19		133	Ref		**0,02**
	TAM & metformin	47	11	1,72	(0.96-3.08)		5	1,11	(0.44-2.77)		9	1,12	(0.57-2.23)	
	EXE only	736	108	0,8	(0.62-1.03)		64	0,74	(0.52-1.04)		100	0,73	(0.56-0.94)	
	EXE & metformin	58	10	0,67	(0.31-1.45)		3	0,38	(0.09-1.56)		5	0,32	(0.10-1.00)	

**adjusted for age, BMI, T-stage, N-stage, histological grade*

Multivariate analyses for OS, BCSS and RFS for hormonal treatment with/without metformin use, stratified for IGF1R expression on the tumor
Abbreviations: TAM: tamoxifen EXE: exemestane

DISCUSSION

This study showed a significantly improved RFS in patients with high IGF1R expression on their breast tumor surface treated with exemestane compared to sequential therapy. Additionally, our data suggested a further enhancement of the RFS when metformin was added to exemestane in these patients, although it must be noted that the number of events in patients who received metformin was low.

The findings of our analyses are interesting, and are in contrast with the main results of the TEAM-trial, which showed no difference in OS, BCSS nor DFS for either one of the two treatment arms [21].

There may be several explanations for the observed benefit of exemestane in patients with high IGF1R expression. Evidence is building for a novel view that that estrogen can, next to binding and activating its classical receptor, the ER, also phosphorylate and activate the IGF1R [15]. In view of our results, we hypothesize that the interaction between the degree of IGF1R expression on the tumor surface and the efficacy of exemestane is mainly induced by the fact that exemestane, an aromatase inhibitor, suppresses estrogen production. Suppression of estrogen production could lead to reduced estrogen induced activation of IGF1R and thus less activation of the mitogen-stimulating pathway. Since this ultimately leads to less proliferation of the BC cells, this can translate into a clinical benefit for the high IGF1R expressing, hormone sensitive BC patients. This hypothesis also supports our finding that patients with high IGF1R expression who were treated with tamoxifen (an ER blocker) for the first 2.5 years following local therapy did not experience clinical benefit, as the unaffected levels of circulating estrogens can still phosphorylate the IGF1R, thereby stimulating breast cancer cell growth. The fact that no clinical benefit of exemestane treatment was observed in patients with tumors harboring low IGF1R expression also supports our proposed hypothesis, as the effect of estrogen induced tumor growth promoting signaling by IGF1R is too small in these tumors.

When metformin was added to the endocrine treatment received, an additional significant benefit was seen with respect to the clinical outcome parameters OS and RFS for patients treated with exemestane and metformin, and non-significant similar estimates were seen for BCSS in patients treated with exemestane and metformin. However, these results must be interpreted with caution, as the number of events was small in patients who were treated with metformin. However, these findings support our hypothesis concerning inhibition of the IGF-1 pathway, as metformin induces lowering of insulin and IGF concentrations [27]. Thus, we propose that metformin induced lowering of the IGF concentration leads to direct loss of IGF1R stimulation. Therefore, our hypothesis states that patients with high IGF1R expression on their tumor surface treated with both exemestane and metformin will encounter dual blockage of IGF1R activation, thus block-

ing both estrogen-driven as well as insulin-driven IGF1R activation. This study showed that dual blockage of the IGF1R results in better clinical outcome. These findings are promising, as several previous observational studies have shown benefits of metformin treatment in cancer patients [28;29]. By stratifying patients according to IGF1R expression of the tumor, which is up-regulated in roughly two-thirds of the postmenopausal breast cancer population and thus widely applicable, it may become possible to identify a subgroup of patients who may benefit of these combined treatments, thereby further individualizing treatment and improving outcomes for particular subgroups within the heterogeneous BC population. Of course, our interesting and promising results need first to be confirmed in other large studies containing HR+ve BC patients treated with AI, such as, for example the ATAC, BIG, or IES study, all with tumor material available, or preferably in a randomized trial setting, before they can be implemented in clinical practice. To our knowledge, there are no ongoing trials that specifically assess the value of metformin added to treatment with an AI, nor are there trials that assess the benefit of AI in relation to IGF1R expression.

The main strength of this study is the fact that we clearly defined hypotheses before data collection and analyses. Biomarker substudies of clinical trials frequently "search" for significant associations between many different subgroups or biomarkers, and present the significant associations only. Although the current study was not a prospectively planned subgroup analysis, it can still be considered a major strength of this study that we only assessed the IGF1R receptor and formulated hypotheses before data collection. Secondly, to our knowledge this is the first study that assessed the relation between adjuvant endocrine therapy in relation to IGF1R expression on the tumor surface of postmenopausal HR+ve, early BC patients. Furthermore, no previous studies assessed the added benefits of metformin in relation to IGF1R expression. Another major strength of this study is the use of data from the TEAM-trial, as this provides well-registered data in a large number of patients.

This study, however, also has its limitations. First, there were no uniform cut-off values for IGF1-R expression available from previous literature. Therefore, we categorized patients by defining a moderate to strong expression in >10% of the tumor cells as high IGF-1R expression, in line with e.g. categorization of endocrine receptor positivity. Furthermore, patients on sequential hormonal therapy received exemestane after the first 2.5 years of tamoxifen treatment. It would be desirable to compare two endocrine treatment regimens, consisting of solely exemestane and solely tamoxifen given for five consecutive years. Also, metformin use was not randomized in this trial, which makes that these analyses must be interpreted with caution, as they may be subjected to confounding by indication. However, RFS and BCSS in relation to metformin use can be considered as unintended effects of metformin, and therefore we believe that it is possible to assess this relation in this study. Furthermore, it is plausible that the patients

using metformin in this study are diabetics. Therefore, it is unclear whether the results concerning the effect of metformin in this specific population can be extrapolated to the non-diabetic population. Finally, the relatively small number of events for BCSS may be considered as a limitation, but the estimates for BCSS strongly resembled the estimates for RFS. Especially for the analyses where the additional value of metformin on clinical outcome was assessed, the small number of events was a strong limitation of this study.

In conclusion, these study results add to the ongoing discussion of the value of optimal endocrine treatment as well as metformin use in BC patients, as it appears that high IGF1R expression on the breast tumor surface is a potential biomarker of improved clinical outcome in HR+ve BC patients treated with exemestane. Combining metformin with exemestane may further add to this effect.

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SUPPLEMENTARY TABLES

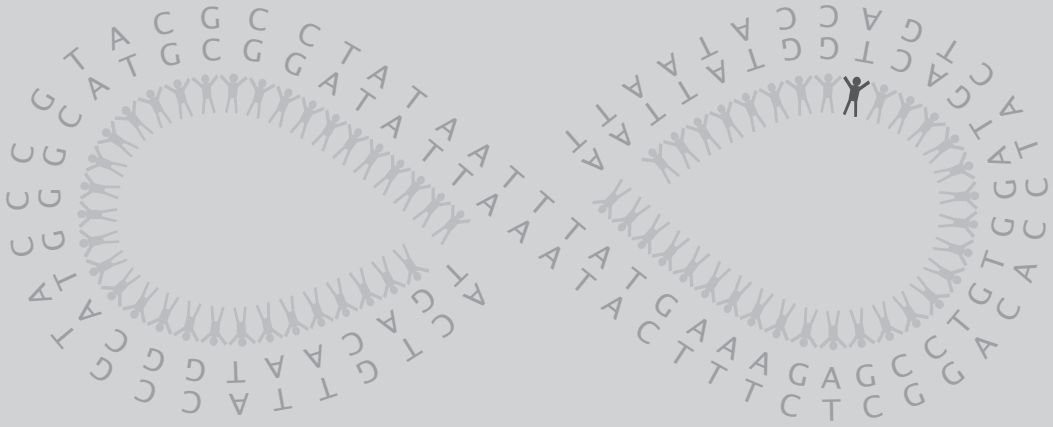
Web-table 1: Patient characteristics of patients from the Netherlands compared to other countries

	Netherlands		Other countries		
	N	**(%)**	**N**	**(%)**	***p-value***
Age					
<55	363	(13.2)	1090	(15.5)	**<0.001**
55-59	551	(20.0)	1344	(19.2)	
60-64	514	(18.7)	1487	(21.2)	
65-69	451	(16.4)	1279	(18.3)	
70-74	401	(14.6)	928	(13.2)	
>=75	473	(17.2)	885	(12.6)	
BMI					
<20	68	(2.5)	109	(1.6)	**<0.001**
20-24	851	(30.9)	872	(12.4)	
25-29	931	(33.8)	831	(11.8)	
>=30	601	(21.8)	502	(7.2)	
Unknown	302	(11.0)	4699	(67.0)	
T-stage					
In Situ	1	(0.0)	5	(0.1)	**<0.001**
T1	1235	(44.9)	4455	(63.5)	
T2	1329	(48.3)	2263	(32.3)	
T3	120	(4.4)	216	(3.1)	
T4	63	(2.3)	58	(0.8)	
Missing	5	(0.2)	16	(0.2)	
N-stage					
N0	834	(30.3)	4278	(61.0)	**<0.001**
N+	1915	(69.3)	2673	(38.1)	
Unknown	4	(0.1)	62	(0.9)	
Histological grade					
Grade 1	420	(15.3)	1257	(17.9)	**<0.001**
Grade 2	1218	(44.2)	3579	(51.0)	
Grade 3	934	(33.9)	1490	(21.2)	
Unknown	181	(6.6)	687	(9.8)	
ER- and/or PR-status					
Negative	6	(0.2)	9	(0.1)	0.40
Positive	2747	(99.8)	7002	(99.8)	
Unknown	0	(0.0)	2	(0.0)	
HER-2 status					
Negative	750	(27.2)	3169	(45.2)	**<0.001**
Positive	49	(1.8)	826	(11.8)	
Unknown	1954	(71.0)	3018	(43.0)	

Abbreviations: BMI: body mass index T-stage: tumor-stage N-stage: nodal stage
ER: estrogen receptor PR: progesterone BCS: breast conserving surgery TAM: tamoxifen EXE: exemestane

Web-table 2: Overall survival of patients included in the TEAM trial

	HR	**95% CI**	***p-value***
The Netherlands	1.0 (ref)		**<0.001**
Germany	0.45	(0.37-0.53)	
UK/Ireland	0.71	(0.61-0.83)	
Greece	0.42	(0.27-0.64)	
France	0.31	(0.24-0.40)	
US	0.60	(0.51-0.72)	
Japan	0.36	(0.20-0.63)	
Belgium / Luxembourg	0.58	(0.44-0.78)	



Chapter 8

The clinical value of HER-2 overexpression and PIK3CA mutations in the older breast cancer population: a FOCUS study analysis

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ABSTRACT

Background

Studies to confirm the effect of acknowledged prognostic markers in older breast cancer patients are scarce. The aim of this study was to evaluate the prognostic value of HER-2 overexpression and PIK3CA mutations in older breast cancer patients.

Design

Female breast cancer patients aged 65 years or older, diagnosed between 1997-2004 in a geographical region in The Netherlands, with an invasive, non-metastatic tumour and tumour material available, were included in the study. The primary endpoint was relapse free period and secondary endpoint was relative survival. Determinants were immunochemical HER-2 scores (0/1+, 2+ or 3+) and PIK3CA as a binary measure.

Results

Overall, 1,698 patients were included, and 103 had a HER-2 score of 3+. HER-2 overexpression was associated with a higher recurrence risk (5 years recurrence risk 34% vs. 12%, adjusted p=0.005), and a worse relative survival (10 years relative survival 48% vs. 84% for HER-2 negative; p=0.004). PIK3CA mutations had no significant prognostic effect.

Conclusion

We showed, in older breast cancer patients, that HER-2 overexpression was significantly associated with a worse outcome, but PIK3CA mutations had no prognostic effect. These results imply that older patients with HER-2 overexpressing breast cancer might benefit from additional targeted anti-HER-2 therapy.

INTRODUCTION

Over the last decades an increased aged population in Western countries paralleled a marked increase in the incidence of age-related tumours, such as breast cancer [1]. In the United States of America, more than 40% of the women diagnosed with breast cancer were 65 years of age or older in 2013 [2]. Due to the continuously increasing life expectancy, it is assumed that this number will further increase the coming years.

Human epidermal growth factor receptor-2 (HER-2) overexpression occurs in approximately 15-20 percent of invasive breast carcinomas [3;4]. Amplification of the HER-2 gene is associated with a more aggressive tumour phenotype [5], and if left untreated, is associated with worse clinical outcomes [6-9]. Detection of the amplification of this oncogene is widely performed and often routinely used in clinical settings [10], mainly for allocation of anti-HER-2 therapy, consisting of trastuzumab or pertuzumab in the (neo)adjuvant setting [11-16] and in the metastatic setting of lapatinib and trastuzumab-emtansine [17;18]. These anti-HER-2 therapies, in addition to cytotoxic chemotherapy, can result in a substantial reduction of recurrences, both in node negative and node positive breast cancer patients [19-21].

A well-known accomplice of HER-2 overexpression is the PIK3CA mutation, leading to aberrant activation of the Phosphatidylinositol 3-kinase (PI3K)/AKT pathway, co-occurring in approximately 40% of HER-2 amplified tumors where it supports tumor growth [22;23]. The aberrant activation of the PI3K pathway correlates with reduced response to HER-2-directed therapies and accelerates HER-2 mediated breast epithelial transformation and metastatic progression [5;24;25]. In the younger population PI3K inhibitors are considered promising novel therapeutic modalities for the treatment of breast cancer.

An important shortcoming in current clinical breast cancer research is that the majority of studies are performed in a relatively young or fit elderly breast cancer population. This hampers the extrapolation of results of prognostic as well as therapeutic studies for older patients [26;27].

The high incidence of cancer in the growing elderly population encourages to investigate the effect of potential prognostic and/or predictive markers in this population specifically. Some studies suggest that HER-2 positive breast cancer occurs less frequently in older patients [28;29], but other studies fail to confirm this [30]. The clinical value of HER-2 overexpression and its treatment in the older population, well-known for competing clinical events, is not known. Because the accrual of older patients in clinical trials for anti-HER-2 therapy was poor, and an increased incidence of cardiac adverse events of trastuzumab was reported, its use is less widespread in older women than younger women [31]. However, evidence for the omission of anti-HER-2 therapy is lacking.

In summary, there are no conclusive data on the prognostic effect of HER-2 overexpression and PIK3CA mutations in the elderly breast cancer patient. Therefore, in order to restore the clinical interest of this neglected, but potentially very valuable treatment target for the elderly breast cancer patients, this study aims to elucidate the prognostic value of HER-2 overexpression, PIK3CA mutations and the interplay between these two markers in a population-based cohort of older breast cancer patients, that was not exposed to any anti-HER-2-therapy.

MATERIAL AND METHODS

Patients and tumors

For this study, patients with invasive, non-metastatic breast cancer from the FOCUS cohort (Female breast cancer in the elderly, Optimizing Clinical guidelines USing clinic-pathological and molecular data) who received surgery and had formalin fixed paraffin embedded (FFPE) intra-operative tumor samples available with successful measurements of the HER-2 status and/or PIK3CA mutations were included. The FOCUS cohort has been described extensively in previous publications [32]. Briefly, the cohort consists of all women aged ≥ 65 years at time of diagnosis, with invasive and *in situ* breast cancer, diagnosed between 1997 and 2004 in the South Western region of The Netherlands. Follow-up on survival status was available until the 1st of January 2013. All tumor samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Immunohistochemistry for HER-2

The immunohistochemical staining against HER-2 was performed on tissue sections of 4µm from intra-operatively derived FFPE tumor material of the FOCUS cohort processed into a Tissue Micro Array (TMA) according to the previously described protocol [33]. Sections were incubated at room temperature with Polyclonal Rabbit Anti-Human c-erbB-2 Oncoprotein (DAKO, Denmark (A0485); 1:100, diluted in 1% PBSA) for 20 minutes, followed by Envision anti-rabbit (DAKO, Denmark, Cytomation K4003) for 20 minutes. DAB was used for visualization of positively stained breast tumor tissue on the TMA and counterstained with haematoxylin. All slides were stained simultaneously to avoid inter-assay variation. Strongly c-erbB-2 Oncoprotein positive brain tumor tissue served as positive- and a negative-control, the latter was obtained by omitting the primary antibody.

Evaluation of immunostaining

Microscopic quantification of the c-erbB-2 Oncoprotein was performed by two independent observers (C.E and A.M.). C-erbB-2 Oncoprotein was scored as follows: 0 for no

staining at all or incomplete or faint/barely perceptible membrane staining in <10% of the invasive tumor cells; 1+ for a faint/barely perceptible partial membrane staining in >10% of the tumor cells; 2+ for weak to moderate complete membrane staining in >10% of the tumor cells; and 3+ for strong to complete membrane staining in >30% of the tumor cells (Figure 1). For all patients, the highest score out of the three punches of the

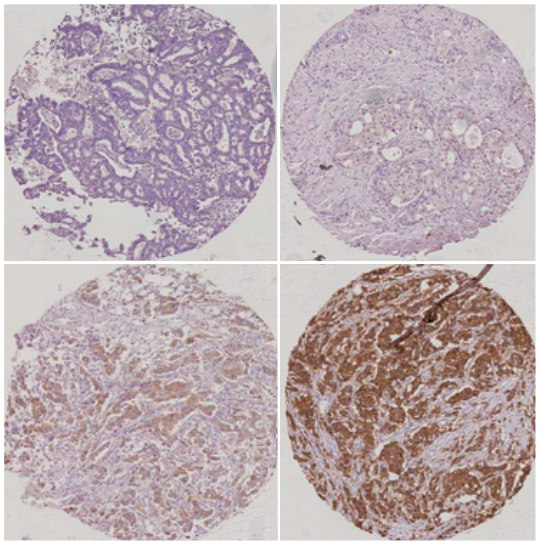

Figure 1: Immunohistochemical staining for HER-2 overexpression (*ERBB2*). Top left: HER2 score 0. Top right: HER2 score 1+. Bottom left: HER2 score 2+; and bottom right: HER2 score 3+.

same tumor was used for statistical analysis. If one or more punches were missing, the highest score of the remaining punch(es) was included for analyses.

PIK3CA mutation analysis

DNA was extracted from 2.0 mm diameter FFPE breast cancer tissue cores of 896 patients, using a fully automated system (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) as described previously [34]. Hydrolysis probes assays were performed for the major known mutations (hotspots) in exon 9, c.1624G>A; *p*.E542K, c.1633G>A; p.E545K and in exon 20 the c.3140A>G; p.H1047R as described before [35]. Hydrolysis probe assays were analyzed using qPCR analysis software (CFX manager version 3/0, Bio-Rad). Mutation detection was performed by two observers (C.E. and R.E.) using DNA variant analysis software (Mutation Surveyor version 4.0.9, Softgenetics, State College, PA, USA). All primers and probes used for the assays are listed in Supplementary Table 1.

Statistical analysis

Statistical analyses were performed using the statistical packages SPSS (version 20.0 for Windows, IBM SPSS statistics) and Stata SE 12.0.

The primary endpoint was Relapse-Free Period (RFP), defined as the time from date of diagnosis until any recurrence (any registered loco-regional recurrence, distant recurrence or contralateral breast cancer). The Cumulative Incidence Competing Risks method was used for calculating the cumulative incidence of recurrence, taking into account the competing risk of death [36]. Fine & Gray competing risks regression analyses were used for univariable and multivariable analysis for RFP, taking into account the competing risk of death without recurrence [37]. Multivariable analyses were adjusted for age, TNM stage, grade, estrogen receptor (ER) and progesterone receptor (PR)) and type of breast surgery, axillary surgery, radiotherapy, endocrine therapy and chemotherapy (no anti-HER-2-therapy). Because the total follow-up time was longer than the follow-up time for RFP, the secondary endpoint was relative survival, calculated as the ratio between the observed survival in the cohort and the expected survival as calculated from the age-, sex- and year-matched background population [38]. Patients with missing data on the determinant of interest due to material handling were excluded from the statistical analyses regarding that determinant. First, it was checked if the patients with missing data had no significant survival difference from the patients without missing values, to confirm that there is no association with general prognosis.

Immunohistochemical HER-2 scores 0 and 1+ were considered HER-2 negative and HER-2 scores 2+ and 3+ were analyzed as separate categories. In clinical practice, tumors with an immunohistochemical HER-2 score of 2+ are considered borderline positive, therefore, it is recommended to validate the immunohistochemical HER-2 2+ results using in situ hybridization [39-41]. Unfortunately, it was not feasible to perform this additional test in our population. PIK3CA mutations were analyzed as dichotomous variable (negative or positive) in all patients, and stratified for HER-2 status.

RESULTS

Patient and tumour characteristics

A total of 1,932 tumour blocks were available for immunohistochemical staining. After material handling and staining, 1,698 patients were available for HER-2 analyses, and 912 patients for PIK3CA analyses. For the combined HER-2/PIK3CA analyses, 896 postmenopausal breast cancer patients were included in the analyses. The weighted Kappa for the HER-2 immunohistochemistry was 0.78 (SE 0.03), indicative of good inter-observer agreement'. There was no significant difference in overall survival between all patients

Table 1: Patient characteristics, tumor characteristics and treatment

	HER-2 negative (N=1,326)		HER-2 2+ (N=269)		HER-2 3+ (N=103)		*P* for difference**
	N	%	N	%	N	%	
Age in years (mean, SD)	76.1 (7.2)		76.0 (7.2)		75.0 (6.9)		0.272
Number of comorbidities							0.481
0-1	627	47.3	586	44.2	113	8.5	
2-4	586	44.2	114	42.4	28	10.4	
5 or more	113	8.5	39	37.9	13	12.6	
TNM stage							**0.050**
I	478	36.0	87	32.3	21	20.4	
II	671	50.6	138	51.3	66	64.1	
III	145	10.9	36	13.4	14	13.6	
Missing	32	2.4	8	3.0	2	1.9	
Grade							**<0.001**
1	184	13.9	27	10.0	4	3.9	
2	422	31.8	87	32.3	16	15.5	
3	316	23.8	90	33.5	62	60.2	
Missing	404	30.5	65	24.2	21	20.4	
Morphology							**0.001**
Ductal	969	73.1	218	81.0	91	88.3	
Lobular	157	11.8	18	6.7	4	3.9	
Other/missing	200	15.1	33	12.3	8	7.8	
ER/PR							**<0.001**
Negative	180	13.6	33	12.3	54	52.4	
Positive	980	73.9	209	77.7	35	34.0	
Missing	166	12.5	27	10.0	14	13.6	
Ki67							**<0.001**
Negative	1094	82.5	228	84.8	75	72.8	
Positive	84	6.3	20	7.4	21	20.4	
Missing	148	11.2	21	7.8	7	6.8	
Breast surgery*							**0.007**
BCS	510	38.5	95	35.3	24	23.3	
Mastectomy	816	61.5	174	64.7	79	76.7	
Axillary surgery*							0.296
No axillary surgery	181	13.7	35	13.0	16	15.5	
Sentinel node	311	23.5	71	26.4	16	15.5	
ALND	834	62.9	163	60.6	71	68.9	
Adjuvant radiotherapy							0.947
No	686	51.7	142	52.8	54	52.4	
Yes	640	48.3	127	47.2	49	47.6	

Table 1: Patient characteristics, tumor characteristics and treatment (continued)

	HER-2 negative (N=1,326)		HER-2 2+ (N=269)		HER-2 3+ (N=103)		*P* for difference**
	N	**%**	**N**	**%**	**N**	**%**	
Adjuvant endocrine therapy							**0.008**
No	728	54.9	127	47.2	66	64.1	
Yes	598	45.1	142	52.8	37	35.9	
Adjuvant chemotherapy							**0.003**
No	1250	94.3	257	95.5	89	86.4	
Yes	76	5.7	12	4.5	14	13.6	

Abbreviations: ER: estrogen receptor PR: progesterone receptor BCS: breast conserving surgery ALND=axillary lymph node dissection

*The most extended therapy was taken into account **p-values are calculated by the Pearson Chi-Square for categorical variables, and with an oneway ANOVA test for continuous variables. P-values in bold font indicate a statistically significant difference between the groups at the p-level of <0.05.

from the original cohort (with or without tumour blocks available) and the patients with available data on respectively HER-2 or PIK3CA (Log Rank p=0.537 and p=0.298).

Patient, tumour and treatment characteristics are shown in Table 1 by HER-2 score. Overall, mean age at diagnosis was 76 years (standard deviation 7.2 years). The majority of patients presented with early stage breast cancer (stage I 34.5%, stage II 51.5%, stage III 11.5%) of ductal morphology (75.3%). In the majority of the patients a mastectomy (63.0%) was performed. Merely six percent of patients were treated with adjuvant chemotherapy (not including anti-HER-2-therapy). In this cohort, 78% of the breast cancers were classified as HER-2 negative (N=1,326). Two hundred sixty-nine patients had a HER-2 score of 2+ (16%), and 103 patients had a HER-2 score of 3+ (6%). HER-2 overexpression was significantly associated with higher tumor grade (p<0.001), ductal tumor morphology (p=0.001), negative hormone receptor status (p<0.001), and a higher Ki-67 proliferation rate (p<0.001*)*. In addition, HER-2 overexpression was associated with undergoing mastectomy rather than breast conserving surgery (p=0.007), less endocrine therapy (p=0.008) and more chemotherapy (without anti-HER2 therapy) (p=0.003).

Among all patients from whom PIK3CA status was available, 30% had a PIK3CA mutation. In our data, PIK3CA mutations were not associated with HER-2 overexpression (p=0.7).

Relapse free period

For RFP, median follow-up was 5.3 years (range: 0-13.5 years). HER-2 negative patients had a cumulative recurrence risk of 12% at 5 years, as compared to 12% for patients with a HER-2 score of 2+ and 34% for patients with a HER-2 score of 3+. In adjusted analysis, patients with a score of 2+ had an equal recurrence free period at 5 years as compared to HER-2 negative patients (adjusted sub-distribution Hazard Ratio (SHR) 0.95, 95% Con-

Table 2: Relapse free period (Fine & Gray regression)

	N of events	Recurrence risk at 5 years (%)	SHR	95% CI lower	95% CI upper	*P*	SHR*	95% CI lower	95% CI upper	*P*
HER2										
negative:										
0/1+	155	12%	1 (Reference)				1 (Reference)			
2+	32	12%	1.06	0.76	1.47	0.747	0.95	0.67	1.35	0.790
3+	34	34%	2.68	1.86	3.86	**<0.001**	1.81	1.19	2.74	**0.005**
PIK3CA										
negative	103	17%	1 (Reference)				1 (Reference)			
positive	42	16%	0.98	0.71	1.35	0.892	1.00	0.72	1.41	0.987

*adjusted for age, morphology, grade, hormone receptor status, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

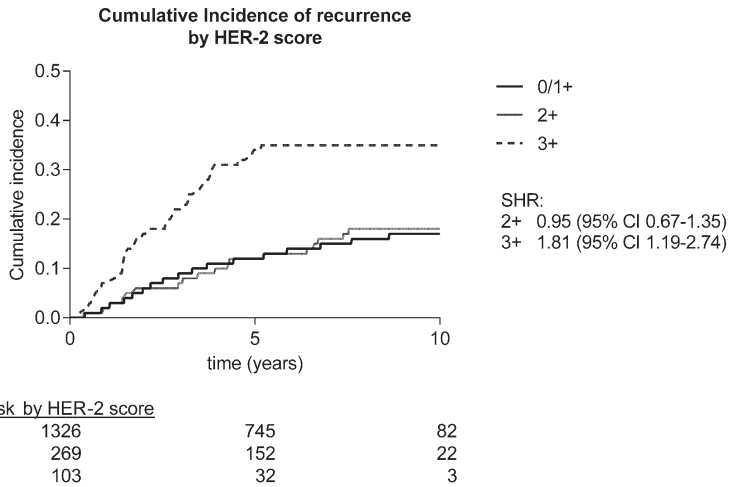

Figure 2: Cumulative incidence of recurrence by HER-2 score.

fidence Interval (CI) 0.67-1.35; *p*=0.8), patients with a score of 3+ had significantly worse relapse free period (adjusted SHR 1.81, 95% CI 1.19-2.74, *p*=0.005) (Table 2). Cumulative incidence curves are depicted in Figure 2. Among patients without PIK3CA mutations, the cumulative incidence of recurrences at 5 years was 17%, as compared to 16% of patients with a PIK3CA mutation. There was no statistically significant difference in RFP between patients with and without a PIK3CA mutation (*p*=0.2), also after stratifying for HER-2 status (Supplementary file 2).

Table 3: Relative survival

	N of observed deaths / N of Expected deaths	Relative survival at 10 years (%)		95% CI				95% CI		
			RER	lower	upper	*P*	RER*	lower	upper	*P*
HER2										
negative: 0/1+	712 / 536	84%	1 (Reference)				1 (Reference)			
2+	135 / 105	88%	0.91	0.46	1.82	0.796	0.78	0.45	1.35	0.373
3+	69 / 22	48%	4.75	3.10	7.28	**<0.001**	2.07	1.26	3.39	**0.004**
PIK3CA										
negative	344 / 250.6	82%	1 (Reference)				1 (Reference)			
positive	149 / 105	81%	0.94	0.50	1.76	0.855	1.24	0.78	2.01	0.194

*adjusted for age, morphology, grade, hormone receptor status, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy

Relative Survival

Median follow-up was 8.9 years (range 0-17.0 years) for vital follow-up status. The results of the relative survival analyses are shown in Table 3. Patients with a HER-2 score of 2+, again showed an equal outcome as compared to the HER-2 negative patients (88% vs. 84% at 10 years; multivariable Relative Excess Risk (RER) 0.78, 95% CI 0.45-1.35; p=0.4). Patients with a HER-2 score of 3+ had a significantly worse relative survival at 10 years (48%; RER 2.07, 95% CI 1.26-3.39; p=0.004). Patients without PIK3CA mutations had a 10 years relative survival of 82%, as compared to 81% of patients with a PIK3CA mutation. There was no statistically significant difference in relative survival between patients with and without a PIK3CA mutation, also after stratifying for HER-2 status.

DISCUSSION

The main finding of our study is that HER-2 overexpression is of significant prognostic value in the older breast cancer population, even when taking the competing risk of mortality into account. Patients with HER-2 3+ tumours showed a significantly higher risk of recurrence, as compared to patients with a HER-2 negative tumour. Interestingly, patients with HER-2 2+ tumours had a similar recurrence risk as patients with HER-2 scores of 0 and 1+, who are considered to have HER-2 negative tumours. In this specific breast cancer population, PIK3CA mutations had no prognostic value.

With the results of this study, we identified a subgroup of older breast cancer patients with a significantly worse clinical outcome. Therefore, our results raise the hypothesis

that women aged 65 and older with HER2 3+ tumours might benefit from additional therapy, which includes anti-HER-2 therapy. In contrast to the study performed by Syed *et al.*, in which they claim a more indolent tumour character in the older breast cancer patient, we show that HER2+ positive tumours in the elderly are significantly associated with higher proliferative Ki67 presence in the tumour [42]. Our results could justify more aggressive anti-cancer treatment in the older breast cancer patients harbouring tumours with disadvantageous characteristics, as they, similar to their presence in the younger breast cancer patients, are associated with worse survival rates.

It is essential to realize that in the FOCUS cohort, it is very unlikely that patients received anti-HER-2-therapy, as the FDA approved trastuzumab for the treatment of breast cancer in 2006, and patients included in the FOCUS cohort were diagnosed and treated for their breast cancer between 1997 and 2004 [43]. Moreover, the receipt of adjuvant chemotherapy was very low among the patients in our cohort, but is similar to previous observational studies in older breast cancer patients [44;45].

An important question that still needs to be addressed is whether older breast cancer patients will benefit from anti-HER-2 therapy. Anti-HER-2 therapy is notorious for serious, mainly cardiac related, adverse events. However, recent studies have shown that cardiac adverse events from anti-HER-2 therapy are less severe and present with a lower incidence than initially assumed [21;46;47]. One of these studies was a side study of the HERA trial, in which the primary aim was to compare 1 versus 2 years of trastuzumab treatment. In this study patients with a median follow-up of 8-years had a relatively low incidence of cardiac events (cumulative incidence of confirmed LVEF decrease was 7% in the 2-year arm, 4% in the 1-year arm and, 1% in the observation arm). Moreover, the majority (87%) of HER-2 therapy induced cardiac events appeared to be reversible after stopping trastuzumab treatment [47]. Chavez-MacGregor *et al.* showed that trastuzumab-related cardiotoxicity did increase according to age. However, also in this older breast cancer population most of the cases were reversible, stressing the need for adequate monitoring [48]. It should also be noted that most (>80%) of the older patients who initiate trastuzumab complete this therapy [49]

In current medical practice, anti-HER-2 therapy is frequently omitted from treatment options in older breast cancer patients. This is probably due to the current standards that advise the combination with chemotherapy, but also due to the fear of cardiac toxicity. One of the major characteristics of the older cancer population is the clinical heterogeneity among patients of the same chronological age. Therefore, merely taking into account chronological age may result in unfair survival chances due to under-treatment of fit elderly. Given the results of our current study, it could be suggested that the fit elderly breast cancer patient, especially when the Left Ventricular Ejection Fraction (LVEF) is acceptable (more than 50%), should be treated with the same adjuvant-regimen as

the younger HER-2 positive breast cancer patients; consisting of a taxane based chemotherapy supplemented with one year of trastuzumab [41]. Moreover, older patients with less desirable clinical conditions, or when there is a strong preference to omit chemotherapy, dual HER-2 blockage (trastuzumab combined with pertuzumab) could be considered. In the neoadjuvant setting, such chemotherapy-free regimens have shown to be associated with very few side effects, but unfortunately also with a lower tumour response [46]. Currently, underrepresentation of elderly cancer patients in clinical trials hampers the implementation of widely agreed clinical practice recommendations, such as for HER-2 overexpressing breast cancer, which might result in under-treatment (sub-optimal clinical outcome) or over-treatment (risk of significant toxicities) in the older breast cancer population. Currently, a randomized clinical trial is recruiting older (70+) patients with metastatic breast cancer disease, in which a chemotherapy-free regimen of trastuzumab and pertuzumab is compared with this combination and the addition of chemotherapy (EORTC-75111-10114) [50]. We believe that clinical trials focusing on such specific therapies can change clinical practice for this specific breast cancer population in the coming years, as finally more insight is provided about an often neglected but increasingly important subgroup of the breast cancer population. It is for this reason that the results of this trial are eagerly awaited.

In our study, no clinically prognostic value was retrieved from PIK3CA mutation analyses in the elderly, HER-2 positive or HER-2 negative breast cancer patients. Currently, it is believed that PIK3CA mutations in the co-occurrence of HER-2 positive breast disease result in poor prognosis [51]. In contrast, other studies have shown good prognosis with mutated PIK3CA in hormone receptor–positive tumors. The potential explanation of this finding is that continued activation of PI3K may have an inhibitory effect on HER-2 signalling [51]. Based on the results from our study, PIK3CA mutations might not have any prognostic value in the elderly breast cancer patient.

The major strength of our study is that we used the largest consecutive series of older breast cancer patients from a population-based cohort, from which tumour material was available. Therefore, our study is not affected by selection bias. Our study is unique because most previous prognostic studies on HER-2 expression are performed in a younger breast cancer population or as part of a clinical trial. It has been shown that older patients who are registered in a clinical trial have a significantly better overall health than patients of the same age in the general population [52]. Therefore, these studies cannot be simply extrapolated to the older breast cancer patients. This observation is in agreement with the American Cancer Society, whom states that older patients (≥65years) represent 45% of all breast cancer cases and are a particularly vulnerable, and underrepresented patient group [53;54]. Furthermore, recent articles by Chavez-MacGregor

et al., and Vaz-Luis *et al.* [49;53] point out that a relatively small portion of older breast cancer patients receive adjuvant chemotherapy (with/without trastuzumab therapy), which is attributed to undertreatment, a well described phenomenon among the elderly in the USA, and also in Europe. Herewith urging the need for better identification of older patients who could benefit from anti-HER2 therapy through real-world information instead of selective clinical trials.

Our present study demonstrates that HER-2 overexpression is a strong and independent predictor of worse prognosis, tested in a representative population of older breast cancer patients and even after taking into account competing risk of mortality due to other reasons. Therefore, from an under-treatment point of view, the results of this study should convince oncologists that anti-HER2 therapy should be at least considered for every HER-2 positive patient, regardless of age.

A limitation of the study is that there was no tumour material available for all patients in the cohort, mostly due to logistical reasons. We minimized the chance that this selection affected our study aims, by ruling out the association of having missing values on the determinants of interest (HER-2 amplification and PIK3CA mutation) with overall mortality. Second, in contrast to clinical practice, no confirmatory in situ hybridization was performed for the HER-2 2+ patients. However, based on the immunohistochemical data, the HER-2 2+ patients did not show a different clinical outcome compared to HER-2 negative patients. Based on these data, one could question the additional value of the costly in situ hybridization in this specific sub-group of the older breast cancer population, as this group does not have a worse prognosis than the HER-2 negative patients.

In conclusion, this population-based study among elderly breast cancer patients showed a strong prognostic effect of HER-2 overexpression, defined as an immunohistochemical score of 3+, on higher recurrence risk as well as worse relative survival. Herewith, we defined a subgroup of older breast cancer patients who are at high risk for worse clinical outcome, with a strong likelihood of under-treatment. Future research should point out whether it is possible to establish an effective anti-HER-2 regimen with minimal toxicity for the elderly breast cancer population.

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SUPPLEMENTARY TABLES

Supplementary table 1: primers used for PIK3CA mutation analysis

Assay Name	Primer Name	Primer Sequence	Reporter 1 Name	Dye	Reporter 1 Sequence	Reporter 2 Name	Dye	Reporter 2 Sequence
p.E542K*	PIK3CA_p.E542K _F PIK3CA_p.E542K _R	AGCTCAAAGCAATTTCTA-CACGAGAT GCACTTACCTGTGACTC-CATAGAAA	PIK3CA_p.E542K _V	VIC	CCTCTCTCTGAAATCA	PIK3CA_p.E542K _M	FAM	CCTCTCTCTAAAATCA
p.E545K*	PIK3CA_p.E545K_F PIK3CA_p.E545K_R	TCAAAGCAATTTCTACAC-GAGATCCT GCACTTACCTGTGACTC-CATAGAAA	PIK3CA_p.E545K_V	VIC	CTCTCTGAAATCACT-GAGCAG	PIK3CA_p.E545K_M	FAM	CTCTGAAATCACTAAGCAG
p.H1047R*	PIK3CA_p.H1047R_F PIK3CA_p.H1047R_R	GCAAGAGGCTTTGGAG-TATTTCATG GCTGTTTAATTGTGTGGAA-GATCCAA	PIK3CA_p. H1047R_V	VIC	CCACCATGATGTGCATC	PIK3CA_p. H1047R_M	FAM	CACCATGACGTGCATC
Exon 9**	PIK3CA_x9_M13F PIK3CA_x9_M13R	TGTAAAACGACGGCCAGT-GGGAAAA TGACAAAGAACAGC CAGGAAACAGCTAT-GACCTCCATTTT AGCACTTACCTGTGAC						
Exon 20**	PIK3CA_x20_M13F PIK3CA_x20_M13R	TGTAAAACGACGGC-CAGTCTGAGCA AGAGGCTTTGGAG CAGGAAACAGCTAT-GACCCCTATGC AATCGGTCTTTGC						
***	PR_M13F	TGTAAAACGACGGCCAGT						
***	PR_M13R	CAGGAAACAGCTATGACC						

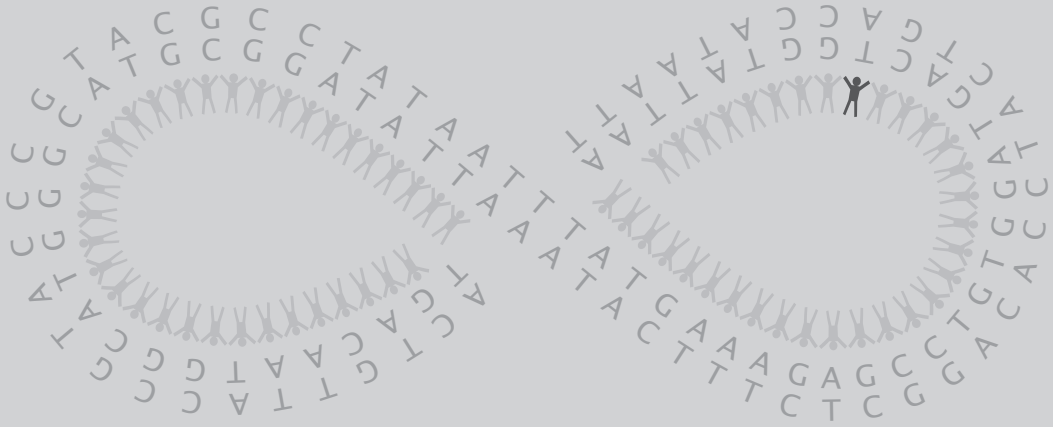
* Hydrolysis probes assays, ** Genomic PCR, *** Sanger sequencing, F=Forward primer, R=Reverse primer

Supplementary table 2: Relapse free period (Fine & Gray regression) and Relative survival, PIK3CA, stratified by HER-2

	N of recurrences	Recurrence risk at 5 years (%)	SHR	95% CI		*P*	SHR*	95% CI		*P*
				lower	upper			lower	upper	
PIK3CA in HER-2 negative patients										
negative	72	15%	1 (Reference)				1 (Reference)			
positive	30	16%	1.00	0.68	1.48	0.987	1.10	0.73	1.65	0.653
PIK3CA in HER-2 2+										
negative	15	16%	1 (Reference)				1 (Reference)			
positive	6	14%	0.86	0.38	1.97	0.737	0.92	0.35	2.44	0.863
PIK3CA in HER-2 3+										
negative	16	35%	1 (Reference)				1 (Reference)			
positive	6	35%	1.05	0.40	2.77	0.921	n/a**			

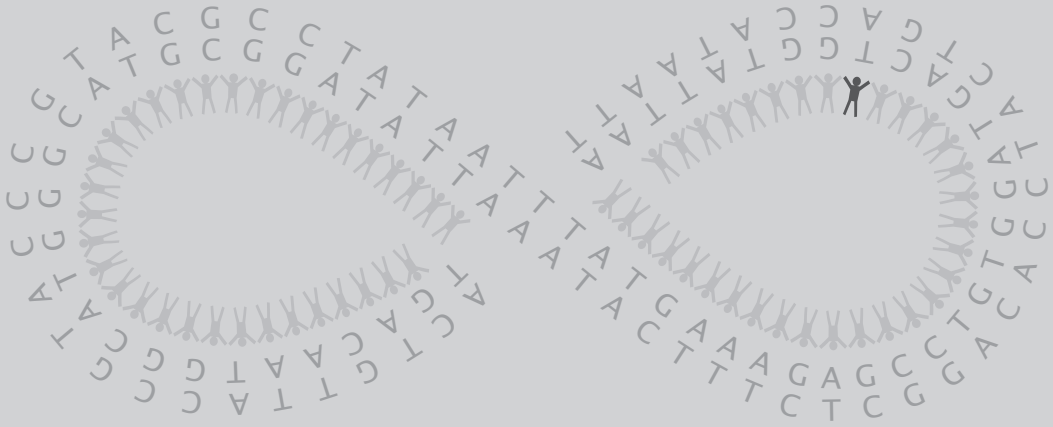
	N of observed deaths / N of expected deaths	Relative survival at 10 years (%)	RER	95% CI		*P*	RER*	95% CI		*P*
				lower	upper			lower	upper	
PIK3CA in HER-2 negative patients										
negative	261 / 198	84%	1 (Reference)				1 (Reference)			
positive	113 / 83	82%	0.89	0.38	2.09	0.795	1.14	0.56	2.32	0.710
PIK3CA in HER-2 2+										
negative	46 / 37	88%	1 (Reference)				1 (Reference)			
positive	24 / 17	81%	0.82	0.16	4.16	0.813	n/a**			
PIK3CA in HER-2 3+										
negative	31 / 13	67%	1 (Reference)				1 (Reference)			
positive	11 / 3	54%	1.46	0.55	3.85	0.443	n/a**			

*adjusted for age, morphology, grade, hormone receptor status, tumor stage, type of breast surgery, type of axillary surgery, radiotherapy, endocrine therapy, chemotherapy. **not applicable due to too few events



Part III

Aging in the breast cancer patient



Chapter 9

HIF-1α and its metabolic targets are highly expressed in breast tumors of patients of 65 years or older but not in patients younger than 65 years of age

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Article submitted

ABSTRACT

Introduction

HIF-1α is over-expressed in the majority of tumors, taking advantage of HIF-1 α responses including angiogenesis and glycolysis. Accumulating evidence indicates that HIF-1α levels increase during normal aging, a process that is also associated with increased cancer risk. In this study, we investigated the difference in mRNA expression of HIF-1α and its target genes in normal and breast tumor tissue of young and old patients. We hypothesized that HIF-1α plays a more important role in breast cancer development in older than in younger individuals.

Material and Methods

Frozen normal and breast tumor tissue of patients treated at the Leiden University Medical Center (n=35) in the Netherlands were used. Total RNA was extracted from these samples after which quantitative RT-PCR was performed for HIF-1α and its target genes. Expression differences between normal and breast tumor tissue were analyzed per primer, after which patients were stratified in young (<65 years (n=16)) and old (≥65 (n=19)).

Results

Significantly higher HIF-1α (p=0.0097) and associated metabolic, angiogenic (VEGFA: p=0.0311) and inflammatory (HMOX1: p=0.0006) target gene expression was seen in tumors compared to normal breast tissue. In the stratified analysis, the same result was seen for the patients ≥ 65 years but not in patients <65 years.

Conclusion

HIF-1α and its target genes are significantly up-regulated in the tumors of breast cancer patients older than 65 years and less so in patients younger than 65 years indicating that oncologic dysregulation of HIF-1α is more likely to occur in older patients, and that anti-HIF-1α therapy might be an effective therapy for breast cancer patients of a more advanced age.

INTRODUCTION

Hypoxia can occur as a result of a decline in tissue oxygen tension in normal tissues and in different diseases such as vascular and pulmonary disease [1]. In cancer, tumors become hypoxic because of a lack in adequate neovascularisation, often with poor vessel-wall quality. Although hypoxia is toxic for most cells, cancer cells can proliferate in these stressful conditions either by adapting genetically or epigenetically to turn on the hypoxic response pathways. These alterations contribute to the malignant phenotype and behavior of the tumor [1]. The major response to low tissue oxygen levels is mediated by up-regulation of the hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer made up of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. In the absence of oxygen, HIF-1 binds to hypoxia-response elements (HREs), which activates the expression of numerous hypoxia response genes [1]. Target genes of HIF-1 are involved in cell proliferation, angiogenesis (VEGFA, EPO), inflammation (HMOX-1), metabolism (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH), apoptosis, immortalization, and migration [1;2]. In the majority of human cancers, HIF-1α is over-expressed and the tumor cells take advantage of some of these responses, for example, angiogenesis induction and metabolic adaptation, and evade others, such as apoptosis [3]. In previous studies it was shown that HIF-1α overexpression in the tumor is associated with treatment failure and increased mortality [4-6]. Currently, the quest proceeds to develop more efficient anti-cancer strategies, which characterize the products of transcription factor activity essential for tumoregenesis [7]. Based on current knowledge, HIF-1α is nominated as a promising novel therapeutic candidate that fulfills these criteria. However, identification of the cancer patients who would benefit most of this novel therapeutic approach is highly warranted, as such aggressive therapy should not be given to patients who would have minimal benefit of its mode of action.

An important link between aging and cancer was recently demonstrated (Gomes *et al.*,). In old mice, HIF-1α was shown to be stabilized, even in healthy tissue as a consequence of cellular aging, a phenomenon they call "pseudophyopxia" [8].

Their results implied that, during aging, the decline in nuclear nicotinamide adenine dinucleotide (NAD^+) levels, leads to a reduction of Sirtuin 1 (SIRT1) activity in the nucleus, causing Von Hippel-Lindau (VHL) to decline and HIF-1α to be stabilized. This age-induced stabilization of HIF-1α, leads to a pseudohypoxic state that disrupts oxidative phosphorylation (OXPHOS), and promotes a Warburg-like state. The subsequent increase of reactive oxygen species (ROS) may establish an environment for subsequent mutations leading to carcinogenesis, which helps to explain why cancer risk increases exponentially as we age [8;9].

We hypothesize that HIF-1α and its related target genes will be highly expressed and involved in tumorigenesis in older breast cancer populations and less so in younger

counterparts. To test this, we have investigated the difference in expression of HIF-1α and its associated target genes in normal breast tissue and in breast tumor tissue of both young and old patients.

MATERIAL AND METHODS

Patients and tumors

For this study, frozen intra-operative breast tumor and normal breast tissue of patients treated at the Leiden University Medical Center (n=35) in the Netherlands were used. All patients were diagnosed and treated between 2006 and 2014. Twenty-one patients had normal breast tissue samples available and 14 patients had breast tumor tissue available. Thirteen patients had both tissue types available. For all patients the following data was retrieved from the central patient database at the Leiden University Medical Center: age at diagnosis, histological tumor grade (classified as Grade I, II or III) and tumor type (ductal, lobular or "other"), estrogen (ER) and progesterone receptor (PGR) status, human-epidermal growth factor receptor-2 (HER-2) status, pathological tumor and nodal stage, adjuvant treatment received, date of loco-regional/distant recurrence, and date and cause of death if relevant.

Gene expression and mtDNA analysis

Total RNA was extracted from the frozen samples using the miRNeasy extraction mini kit (QIAGEN) according to the supplier's instructions. RNA samples were quantified using the Nanodrop 1000spectrophotometer (Thermo Scientific). cDNA was synthesized with the iSCRIPT cDNA synthesis kit (BioRad) using 100ng of RNA. Quantitative RT-PCR reactions were performed using 1uM of primers and LightCycler® 480 SYBR Green Master (Roche) on a lightcycler® 480 detection system (Roche). Calculations were performed by a comparative method ($2^{-\Delta CT}$) using 18S as an internal control. HIF-1α target genes were chosen based on the most well-known changes occuring in aging and oncogenesis due to HIF-1α stabilization, namely, metabolic adaptation (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH), inflammation (HMOX-1), angiogenesis (VEGFA, EPO), and mitochondrial dysfunction (ATP-6, COX-1, CYTB and ND-1), and were designed using the IDT software. Primer sequences can be found in Table 1.

Statistics

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics), Microsoft Excel and Graphpad Prism 6. Hypotheses and analysis plan were drafted before the pathological data was available. Patients with missing RT-PCR data were excluded from statistical analyses as it can be assumed that

these data were "missing at random". The Mann-Whitney U test was used to evaluate the difference in mRNA expression of the specific primers between normal and tumor tissue for the whole cohort, and between normal and tumor tissue for the two age groups (<65 years and ≥65 years). This arbitrary age cut-off was chosen based on epidemiologic literature, in which the age of 65 years is usually considered a cut-off point to identify an elderly population [10]. First, we assessed the difference between primer specific RT-PCR mRNA expression in the two tissue types: normal breast and breast tumor tissue. Next, the same analyses were performed, however patients were now stratified in two age groups, namely, younger than 65 years of age (n=16) and 65 years or older (n=19).

The χ^2 test was used to evaluate associations between various clinico-pathological parameters and primer specific RT-PCR data of the breast tumor tissue.

Table 1: primer sequences used for RT-PCR

		Forward	Reverse
HKG	18S	GAGACTCTGGCATGCTAACTAG	GGACATCTAAGGGCATCACAG
MITO	ATP-6	ACACCCCTTATCCCCATACTAG	ATGGTTGATATTGCTAGGGTGG
MITO	COX-1	GCCATAACCCAATACCAAACG	TTGAGGTTGCGGTCTGTTAG
MITO	CYTB	CAATTATACCCTAGCCAACCCC	GGATAGTAATAGGGCAAGGACG
MITO	ND-1	TCAACCTCAAACTACGCCCTG	GTTGTGATAAGGGTGGAGAGG
HYPOXIA	HIF1α	CCGCTGGAGACACAATCATATC	ACTTCCTCAAGTTGCTGGTC
GLYCO	GLUT-1	TCTGGCATCAACGCTGTCTTC	CGATACCGGAGCCAATGGT
GLYCO	PFKL	GCTGGGCGGCACTATCATT	TCAGGTGCGAGTAGGTCCG
GLYCO	GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG
GLYCO	HK-2	GAGCCACCACTCACCCTACT	CCAGGCATTCGGCAATGTG
GLYCO	LDHA	AGATAAGGAACAGTGGAAAGA	CCAATAGCCCAGGATGTGTAG
GLYCO	PKM-1	ACCGCAAGCTGTTTGAAGAA	TCCATGAGGTCTGTGGAGTG
GLYCO	PKM-2	GAGGCCTCCTTCAAGTGCT	CCAGACTTGGTGAGGACGAT
GLYCO	PDK-1	GGCTGGTTTTGGTTATGGATTG	CTGGGAGTCTTTCTATTGAGTCTG
GLYCO	VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
GLYCO	EPO	TGTGGATAAAGCCGTCAGTG	GGAAGAGTTTGCGGAAAGTG
GLYCO	HMOX1	TCAGGCAGAGGGTGATAGAAG	TTGGTGTCATGGGTCAGC

Abbreviations: HKG: House Keeping Gene MITO: Mitochondrial gene GLYCO: Glycolysis related gene

RESULTS

Patient characteristics

The mean age of this cohort was 62 years (range: 27-91 years). From the 35 samples, 16 samples belonged to patients younger than 65 years and 19 samples were from patients equal to, or older than 65 years. Twenty-one samples (11 samples <65yrs, 10 samples

≥65yrs (mean-age: 59yrs)) were of normal breast histology and 14 samples (5 samples <65yrs, 9 samples ≥65yrs (mean-age: 67yrs)) of breast tumor histology.

Only for ER status a statistical difference was found between patients <65years and patients ≥65years. Conform current practice and observations, significantly more ER positive tumors were seen in the older population (82.4%) compared to the patients younger than 65 years of age (33.3%) (p=0.007, Table 2).

Table 2: tumor and patient characteristics per age group

		<65yrs	≥65yrs	
		n (%)	**n (%)**	***p*-value**
pT				
	1	2 (40.0)	4 (50.0)	0,91
	2	2 (40.0)	3 (37.5)	
	3	1 (20.0)	1 (12.5)	
pN				
	0	2 (40.0)	2 (28.6)	0,83
	1	2 (40.0)	3 (42.9)	
	2	1 (20.0)	1 (14.3)	
	3	0 (0.0)	1 (14.3)	
Tumor grade				
	1	0 (0.0)	0 (0.0)	0,73
	2	2 (40.0)	4 (50.0)	
	3	3 (60.0)	4 (50.0)	
Tumor morphology				
	Ductal	4 (80.0)	6 (66.7)	0,73
	Lobular	0 (0.0)	1 (11.1)	
	Other	1 (20.0)	2 (22.2)	
ER status				
	Negative	3 (60.0)	2 (25.0)	0,21
	Positive	2 (40.0)	6 (75.0)	
PR status				
	Negative	3 (60.0)	3 (37.5)	0,43
	Positive	2 (40.0)	5 (62.5)	

Abbreviations: pT: pathological tumor stage pN: pathological nodal stage ER: estrogen receptor PR: progesterone

Breast cancer vs. normal breast tissue mRNA expression

It was previously reported that cancer is associated with an increase in HIF-1α. Consistent with these reports, we, in this study, show an increase in the HIF-1α mRNA expression in the breast tumor, compared to the normal breast tissue (p=0.0097)(Figure 1). All

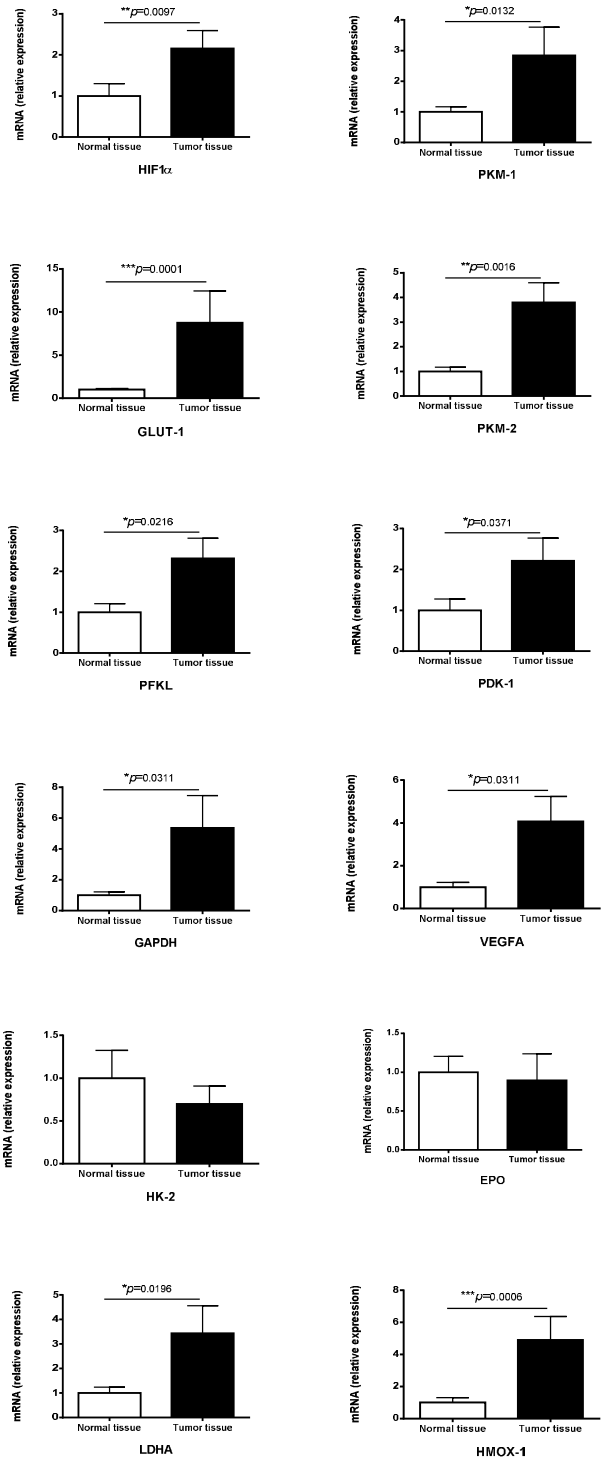

Figure 1:
Column A: Normal breast versus breast tumor mRNA expression per primer.

B

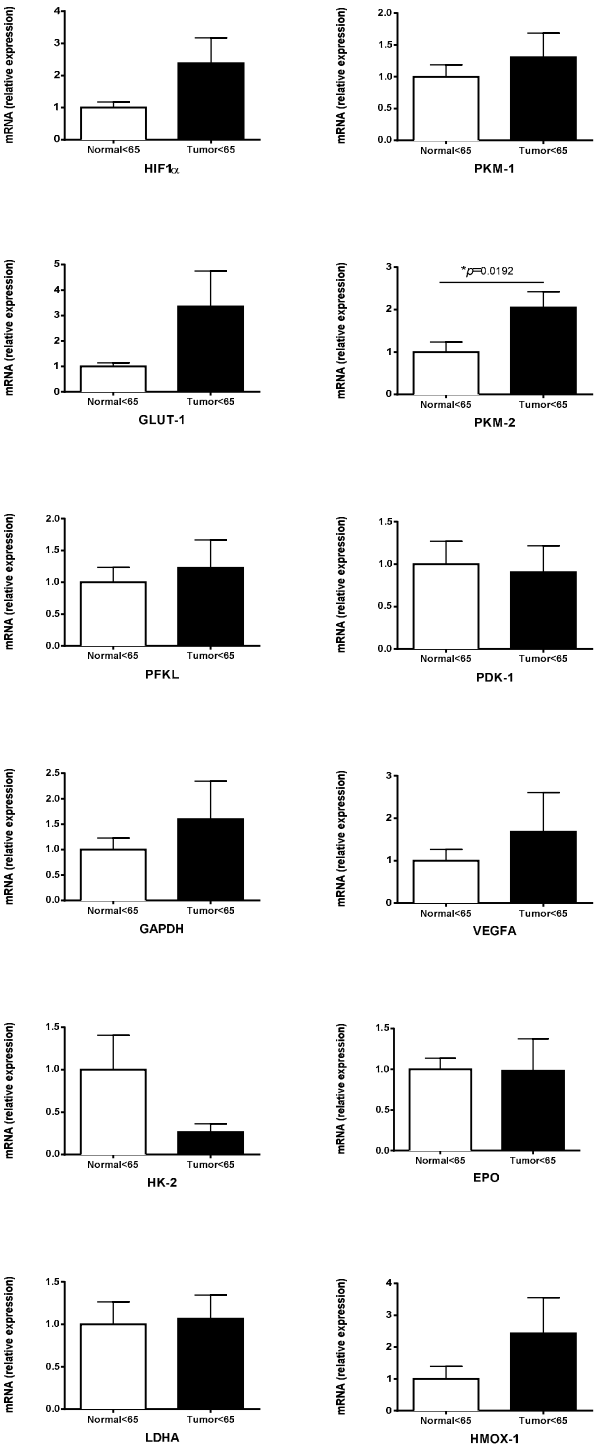

Figure 1:
Column B: Normal breast versus breast tumor mRNA expression per primer for patients younger than 65 years of age.

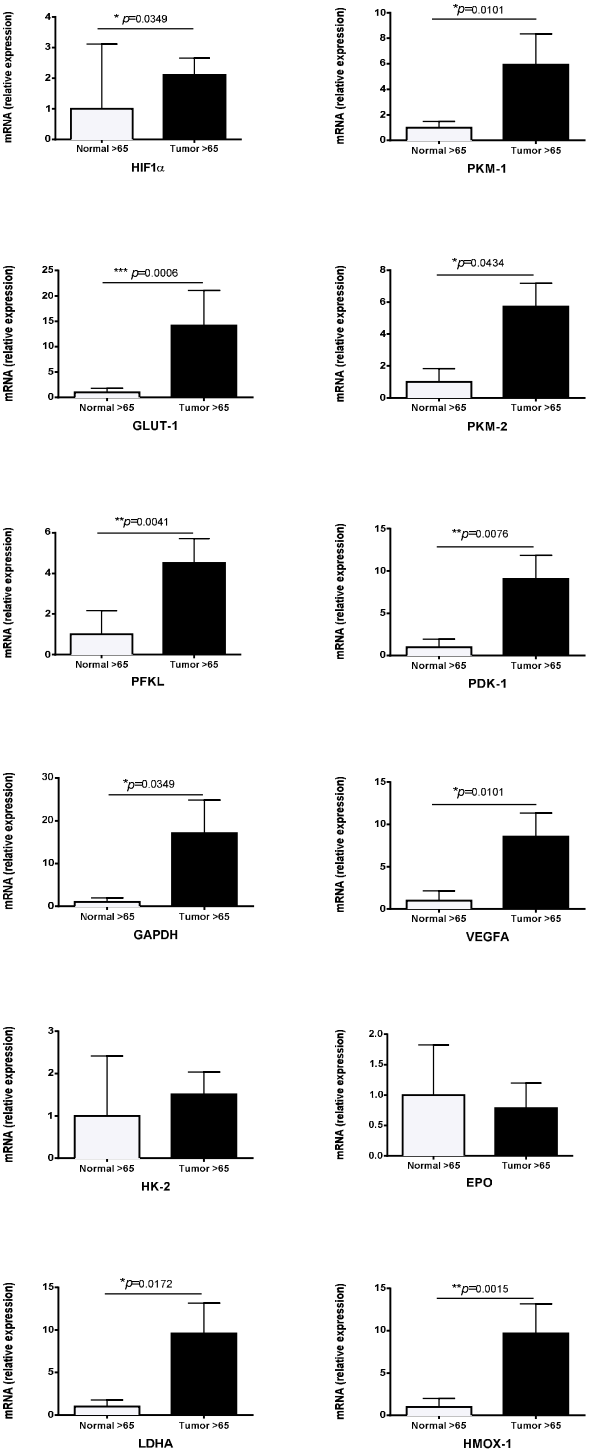

Figure 1:
Column C: Normal breast versus breast tumor mRNA expression per primer for patients ≥65 years of age.

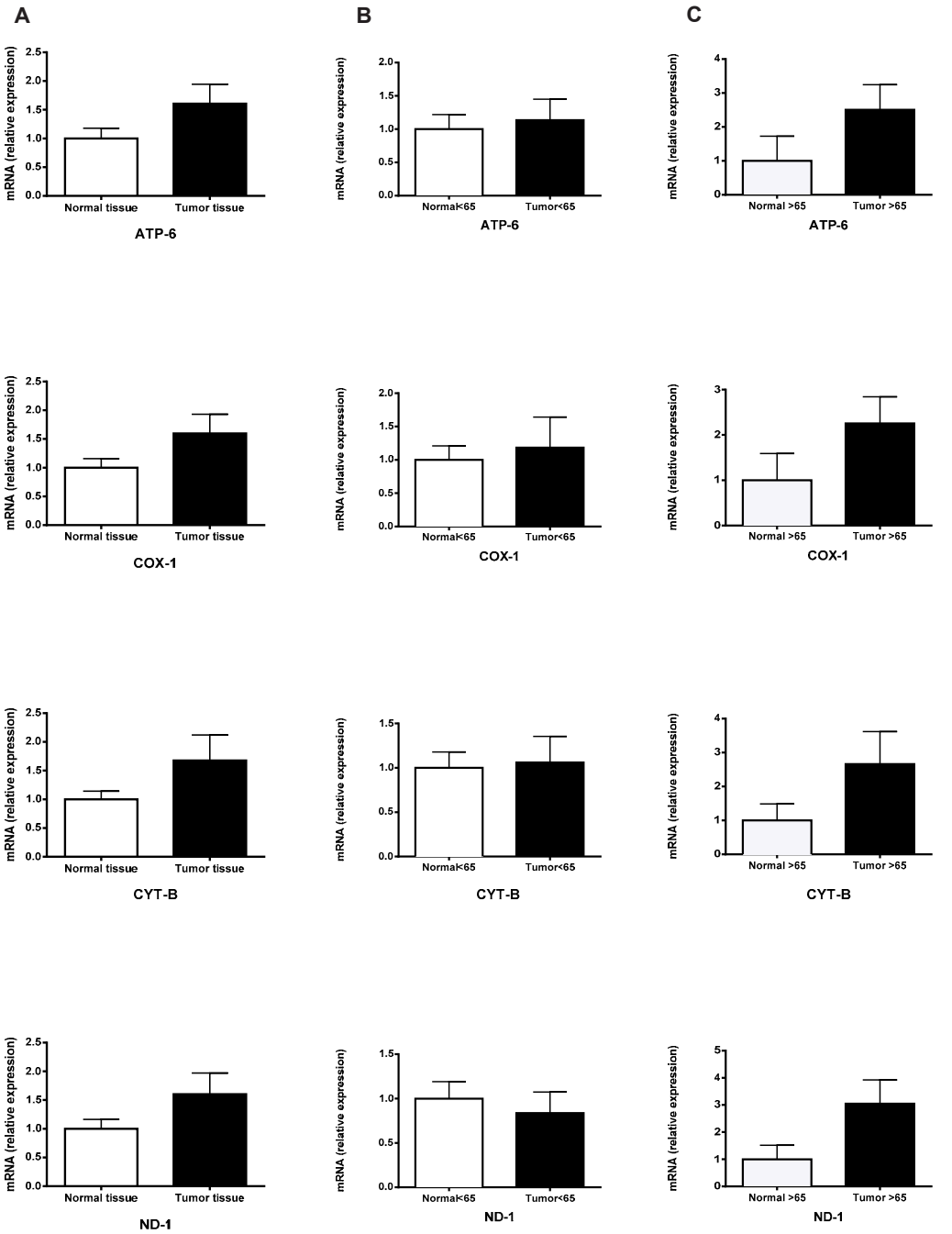

Figure 2:

Column A: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression.

Column B: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression for patients younger than 65 years of age.

Column C: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression for patients ≥65 years of age.

Table 3: Chi-square associations for patient and tumor characteristics and mRNA expression in the tumor tissue

		ATP6			COX1			CYTB			ND1		
		Low	High		Low	High		Low	High		Low	High	
		n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
Age													
	<65	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58
	≥65	4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)	
Number of comorbid diseases													
	0	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5
	1	2(28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
	2 or more	2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)	
Tumor stage													
	1	4 (66.7)	2 (28.6)	0,3	3 (50.0)	3 (42.9)	0,15	4 (66.7)	2 (28.6)	0,3	3 (50.0)	3 (42.9)	0,15
	2	1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)	
	3	1 (16.7)	1 (14.3)		2 (33.3)	0 (0.0)		1 (16.7)	1 (14.3)		2 (33.3)	0 (0.0)	
Tumor grade													
	1	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8
	2	3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)	
	3	3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)	
Tumor morphology													
	Ductal	4 (57.1)	6 (85.7)	0,11	5 (71.4)	5 (71.4)	0,51	4 (57.1)	6 (85.7)	0,11	5 (71.4)	5 (71.4)	0,51
	Lobular	0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)	
	Other	3 (42.9)	0 (0.0)		2 (28.6)	1 (14.3)		3 (42.9)	0 (0.0)		2 (28.6)	1 (14.3)	
ER status													
	Negative	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73
	Positive	3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)		3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)	
PR status													
	Negative	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39
	Positive	3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)	

HIF1α			GLUT1			GAPDH			HK2			LDHA		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
2 (28.6)	3 (42.9)	0,58	4 (57.1)	1 (14.3)	0,09	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58
5 (71.4)	4 (57.1)		3 (42.9)	6 (85.7)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)	
1 (14.3)	4 (57.1)	0,17	3 (42.9)	2 (28.6)	0,82	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5	3 (42.9)	2 (28.6)	0,82
4 (57.1)	1 (14,3)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)	
1 (16.7)	5 (71.4)	0,09	3 (50.0)	3 (42.9)	0,15	3 (50.0)	3 (42.9)	0,15	3 (50.0)	3 (42.9)	0,15	2 (33.3)	4 (57.1)	0,25
3 (50.0)	2 (28.6)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		2 (33.3)	3 (42.9)	
2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)	
0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39
3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)	
3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)	
4 (57.1)	6 (85.7)	0,42	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06
1 (14.3)	0 (0.0)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)	
2 (28.6)	1 (14.3)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)	
2 (33.3)	3 (42.9)	0,73	2 (33.3)	3 (42.9)	0,73	1 (16.7)	4 (57.1)	0,14	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73
4 (66.7)	4 (57.1)		4 (66.7)	4 (57.1)		5 (83.3)	3 (42.9)		3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)	
2 (33.3)	4 (57.1)	0,39	2 (33.3)	4 (57.1)	0,39	1 (16.7)	5 (71.4)	**0,05**	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39
4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		5 (83.3)	2 (28.6)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)	

PDK1			PFKL			PKM1			PKM2			VEGFA		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	4 (57.1)	1 (14.3)	0,09	3 (42.9)	2 (28.6)	0,58
4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		3 (42.9)	6 (85.7)		4 (57.1)	5 (71.4)	
1 (14.3)	4 (57.1)	0,22	3 (42.9)	2 (28.6)	0,22	3 (42.9)	2 (28.6)	0,22	3 (42.9)	2 (28.6)	0,82	3 (42.9)	2 (28.6)	0,82
3 (42.9)	2 (28.6)		1 (14.3)	4 (57.1)		1 (14.3)	4 (57.1)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
3 (42.9)	1 (14.3)		3 (42.9)	1 (14.3)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)	
4 (57.1)	2 (33.3)	0,11	4 (66.7)	2 (28.6)	**0,02**	4 (66.7)	2 (28.6)	**0,02**	2 (33.3)	4 (57.1)	0,25	3 (50.0)	3 (42.9)	0,15
1 (14.3)	4 (66.7)		0 (0.0)	5 (71.4)		0 (0.0)	5 (71.4)		2 (33.3)	3 (42.9)		1 (16.7)	4 (57.1)	
2 (28.6)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)	
0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,8
4 (57.1)	2 (33.3)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)		3 (50.0)	3 (42.9)	
3 (42.9)	4 (66.7)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)	
6 (85.7)	4 (57.1)	0,42	4 (57.1)	6 (85.7)	0,11	4 (57.1)	6 (85.7)	0,11	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06
0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)	
1 (14.3)	2 (28.6)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)	
2 (28.6)	3 (50.0)	0,43	2 (33.3)	3 (42.9)	0,73	2 (33.3)	3 (42.9)	0,73	3 (50.0)	2 (28.6)	0,43	1 (16.7)	4 (57.1)	0,14
5 (71.4)	3 (50.0)		4 (66.7)	4 (57.1)		4 (66.7)	4 (57.1)		3 (50.0)	5 (71.4)		5 (83.3)	3 (42.9)	
2 (28.6)	4 (66.7)	0,17	2 (33.3)	4 (57.1)	0,39	2 (33.3)	4 (57.1)	0,39	3 (50.0)	3 (42.9)	0,8	1 (16.7)	5 (71.4)	**0,05**
5 (71.4)	2 (33.3)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)		5 (83.3)	2 (28.6)	

HMOX1			EPO		
Low	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (42.9)	2 (28.6)	0,58	2 (33.3)	3 (37.5)	0,87
4 (57.1)	5 (71.4)		4 (66.7)	5 (62.5)	
1 (14.3)	4 (57.1)	0,22	2 (33.3)	3 (37.5)	0,94
3 (42.9)	2 (28.6)		2 (33.3)	3 (37.5)	
3 (42.9)	1 (14.3)		2 (33.3)	2 (25.0)	
3 (42.9)	3 (50.0)	0,34	3 (60.0)	3 (37.5)	0,45
2 (28.6)	3 (50.0)		2 (40.0)	3 (37.5)	
2 (28.6)	0 (0.0)		0 (0.0)	2 (25.0)	
0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,43
4 (57.1)	2 (33.3)		3 (60.0)	3 (37.5)	
3 (42.9)	4 (66.7)		2 (40.0)	5 (62.5)	
4 (57.1)	6 (85.7)	0,42	4 (66.7)	6 (75.0)	0,48
1 (14.3)	0 (0.0)		1 (16.7)	0 (0.0)	
2 (28.6)	1 (14.3)		1 (16.7)	2 (25.0)	
2 (28.6)	3 (50.0)	0,43	2 (40.0)	3 (37.5)	0,93
5 (71.4)	3 (50.0)		3 (60.0)	5 (62.5)	
2 (28.6)	4 (66.7)	0,17	2 (40.0)	4 (50.0)	0,73
5 (71.4)	2 (33.3)		3 (60.0)	4 (50.0)	

HIF-1α targets regulating glucose metabolism, except for HK-2, are upregulated in the breast tumor tissue, compared to the normal breast tissue (Figure 1). mRNA expression of *VEGFA* was significantly increased in the tumor tissue compared to the normal tissue (p=0.0311). This was also seen for *HMOX1* (p=0.0006). In contrary to the results presented in the aging study from Gomes *et al.*, in which it was shown that mitochondrially-encoded OXPHOS mRNAs (*ND1, Cytb, COX1 and ATP6*) were significantly lower in aged mice compared to their younger counterparts [8], our data did not show a significant difference between healthy and diseased tissue (Figure 2).

Breast cancer vs. normal breast tissue mRNA expression by age groups

When the cohort was stratified by age, defined as younger than 65 years or 65 years or older, our data showed that HIF-1α was only significantly higher expressed in patients ≥65 years (Figure 1). This was further supported by the significant increase of the HIF-1α targets regulating glucose metabolism (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH) in the patients ≥65 years, but not in patients <65 years, except for PKM-2, which showed a significant difference in both age groups (Figure 1). A significant increase was also only seen in the elderly patients for mRNA expression of angiogenesis inducing VEGFA and inflammation regulating HMOX-1.

Although no significance was seen for the comparison of tumor tissue mRNA expression for young and old breast cancer patients, a trend was seen for higher mRNA expression of HIF-1α and its related genes in the old (≥65 years) patients compared to the young (<65 years) (Table 3). We strongly believe that the lack of significance in this analysis can be attributed to the relatively small sample size.

DISCUSSION

Results of this study imply that, in elderly patients, HIF-1α and its targets are significantly up-regulated in breast cancer compared to normal breast tissue. This increase of HIF-1α and its metabolic and angiogenic targets was not seen comparing breast tumor tissue to normal breast tissue of younger patients, even though there was no significant difference in the pathological tumor stage, grade and tumor morphology of the two age groups. It should be noted that a same trend as the ≥ 65 year old cohort, was seen between normal and breast cancer tissue in the younger breast cancer patients, although it was non-significant. These results imply that HIF-1α and its targets certainly play role in tumor development of the younger breast cancer patients, but are less pronounced when compared to the patients above the age of 65 years. This finding indicated that this oncogenic mechanism may be less important in the young. Therefore, it could be postulated that the mode of carcinogenesis is dependent on different mechanisms in the young compared to older breast cancer patients.

The data also supports the possibility that cells from older patients may already be primed high HIF-1α expression due to the so-called age-induced HIF-1α stabilized pseudohypoxic state, as proposed by Gomes *et al.* [8]. This age-related HIF-1α-induced pseudohypoxic state is thought to be responsible for the shift in glucose metabolism primarily performed by the oxygen-dependent tricarboxylic acid (TCA) cycle to glycolysis, the oxygen independent metabolic pathway, also responsible for ATP production [1]. Tumor development, which is known for high HIF-1α expression within the tumor [11], in

an already HIF-1α primed environment, would lead to an additive increase of HIF-1α in the tumor, especially compared to the younger patients.

An emerging paradigm is that the decline in metabolic homeostasis during aging induces a pro-carcinogenic environment, and may be one of the main reasons for the increase in cancer incidence with age [8;9]. We believe that there is also a probability that the significantly higher expression of HIF-1α in the breast tumor compared to the normal tissue of the elderly breast cancer patients plays an important role in the more aggressive, and less therapy-sensitive character of breast cancer in the old, leading to unfavorable outcome in this breast cancer sub-group [12]. In all probability, the mechanism of this more aggressive character lies in the observed metabolic shift, allowing tumor cells to thrive in a low oxygen environment and stimulate angiogenesis [11]. This could also explain the observation of significantly higher VEGF expression in the tumor tissue compared to the normal breast tissue of the older patients, which was not seen in the younger patients.

The ability to survive under hypoxic conditions, characterized by HIF-1α overexpression, is one of the fundamental physiological differences between tumor and healthy cells. Therefore, targeting HIF-1α in the adjuvant setting of cancer treatment has gained substantial ground over the last few years. Particularly, in the older patients, whom are at increased risk of toxicity and adverse events, resulting in non-persistence of the current adjuvant standards, chemotherapy and hormonal therapy [13;14], the demand for targeted therapy increased. In addition, breast cancer mortality increases with age, which may be explained by both undertreatment and overtreatment [12]. New treatment strategies for this group of patients are therefore highly warranted, preferably with a low toxicity profile. Previous studies [15], and also the results of this current study, suggest that HIF-1α overexpression is closely related with increased tumor vascularization, by means of VEGF up-regulation and metabolic adaptation, which in turn is essential for tumor progression. However, only in patients above the age of 65 years the difference in HIF-1α and it's targets expression was significantly different, implying that the greatest effect of HIF-1α blockage will be observed in this sub-group of breast cancer patients. If hypoxia is indeed the reason for the aggressive tumor phenotypes seen in the elderly breast cancer population, blockage of HIF would result in a reduction of tumor growth, due to a disruption in the neovascularization and the metabolic reprogramming, which will subsequently lead to less disease specific mortality in this group of breast cancer patients. Currently, the quest for anti HIF-1α treatment is ongoing. Antisense HIF-1α, such as EZN-2968, by means of down-regulation of HIF-1α and its related genes, was the first molecule studied in hypoxia related gene therapy for cancer [16]. The same research group later reported that expression of the VHL gene suppresses tumor formation by

binding HIF-1α, responsible for stimulating tumor angiogenesis and glycolysis [17]. These results were later also confirmed by Ogura *et al.* [18]. Over the last few years, great improvements have been made on the level of RNA interference techniques, which showed to effectively suppress *in vitro* and *in vivo* growth of hepatobiliary tumors and metastasis, by down-regulating HIF-1α expression [19;20]. Nevertheless, validation of available small molecules in clinical trials to test pharmacological inhibition of the hypoxia-induced pathway is still eagerly awaited. In summary, this very promising novel pharmacologic approach to cancer therapy will, based on the expression profiles presented in this current study, be of special interest for the elderly breast cancer patients, as they present themselves as the breast cancer sub-population who could potentially optimally benefit from this specific pathway blockage.

This is the first study to assess the relationship between HIF-1α and its downstream genes in normal and the cancerous tissue of young and elderly patients with breast cancer. This study, however, also has its limitations. First, this study group did not contain enough events (death or relapse) to reliably report on the clinical significance of the expression of HIF-1α and its related markers in the two age groups. Furthermore, the overall patient count is small, resulting in a lack of power to show significant associations in the chi-square tests performed for the mRNA expression per marker for the different age groups in the breast tumor tissue. Even though the registered values in table 3 indicate high probability of mRNA expression difference between the two age groups.

In conclusion, this work shows that HIF-1α and its related genes are significantly up-regulated in the tumor tissue of breast cancer patients 65 years or older but not in patients younger than 65 years, consistent with HIF-1a playing a more critical role in the tumors of elderly patients and providing evidence for the geroncogenesis theory [9]. Although more work is necessary to verify our findings, these results indicate that small compounds that prevent HIF-1α stabilization or promote its degradation might be an effective therapy particularly for breast cancer patients of a more advanced age.

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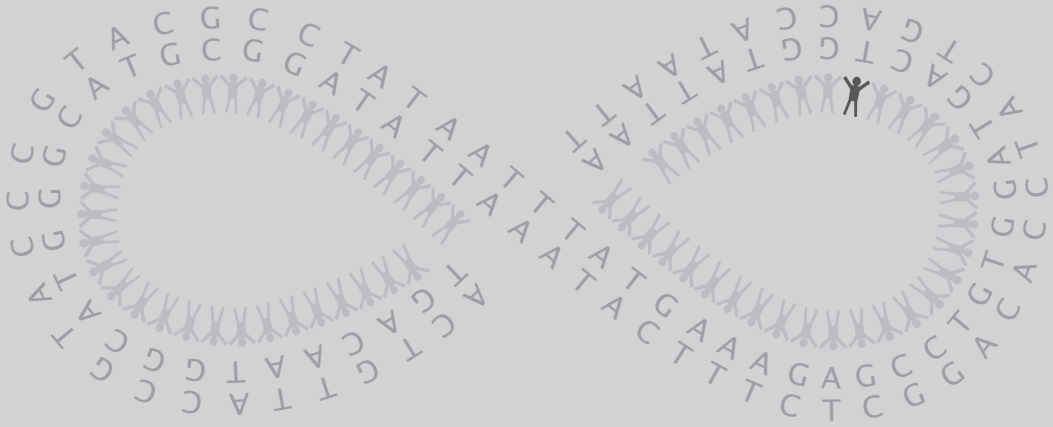

Chapter 10

HIF-1α and PKM2 are important drivers of age associated clinical functional decline and disease in the elderly breast cancer population: A FOCUS study analysis

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ABSTRACT

Introduction

HIF-1α is over-expressed in the majority of tumors. Evidence exists for HIF-1α accumulation during aging, a process that is also associated with higher cancer risk. In this study, we investigated the difference in expression of HIF-1α and its associated target genes in both normal and cancer tissue from middle-aged and old breast cancer patients. The aim of this study was to determine whether the level of expression is associated with patient characteristics associated with aging and outcome.

Material and Methods

120 patients, aged ≥ 65 years, with invasive, non-metastatic breast cancer with formalin fixed paraffin embedded tumor and normal breast samples available were included. On both tumor and normal tissue, total RNA was extracted and RT-PCR was performed for determination of HIF-1α and its associated target genes. Immunohistochemical stainings for HIF1-α and PKM2 were performed on both tumor and normal tissue. Based on the mean value, patients were stratified into two age groups: 65 to 80 years and ≥ 80 years. The difference in mRNA expression per primer between middle-aged and old patients per tissue type and associations with clinicopathological parameters were evaluated. Clinical endpoints examined were Overall Survival, Disease Free Survival, and Relapse Free Period.

Results

Higher mRNA expression of HIF1-α (p=0.017), GAPDH (p=0.003), PKM2 (p=0.069) and VEGFA (p=0.071 was seen in normal breast tissue of the older patients compared to the middle-aged. Upregulation of HIF1-α targets in normal breast tissue was significantly associated with different patient characteristics associated with clinical deterioration. Compared to normal breast tissue, tumor tissue of middle-aged patients showed a significant increase of HIF1-α (p=0.0011), GAPDH (p=0.0260) and TFAM (p=0.0171). This significant increase in the tumor tissue was also seen in patients older than 80 years for HIF1-α (p=0.0242) and TFAM (p=0.0041). High HIF1-α (HR1.65, 95%CI: 0.77-12.08, p=0.06) and PKM2 (HR1.69, 95CI: 0.95-3.03, p=0.08) mRNA expression in normal breast tissue showed a statistical trend for overall survival. High PKM2 (HR1.72, 95%CI:0.92-3.22, p=0.087) and VEGFA (HR2.07, 95%CI:1.01-4.14, p=0.039) mRNA expression in the breast tumor were associated with overall survival in univariate analyses, but lost their significance in the adjusted analyses.

Conclusion

This study supports the hypothesis that reversing or halting metabolic changes during aging possess the potential to benefit individuals as they reach an age where the chance of tumorigenesis increases exponentially. More research is needed to elucidate the potential contribution of age-related changes in HIF1-α and PKM2.

INTRODUCTION

Of all the factors that contribute to cancer, aging is the most potent but the reasons are still debated [1]. The most prominent explanation is the so-called multi-hit or Knudson hypothesis, which states that cancer occurs more frequently as we age because time is necessary for genetic mutations to accumulate and exceed a mutagenic threshold [2]. This hypothesis however fails to adequately explain why cancer risk is greatly reduced by calorie restriction and physical exercise [3]. Wu *et al.* proposed that a decline in metabolic homeostasis with age is a major contributor to increased cancer rate during aging [4]. In support of this hypothesis is the strong association between cancer and type 2 diabetes, obesity, and molecules that modulate energy utilization, such as metformin and resveratrol [3;5-9].

Under normal conditions healthy cells metabolize glucose by oxidative phosphorylation for efficient energy production whereas tumor cells preferentially metabolize glucose by aerobic glycolysis, also known as the Warburg effect. This produces less energy but facilitates rapid proliferation by enabling cells to incorporate metabolites from glycolysis [10;11].

Recently, evidence has emerged that the age related decline of metabolic homeostasis in healthy tissue is a driver of tumorigenesis. Gomes *et al.*, showed that in old mice a pseudohypoxic state causes Warburg-like metabolic reprogramming in normal tissue, resulting in disruption of mitochondrial homeostasis [12], a hallmark of aging [13]. Normally, in the absence of oxygen, the hypoxia-inducible factor HIF-1 binds to hypoxia-response elements (HREs), and activates the expression of numerous hypoxia-response genes [14]. Gomes *et al.* have suggested that this age-related decline in metabolic homeostasis induces a carcinogenic environment, partly due to an increase of reactive oxygen species (ROS), well known for its mutagenic potential, and thus might be an important reason for the high cancer incidence seen in the older population [4;12].

The metabolic shift away from oxidative phosphorylation to aerobic glycolysis is partly achieved and dependent on the glycolytic enzyme pyruvate kinase (PK) [10]. The existence of different PK isoforms (L, R, M1 and M2) reflects the importance of the last step of glycolysis to cope with the differential metabolic requirements of the cells [15]. The PKM1/M2 isoforms are generated through alternative splicing of two mutually exclusive exons by heterogenous nuclear ribonuclearprotein (hnRNP (hnRNPA1 and hnRNPA2)) and polypyrimidine tract binding protein (PTB) [10]. Normal cells express the pyruvate kinase M1 isoform (PKM1). As tumor cells shift away from oxidative phosphorylation (OXPHOS) toward anaerobic glycolysis, they predominantly express the M2 isoform (PKM2). The latter catalyzes the last step of glycolysis and reprograms the glycolytic flux to feed the special metabolic demands of proliferating cells [10]. It is for this reason that, over the last

decades, PKM2 has identified itself as a promising therapeutic target for cancer treatment, but could also potentially contribute to anti-aging interventions.

In this current study, we investigated the difference in expression of HIF-1α and its associated target genes, including PKM1 and 2, in both normal and cancer tissue from young and old breast cancer patients to determine whether their level of expression is associated with clinical characteristics associated with aging and outcome.

MATERIAL AND METHODS

Patients and tumors

For this study, 120 patients with invasive, non-metastatic breast cancer from the FOCUS cohort (Female breast cancer in the elderly, Optimizing Clinical guidelines USing clinic-pathological and molecular data) who received surgery and had formalin fixed paraffin embedded (FFPE) intra-operative tumor and normal breast samples available with successful determination of HIF-1α and its associated target genes were included. The FOCUS cohort has been described extensively in previous publications [16]. In brief, the cohort consists of all post-menopausal women, aged ≥ 65 years at time of diagnosis, with invasive and *in situ* breast cancer, diagnosed and treated between 1997 and 2004 in the South Western region of The Netherlands. Follow-up on survival status was available until the 1st of January 2013. All tissue samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Gene expression and mtDNA analysis

Total RNA from FFPE normal breast tissue and breast tumor tissue was extracted using the RNeasy FFPE kit (QIAGEN) according to the supplier's instructions. RNA samples were quantified using the Nanodrop 1000spectrophotometer (Thermo Scientific). cDNA was synthesized with the iSCRIPT cDNA synthesis kit (BioRad) using 200ng of RNA. Quantitative Real Time-PCR (RT-PCR) reactions were performed using 1uM of primers and LightCycler® 480 SYBR Green Master (Roche) on a lightcycler® 480 detection system (Roche). Calculations were performed by a comparative method ($2^{-\Delta CT}$) using Tubulin as an internal control. Primers for HIF-1α and its associated metabolic target genes, and mitochondrially and nuclear encoded OXPHOS genes were designed using the IDT software [14;17]. Primer sequences can be found in Table 1.

Immunohistochemistry for HIF1-α and PKM2

The immunohistochemical staining against HIF1-α was performed on tissue sections of intra-operatively derived FFPE tumor material and normal tissue of the FOCUS cohort.

Table 1: primer sequences used for RT-PCR

	Forward	Reverse
ATP-6	ACACCCCTTATCCCCATACTAG	AGTAATGTTAGCGGTTAGGCG
COX-1	CTTCGTCTGATCCGTCCTAATC	TTGAGGTTGCGGTCTGTTAG
CYTB	CCATACATTGGGACAGACCTAG	AGGGCAAGATGAAGTGAAAGG
ND1	CCTCTCCACCCTTATCACAAC	GTTGGTCTCTGCTAGTGTGG
GAPDH	AGAACGGGAAGCTTGTCATC	CATCGCCCCACTTGATTTTG
HIF1α	CCGCTGGAGACACAATCATATC	ACTTCCTCAAGTTGCTGGTC
HK-2	GAATTTGATGTGGCTGTGGATG	GTTACGGACAATCTCACCCAG
HMOX-1	TCAGGCAGAGGGTGATAGAAG	TTGGTGTCATGGGTCAGC
LDHA	AGATAAGGAACAGTGGAAAGAGG	CCAATAGCCCAGGATGTGTAG
PKM-1	ACCGCAAGCTGTTTGAAGAA	TCCATGAGGTCTGTGGAGTG
PKM-2	GAGGCCTCCTTCAAGTGCT	CCAGACTTGGTGAGGACGAT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
TFAM	CTACAGAACTAATTAGAAGAATTGCCC	CTCCGCCCTATAAGCATCTTG
COX-4	CCATGGATGAGAAAGTCGAGT	CCACAACCGTCTTCCACTC
UQCRC	TCCGAGCAGTCCTCTCAG	TCTCAGTCTCAAAACGGCTG
18S	GAGACTCTGGCATGCTAACTAG	GGACATCTAAGGGCATCACAG
TUBULIN	GGCCAGATCTTTAGACCAGAC	CCTTCCGTACCACATCCAG
ACTIN-B	GCACTCTTCCAGCCTTCC	TGTCCACGTCACACTTCATG
RPL19	ATGCCAGAGAAGGTCACATG	ACACATTCCCCTTCACCTTC

The tissue sections were deparaffinized and antigen retrieval was performed at 100 °C for 15 minutes using 0.1M Citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with hydrogen peroxidase 0.3% in PBS for 20 minutes. Sections were incubated at room temperature with monoclonal mouse- anti-human HIF1-α (Abcam, USA (ab8366); 1:1500, diluted in 1% PBSA) overnight. Consecutively, all slides were washed in PBS and incubated with Envision anti-mouse (DAKO, Denmark, Cytomation K4000) for 20 minutes at room temperature. DAB was used for visualization of positively stained breast tissue on the slides and counterstained with haematoxylin, dehydrated and finally mounted with pertex. All slides were stained simultaneously to avoid inter-assay variation. Known highly HIF1-α positive breast tumor tissue served as positive- and a negative-control, the latter was obtained by omitting the primary antibody. Fixed sections were also stained for PKM2 protein expression (Cell Signaling Technology #4053).

Evaluation of immunostaining

Microscopic quantification of HIF1-α-positive breast and/or tumor cells for was performed by two independent, blinded observers (C.E. and T.B.). HIF1-α was scored in the nucleus and cytoplasm, on intensity and percentage, separately. Intensity scores were

as follows: 0 for no staining at all; 1+ for a faint/barely perceptible staining; 2+ for weak to moderate staining; and 3+ for strong staining. Percentage scores were categorized in: 0 for no staining; 1+ for 1-25% of the breast cells/tumor cells stained; 2+ for 26-50% stained and 3+ for ≥50% of the cells stained. For all patients, the product of the intensity score and the percentage score was calculated. Ultimately, the calculated scores for the nucleus and the cytoplasm were summed up for normal and the tumor tissue separately, and dichotomized into low and high expression (<8 and ≥8 for tumor and <6 and ≥6 for the normal tissue) of HIF1-α, based on mean expression scores. PKM2 staining intensity was quantified by two independent observers (S.D. and D.d.V) and dichotomized in to low vs. high expression.

Statistics

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics), Microsoft Excel and Graphpad Prism 6. Patients with missing data were excluded from statistical analyses as it can be assumed that these data were "missing at random". Patients were stratified into two age-groups: 65 to 80 years and ≥ 80 years of age, based on the mean age of the population (mean: 79yrs, range: 65-97yrs). The Mann-Whitney U test was used to evaluate the difference in mRNA expression of the specific primers between young and old patients for the normal and tumor tissue. The χ^2 test was used to evaluate associations between various clinico-pathological parameters and primer specific RT-PCR (dichotomized based on the median value) and immunohistochemical data for the breast tumor and normal breast tissue. The clinical endpoints examined were Overall Survival (OS), defined as the time from date of operation until death by any reason; Disease Free Survival (DFS), defined as date of operation until locoregional recurrence, distant recurrence or breast cancer death (whichever came first), and Relapse Free Period (RFP), defined as date of operation until an event (locoregional recurrence and/or distant recurrence, whichever came first). The Kaplan–Meier method was used to compose survival plots, and the log-rank test was performed for comparison of OS, DFS and RFP curves. Cox Proportional Hazard analyses were used to calculate corresponding Hazard Ratio's (HRs), using univariate analyses. These analyses were additionally adjusted for clinically relevant confounders (normal tissue: number of comorbidities, polypharmacy, dementia, TNM classification and age; for the tumor tissue: latter, plus tumor grade and hormone receptor (HR) status).

RESULTS

Patient and tumor characteristics

One hundred and twenty patients were randomly selected from the original FOCUS cohort (N=3.672). Patients with in situ or metastatic disease, and patients who did not receive breast surgery were excluded. Patient, tumor and treatment characteristics are shown in Table 2. Mean age at diagnosis was 79 years (standard deviation 8.4 years). The majority of patients presented with early stage breast cancer (stage I 30.1%, stage II 51.3%, stage III 15.0%) of ductal morphology (74.1%). No significant associations were seen for mRNA expression in the breast tumor of HIF1-α and its target genes in relation with classic patient and tumor characteristics (Table 3). mRNA expression of these markers in the normal breast tissue showed significant association with age for HIF1-α (p=0.017), GAPDH (p=0.003) and a statistical trend was seen for PKM2 (p=0.069) and VEGFA (p=0.071), all showing higher mRNA expression in the older patients compared to the patients younger than 80 years of age (Table 4). Residing in a nursing home showed a statistical trend with high TFAM mRNA expression (p=0.082) (Table 4). Furthermore, PKM2 (p=0.04), LDHA (p=0.023), COX4 (p=0.035) and UQCRC (p=0.08), all showed significant association with polypharmacy (Table 4). A trend was seen for difficulty with walking and high PKM2 mRNA expression (p=0.066)(Table 4).

High mRNA HIF1-α expression in the normal tissue was significantly associated with the tumor grade of the patient (p=0.045), showing a tendency for higher tumor grades when HIF1-α expression was high in the healthy tissue (Table 4). Lastly, LDHA (p=0.04) and HIF1-α (p=0.024) mRNA expression in the normal tissue were significantly associated with more hormone receptor negative breast tumors (Table 4).

mRNA expression per tissue type

In the normal breast tissue, mRNA expression was significantly higher in the older (≥80 years) compared to the younger patients (65-80 years) for HIF1-α (p=0.0034), GAPDH (p=0.0013), PKM2 (p=0.0135) and VEGFA (p=0.0186) (Figure 1A). Except for a statistical trend for CytB (p=0.0511), we did not observe a significant difference in mitochondrially encoded OXPHOS mRNAs in the normal tissue of the two age groups (Figure 1B). Results showed a non-significant increase in the nuclear encoded OXPHOS mRNAs (COX4 and UQCRC) of the older patients compared to the young.

In the breast tumor tissue no significant difference was seen for the mRNA expression of HIF1-α or any of its targets (Figure 1A). Despite no significant association, we did observe lower expression of the mitochondrially encoded OXPHOS mRNAs (ATP6, COX1 and ND1), with, as is also seen in the healthy breast tissue, an increase of the nuclear encoded OXPHOS mRNAs in the older patients (Figure 1B).

Table 2: Patient and tumor characteristics

	All patients (N=120)	
	N	**%**
Age in years (mean, SD)	79.0 (8.3)	
Number of comorbidities		
0	33	28.4
1	19	16.4
2 or more	64	55.2
Nursing home resident		
No	98	84.5
Yes	18	15.5
Polypharmacy		
No	97	83.6
Yes	19	16.4
Difficulty Walking		
No	97	83.6
Yes	19	16.4
Dementia/Alzheimer		
No	111	95.7
Yes	5	4.3
Tumor stage		
I	34	30.1
II	58	51.3
III	17	15.0
Missing	4	3.5
Tumor grade		
1	20	17.2
2	30	25.9
3	23	19.8
Missing	43	37.1
Tumor morphology		
Ductal	86	74.1
Lobular	16	13.8
Other	14	12.1
HR status		
Negative	26	22.4
Positive	76	65.5
Unknown	14	12.1

Table 3: mRNA expression in tumor tissue and patient and tumor characteristics

	HMOX1			ATP6			COX1			CYTB		
	Low	High		Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
Age												
<80	10 (43.5)	12 (54.5)	0.46	17 (53.1)	14 (43.8)	0.45	15 (48.4)	15 (46.9)	0.90	17 (54.8)	13 (41.9)	0.31
>=80	13 (56.5)	10 (45.5)		15 (46.9)	18 (56.2)		16 (51.6)	17 (53.1)		14 (45.2)	18 (58.1)	
Tumor stage												
1	4 (18.2)	8 (38.1)	0.27	8 (26.7)	11 (34.4)	0.45	7 (24.1)	11 (34.4)	0.80	8 (27.6)	11 (35.5)	0.44
2	15 (68.2)	11 (52.4)		18 (60.0)	16 (50.0)		18 (62.1)	16 (50.0)		17 (58.6)	15 (48.4)	
3	3 (13.6)	1 (4.8)		4 (13.3)	3 (9.4)		3 (10.3)	4 (12.5)		4 (13.8)	3 (9.7)	
Missing	0 (0.0)	1 (4.8)		0 (0.0)	2 (6.2)		1 (3.4)	1 (3.1)		0 (0.0)	2 (6.5)	
Tumor grade												
1	4 (17.4)	3 (13.6)	0.96	10 (31.2)	2 (6.2)	0.06	9 (29.0)	3 (9.4)	0.09	8 (25.8)	4 (12.9)	0.06
2	6 (26.1)	6 (27.3)		8 (25.0)	8 (25.0)		9 (29.0)	7 (21.9)		11 (35.5)	5 (16.1)	
3	4 (17.4)	5 (22.7)		4 (12.5)	8 (25.0)		3 (9.7)	9 (28.1)		3 (9.7)	9 (29.0)	
Missing	9 (39.1)	8 (36.4)		10 (31.2)	14 (43.8)		10 (32.3)	13 (40.6)		9 (29.0)	13 (41.9)	
Tumor morphology												
Ductal	15 (65.2)	17 (77.3)	0.11	27 (84.4)	22 (68.8)	0.32	25 (80.6)	23 (71.9)	0.33	25 (80.6)	22 (71.0)	0.48
Lobular	6 (26.1)	1 (4.5)		3 (9.4)	5 (15.6)		2 (6.5)	6 (18.8)		4 (12.9)	4 (12.9)	
Other	2 (8.7)	4 (18.2)		2 (6.2)	5 (15.6)		4 (12.9)	3 (9.4)		2 (6.5)	5 (16.1)	
ER/PR status												
Negative	7 (30.4)	4 (18.2)	0.40	5 (15.6)	9 (28.1)	0.43	4 (12.9)	9 (28.1)	0.09	4 (12.9)	9 (29.0)	0.27
Positive	16 (69.6)	17 (77.3)		25 (78.1)	22 (68.8)		24 (77.4)	23 (71.9)		25 (80.6)	21 (67.7)	
Unknown	0 (0.0)	1 (4.5)		2 (6.2)	1 (3.1)		3 (9.7)	0 (0.0)		2 (6.5)	1 (3.2)	

p-values calculated with Chi-square test

Abbreviations: ER: estrogen receptor PR: progesterone receptor

ND1			GAPDH			LDHA			PKM1		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
16 (50.0)	14 (45.2)	0.70	13 (41.9)	17 (53.1)	0.37	11 (40.7)	13 (50.0)	0.50	12 (50.0)	9 (37.5)	0.38
16 (50.0)	17 (54.8)		18 (58.1)	15 (46.9)		16 (59.3)	13 (50.0)		12 (50.0)	15 (62.5)	
9 (30.0)	10 (32.3)	0.34	7 (24.1)	11 (34.4)	0.54	7 (26.9)	7 (28.0)	0.76	4 (17.4)	6 (26.1)	0.40
16 (53.3)	17 (54.8)		16 (55.2)	18 (56.2)		15 (57.7)	14 (56.0)		16 (69.6)	12 (52.2)	
5 (16.7)	2 (6.5)		5 (17.2)	2 (6.2)		4 (15.4)	3 (12.0)		3 (13.0)	3 (13.0)	
0 (0.0)	2 (6.5)		1 (3.4)	1 (3.1)		0 (0.0)	1 (4.0)		0 (0.0)	2 (8.7)	
9 (28.1)	3 (9.7)	0.19	8 (25.8)	4 (12.5)	0.33	4 (14.8)	5 (19.2)	0.61	6 (25.0)	2 (8.3)	0.058
9 (28.1)	7 (22.6)		6 (19.4)	9 (28.1)		8 (29.6)	5 (19.2)		5 (20.8)	8 (33.3)	
4 (12.5)	7 (22.6)		4 (12.9)	8 (25.0)		4 (14.8)	7 (26.9)		7 (29.2)	2 (8.3)	
10 (31.2)	14 (45.2)		13 (41.9)	11 (34.4)		11 (40.7)	9 (34.6)		6 (25.0)	12 (50.0)	
26 (81.2)	22 (71.0)	0.62	23 (74.2)	25 (78.1)	0.90	19 (70.4)	19 (73.1)	0.20	19 (79.2)	17 (70.8)	0.36
3 (9.4)	5 (16.1)		4 (12.9)	4 (12.5)		6 (22.2)	2 (7.7)		4 (16.7)	3 (12.5)	
3 (9.4)	4 (12.9)		4 (12.9)	3 (9.4)		2 (7.4)	5 (19.2)		1 (4.2)	4 (16.7)	
4 (12.5)	10 (32.3)	0.16	5 (16.1)	9 (28.1)	0.46	5 (18.5)	8 (30.8)	0.31	4 (16.7)	6 (25.0)	0.24
26 (81.2)	20 (64.5)		24 (77.4)	22 (68.8)		22 (81.5)	17 (65.4)		20 (83.3)	16 (66.7)	
2 (6.2)	1 (3.2)		2 (6.5)	1 (3.1)		0 (0.0)	1 (3.8)		0 (0.0)	2 (8.3)	

PKM2			VEGFA			HIF1A			TFAM		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
16 (53.3)	12 (40.0)	0.30	16 (59.3)	10 (37.0)	0.10	16 (51.6)	15 (45.5)	0.62	13 (48.1)	12 (42.9)	0.69
14 (46.7)	18 (60.0)		11 (40.7)	17 (63.0)		15 (48.8)	18 (54.5)		14 (51.9)	16 (57.1)	
8 (28.6)	7 (23.3)	0.50	10 (37.0)	6 (23.1)	0.45	8 (26.7)	11 (34.4)	0.45	8 (30.8)	9 (33.3)	0.97
14 (50.0)	20 (66.7)		15 (55.6)	15 (57.7)		18 (60.0)	16 (50.0)		14 (53.8)	15 (55.6)	
5 (17.9)	2 (6.7)		2 (7.4)	4 (15.4)		4 (13.3)	3 (9.4)		3 (11.5)	2 (7.4)	
1 (3.6)	1 (3.3)		0 (0.0)	1 (3.8)		0 (0.0)	2 (6.2)		1 (3.8)	1 (3.7)	
8 (26.7)	4 (13.3)	0.27	6 (22.2)	4 (14.8)	0.40	8 (25.8)	4 (12.1)	0.11	6 (22.2)	3 (10.7)	0.41
6 (20.0)	9 (30.0)		9 (33.3)	5 (18.5)		9 (29.0)	7 (21.2)		8 (29.6)	6 (21.4)	
3 (10.0)	7 (23.3)		5 (18.5)	6 (22.2)		7 (22.6)	5 (15.2)		5 (18.5)	5 (17.9)	
13 (43.3)	10 (33.3)		7 (25.9)	12 (44.4)		7 (22.6)	17 (51.5)		8 (29.6)	14 (50.0)	
23 (76.7)	22 (73.3)	0.92	23 (85.2)	19 (70.4)	0.31	25 (80.6)	24 (72.7)	0.13	22 (81.5)	20 (71.4)	0.51
4 (13.3)	4 (13.3)		3 (11.1)	4 (14.8)		5 (16.1)	3 (9.1)		3 (11.1)	3 (10.7)	
3 (10.0)	4 (13.3)		1 (3.7)	4 (14.8)		1 (3.2)	6 (18.2)		2 (7.4)	5 (17.9)	
3 (10.0)	10 (33.3)	0.09	5 (18.5)	9 (33.3)	0.46	5 (16.1)	9 (27.3)	0.45	2 (7.4)	9 (32.1)	0.07
25 (83.3)	19 (63.3)		21 (77.8)	17 (63.0)		25 (80.6)	22 (66.7)		24 (88.9)	18 (64.3)	
2 (6.7)	1 (3.3)		1 (3.7)	1 (3.7)		1 (3.2)	2 (6.1)		1 (3.7)	1 (3.6)	

COX4			UQCRC		
Low	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (75.0)	1 (20.0)	0.09	11 (55.0)	9 (42.9)	0.44
1 (25.0)	4 (80.0)		9 (45.0)	12 (57.1)	
1 (25.0)	1 (20.0)	0.89	6 (30.0)	4 (19.0)	0.59
2 (50.0)	2 (40.0)		11 (55.0)	14 (66.7)	
1 (25.0)	2 (40.0)		3 (15.0)	2 (9.5)	
0 (0.0)	0 (0.0)		0 (0.0)	1 (4.8)	
1 (25.0)	1 (20.0)	0.54	5 (25.0)	3 (14.3)	0.17
0 (0.0)	0 (0.0)		6 (30.0)	4 (19.0)	
1 (25.0)	3 (60.0)		6 (30.0)	4 (19.0)	
2 (50.0)	1 (20.0)		3 (15.0)	10 (47.6)	
3 (75.0)	3 (60.0)	0.638	18 (90.0)	10 (47.6)	**0.01**
1 (25.0)	1 (20.0)		1 (5.0)	6 (28.6)	
0 (0.0)	1 (20.0)		1 (5.0)	5 (23.8)	
0 (0.0)	1 (20.0)	0.34	3 (15.0)	9 (42.9)	0.10
4 (100.0)	4 (80.0)		16 (80.0)	12 (57.1)	
0 (0.0)	0 (0.0)		1 (5.0)	0 (0.0)	

Table 4: mRNA expression in normal breast tissue and patient and tumor characteristics

	HMOX1			VEGFA			PKM2		
	Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
Age									
<80	10 (43.5)	12 (54.5)	0.46	25 (62.5)	16 (42.1)	0.07	28 (63.6)	20 (44.4)	0.07
>=80	13 (56.5)	10 (45.5)		15 (37.5)	22 (57.9)		16 (36.4)	25 (55.6)	
Number of comorbid diseases									
0	6 (26.1)	8 (36.4)	0.59	12 (30.0)	7 (18.4)	0.13	10 (22.7)	14 (31.1)	0.40
1	3 (13.0)	4 (18.2)		4 (10.0)	10 (26.3)		9 (20.5)	5 (11.1)	
2 or more	14 (60.9)	10 (45.5)		24 (60.0)	21 (55.3)		25 (56.8)	26 (57.8)	
Nursing home resident									
No	20 (76.9)	21 (80.8)	0.73	34 (85.0)	33 (86.8)	0.82	41 (93.2)	38 (84.4)	0.19
Yes	6 (23.1)	5 (19.2)		6 (15.0)	5 (13.2)		3 (6.8)	7 (15.6)	
Polypharmacy									
No	22 (84.6)	20 (76.9)	0.48	34 (85.0)	31 (81.6)	0.69	41 (93.2)	35 (77.8)	**0.04**
Yes	4 (15.4)	6 (23.1)		6 (15.0)	7 (18.4)		3 (6.8)	10 (22.2)	
Difficulty walking									
No	21 (80.8)	19 (73.1)	0.51	33 (82.5)	30 (78.9)	0.69	39 (88.6)	33 (73.3)	0.07
Yes	5 (19.2)	7 (26.9)		7 (17.5)	8 (21.1)		5 (11.4)	12 (26.7)	
Dementia / Alzheimer									
No	23 (88.5)	25 (96.2)	0.30	36 (90.0)	37 (97.4)	0.18	43 (97.7)	41 (91.1)	0.18
Yes	3 (11.5)	1 (3.8)		4 (10.0)	1 (2.6)		1 (2.3)	4 (8.9)	
Tumor stage									
1	6 (25.0)	9 (34.6)	0.45	13 (32.5)	7 (18.4)	0.46	14 (32.6)	12 (27.3)	0.90
2	14 (53.8)	13 (50.0)		19 (47.5)	21 (55.3)		22 (51.2)	26 (59.1)	

Table 4: (continued)

	HMOX1			VEGFA			PKM2		
	Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3	4 (15.4)	4 (15.4)		5 (12.5)	8 (21.1)		6 (14.0)	5 (11.4)	
Missing	2 (7.7)	0 (0.0)		2 (5.0)	2 (5.3)		1 (2.3)	1 (2.3)	
Tumor grade									
1	7 (26.9)	4 (15.4)	0.76	6 (15.0)	9 (23.7)	0.79	9 (20.5)	6 (13.3)	0.44
2	7 (26.9)	8 (30.8)		9 (22.5)	8 (21.1)		14 (31.8)	10 (22.2)	
3	6 (23.1)	6 (23.1)		7 (17.5)	5 (13.2)		6 (13.6)	10 (22.2)	
Missing	6 (23.1)	8 (30.8)		18 (45.0)	16 (42.1)		15 (34.1)	19 (42.2)	
Tumor morphology									
Ductal	18 (69.2)	19 (73.1)	0.67	27 (67.5)	29 (76.3)	0.69	36 (81.8)	30 (66.7)	0.26
Lobular	4 (15.4)	5 (19.2)		7 (17.5)	5 (13.2)		5 (11.4)	9 (20.0)	
Other	4 (15.4)	2 (7.7)		6 (15.0)	4 (10.5)		3 (6.8)	6 (13.3)	
ER/PR status									
Negative	7 (26.9)	6 (23.1)	0.17	7 (17.5)	9 (23.7)	0.68	10 (22.7)	9 (20.0)	0.53
Positive	16 (61.5)	20 (76.9)		28 (70.0)	26 (68.4)		27 (61.4)	32 (71.1)	
Unknown	3 (11.5)	0 (0.0)		5 (12.5)	3 (7.9)		7 (15.9)	4 (8.9)	

p-values calculated with Chi-square test

Abbreviations: ER: estrogen receptor PR: progesterone receptor

PKM1			LDHA			ND1			CYTB		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
20 (57.1)	14 (40.0)	0.15	24 (61.5)	17 (43.6)	0.11	26(52.0)	26 (52.0)	1.0	31 (62.0)	22 (44.0)	0.07
15 (42.9)	21 (60.0)		15 (38.5)	22 (56.4)		24 (48.0)	24 (48.0)		19 (38.0)	28 (56.0)	
6 (17.1)	11 (31.4)	0.24	13 (33.3)	10 (25.6)	0.69	15 (30.0)	14 (28.0)	0.25	11 (22.0)	18 (36.0)	0.30
8 (22.9)	4 (11.4)		5 (12.8)	7 (17.9)		5 (10.0)	11 (22.0)		9 (18.0)	7 (14.0)	
21 (60.0)	20 (57.1)		21 (53.8)	22 (56.4)		30 (60.0)	25 (50.0)		30 (60.0)	25 (50.0)	
31 (88.6)	29 (82.9)	0.50	34 (87.2)	32 (82.1)	0.53	44 (88.0)	42 (84.0)	0.56	44 (88.0)	42 (84.0)	0.56
4 (11.4)	6 (17.1)		5 (12.8)	7 (17.9)		6 (12.0)	8 (16.0)		6 (12.0)	8 (16.0)	
30 (85.7)	28 (80.0)	0.53	37 (94.9)	30 (76.9)	**0.02**	41 (82.0)	43 (86.0)	0.59	42 (84.0)	43 (86.0)	0.78
5 (14.3)	7 (20.0)		2 (5.1)	9 (23.1)		9 (18.0)	7 (14.0)		8 (16.0)	7 (14.0)	
25 (71.4)	30 (85.7)	0.15	31 (79.5)	31 (79.5)	1.0	42 (84.0)	40 (80.0)	0.60	41 (82.0)	40 (80.0)	0.80
10 (28.6)	5 (14.3)		8 (20.5)	8 (20.5)		8 (16.0)	10 (20.0)		9 (18.0)	10 (20.0)	
32 (91.4)	33 (94.3)	0.64	38 (97.4)	36 (92.3)	0.31	49 (98.0)	47 (94.0)	0.31	48 (96.0)	47 (94.0)	0.65
3 (8.6)	2 (5.7)		1 (2.6)	3 (7.7)		1 (2.0)	3 (6.0)		2 (4.0)	3 (6.0)	
12 (35.3)	9 (26.5)	0.66	11 (28.9)	11 (28.9)	0.36	12 (24.5)	15 (30.6)	0.36	17 (34.7)	11 (22.4)	0.21
15 (44.1)	20 (58.8)		19 (50.0)	21 (55.3)		30 (61.2)	22 (44.9)		26 (53.1)	25 (51.0)	

PKM1			**LDHA**			**ND1**			**CYTB**		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
5 (14.7)	4 (11.8)		5 (13.2)	6 (15.8)		5 (10.2)	10 (20.4)		4 (8.2)	11 (22.4)	
2 (5.9)	1 (2.9)		3 (7.9)	0 (0.0)		2 (4.1)	2 (4.1)		2 (4.1)	2 (4.1)	
5 (14.3)	6 (17.1)	0.39	5 (12.8)	8 (20.5)	0.45	6 (12.0)	12 (24.0)	0.46	7 (14.0)	11 (22.0)	0.22
12 (34.3)	7 (20.0)		14 (35.9)	8 (20.5)		15 (30.0)	12 (24.0)		18 (36.0)	9 (18.0)	
7 (20.0)	5 (14.3)		6 (15.4)	8 (20.5)		10 (20.0)	8 (16.0)		8 (16.0)	11 (22.0)	
11 (31.4)	17 (48.6)		14 (35.9)	15 (38.5)		19 (38.0)	18 (36.0)		17 (34.0)	19 (38.0)	
29 (82.9)	22 (62.9)	0.10	29 (74.4)	30 (76.9)	0.94	30 (60.0)	43 (86.0)	**0.01**	36 (72.0)	38 (76.0)	0.82
2 (5.7)	8 (22.9)		5 (12.8)	4 (10.3)		11 (22.0)	4 (8.0)		7 (14.0)	7 (14.0)	
4 (11.4)	5 (14.3)		5 (12.8)	5 (12.8)		9 (18.0)	3 (6.0)		7 (14.0)	5 (10.0)	
7 (20.0)	7 (20.0)	0.75	6 (15.4)	10 (25.6)	**0.04**	13 (26.0)	8 (16.0)	0.23	12 (24.0)	10 (20.0)	0.50
23 (65.7)	25 (71.4)		25 (64.1)	28 (71.8)		30 (60.0)	38 (76.0)		31 (62.0)	36 (72.0)	
5 (14.3)	3 (8.6)		8 (20.5)	1 (2.6)		7 (14.0)	4 (8.0)		7 (14.0)	4 (8.0)	

COX1			ATP6			GAPDH			HIF1A		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
28 (57.1)	24 (49.0)	0.42	29 (56.9)	24 (48.0)	0.37	33 (68.8)	19 (38.8)	**0.003**	33 (64.7)	20 (40.8)	**0.02**
21 (42.9)	25 (51.0)		22 (43.1)	26 (52.0)		15 (31.2)	30 (61.2)		18 (35.3)	29 (59.2)	
15 (30.6)	12 (24.5)	0.51	13 (25.5)	16 (32.0)	0.55	15 (31.2)	13 (26.5)	0.10	12 (23.5)	17 (34.7)	0.43
6 (12.2)	10 (20.4)		7 (13.7)	9 (18.0)		4 (8.3)	12 (24.5)		8 (15.7)	8 (16.3)	
28 (57.1)	27 (55.1)		31 (60.8)	25 (50.0)		29 (60.4)	24 (49.0)		31 (60.8)	24 (49.0)	
45 (91.8)	40 (81.6)	0.14	46 (20.2)	41 (82.0)	0.23	43 (89.6)	41 (83.7)	0.39	44 (86.3)	43 (87.8)	0.83
4 (8.2)	9 (18.4)		5 (9.8)	9 (18.0)		5 (10.4)	8 (16.3)		7 (13.7)	6 (12.2)	
40 (81.6)	42 (85.7)	0.59	41 (80.4)	44 (88.0)	0.30	41 (85.4)	40 (81.6)	0.62	43 (84.3)	41 (83.7)	0.93
9 (18.4)	7 (14.3)		10 (19.6)	6 (12.0)		7 (14.6)	9 (18.4)		8 (15.7)	8 (16.3)	
43 (87.8)	36 (73.5)	0.07	44 (86.3)	38 (76.0)	0.19	40 (83.3)	39 (79.6)	0.64	41 (80.4)	40 (81.6)	0.87
6 (12.2)	13 (26.5)		7 (13.7)	12 (24.0)		8 (16.7)	10 (20.4)		10 (19.6)	9 (18.4)	
48 (98.0)	45 (91.8)	0.17	50 (98.0)	46 (92.0)	0.16	44 (91.7)	48 (98.0)	0.16	47 (92.2)	48 (98.0)	0.18
1 (2.0)	4 (8.2)		1 (2.0)	4 (8.0)		4 (8.3)	1 (2.0)		4 (7.8)	1 (2.0)	
12 (25.0)	16 (33.3)	0.64	15 (30.0)	13 (26.5)	0.55	16 (34.0)	12 (25.0)	0.58	14 (28.0)	14 (29.2)	0.24
28 (58.3)	23 (47.9)		28 (56.0)	24 (49.0)		22 (46.8)	26 (54.2)		24 (48.0)	27 (56.2)	

COX1			ATP6			GAPDH			HIF1A		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
6 (12.5)	8 (16.7)		5 (10.0)	10 (20.4)		8 (17.0)	7 (14.6)		8 (16.0)	7 (14.6)	
2 (4.2)	1 (2.1)		2 (4.0)	2 (4.1)		1 (2.1)	3 (6.2)		4 (8.0)	0 (0.0)	
6 (12.2)	11 (22.4)	0.37	9 (17.6)	9 (18.0)	0.47	9 (16.7)	9 (18.4)	0.97	13 (25.5)	5 (10.2)	**0.05**
16 (32.7)	11 (22.4)		17 (33.3)	10 (20.0)		12 (25.0)	13 (26.5)		13 (25.5)	13 (26.5)	
11 (22.4)	8 (16.3)		8 (15.7)	11 (22.0)		9 (18.8)	10 (20.4)		5 (9.8)	14 (28.6)	
16 (32.7)	19 (38.8)		17 (33.3)	20 (40.0)		19 (39.6)	17 (34.7)		20 (39.2)	17 (34.7)	
32 (65.3)	40 (81.6)	0.15	36 (70.6)	38 (76.0)	0.80	33 (68.8)	(37 (75.5)	0.73	39 (76.5)	34 (69.4)	0.70
9 (18.4)	6 (12.2)		8 (15.7)	7 (14.0)		8 (16.7)	7 (14.3)		7 (13.7)	8 (16.3)	
8 (16.3)	3 (6.1)		7 (13.7)	5 (10.0)		7 (14.6)	5 (10.2)		5 (9.8)	7 (14.3)	
13 (26.5)	8 (16.3)	0.23	11 (21.6)	11 (22.0)	0.29	11 (22.9)	11 (22.4)	0.77	7 (13.7)	15 (30.6)	**0.02**
29 (59.2)	37 (75.5)		32 (62.7)	36 (72.0)		31 (64.6)	34 (69.4)		35 (68.6)	32 (65.3)	
7 (14.3)	4 (8.2)		8 (15.7)	3 (6.0)		6 (12.5)	4 (8.2)		9 (17.6)	2 (4.1)	

TFAM			COX4			UQCRC		
Low	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
11 (52.4)	9 (47.4)	0.75	5 (45.5)	3 (25.0)	0.30	17 (56.7)	13 (41.9)	0.25
10 (47.6)	10 (52.6)		6 (54.5)	9 (75.0)		13 (43.3)	18 (58.1)	
3 (14.3)	5 (26.3)	0.49	3 (27.3)	1 (8.3)	0.32	8 (26.7)	10 (32.3)	0.85
6 (28.6)	3 (15.8)		2 (18.2)	1 (8.3)		5 (16.7)	4 (12.9)	
12 (57.1)	11 (57.9)		6 (54.5)	10 (83.3)		17 (56.7)	17 (54.8)	
19 (90.5)	13 (68.4)	0.08	9 (81.8)	9 (75.0)	0.69	26 (86.7)	26 (83.9)	0.76
2 (9.5)	6 (31.6)		2 (18.2)	3 (25.0)		4 (13.3)	5 (16.1)	
17 (81.0)	17 (89.5)	0.45	11 (100.0)	8 (66.7)	**0.04**	28 (93.3)	24 (77.4)	0.08
4 (19.0)	2 (10.5)		0 (0.0)	4 (33.3)		2 (6.7)	7 (22.6)	
18 (85.7)	14 (73.7)	0.34	9 (81.8)	10 (83.3)	0.92	23 (76.7)	24 (77.4)	0.94
3 (14.3)	5 (26.3)		2 (18.2)	2 (16.7)		7 (23.3)	7 (22.6)	
20 (95.2)	16 (84.2)	0.25	9 (81.8)	12 (100.0)	0.12	28 (93.3)	29 (93.5)	0.97
1 (4.8)	3 (15.8)		2 (18.2)	0 (0.0)		2 (6.7)	2 (6.5)	
8 (40.0)	4 (21.1)	0.61	5 (45.5)	4 (33.3)	0.59	11 (37.9)	9 (30.0)	0.79
10 (50.0)	12 (63.2)		4 (36.4)	7 (58.3)		13 (44.8)	17 (56.7)	

TFAM			COX4			UQCRC		
Low	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
1 (5.0)	2 (10.5)		1 (9.1)	0 (0.0)		3 (10.3)	3 (10.0)	
1 (5.0)	1 (5.3)		1 (9.1)	1 (8.3)		2 (6.9)	1 (3.3)	
3 (14.3)	5 (26.3)	0.29	1 (9.1)	3 (25.0)	0.69	6 (20.0)	8 (25.8)	0.47
5 (23.8)	8 (42.1)		4 (36.4)	5 (41.7)		9 (30.0)	8 (25.8)	
4 (19.0)	2 (10.5)		3 (27.3)	2 (16.7)		4 (13.3)	8 (25.8)	
9 (42.9)	4 (21.1)		3 (27.3)	2 (16.7)		11 (36.7)	7 (22.6)	
12 (57.1)	15 (78.9)	0.22	6 (54.5)	8 (66.7)	0.54	22 (73.3)	24 (77.4)	0.91
5 (23.8)	1 (5.3)		1 (9.1)	2 (16.7)		3 (10.0)	3 (9.7)	
4 (19.0)	3 (15.8)		4 (36.4)	2 (16.7)		5 (16.7)	4 (12.9)	
5 (23.8)	4 (21.1)	0.59	4 (36.4)	3 (25.0)	0.08	5 (16.7)	7 (22.6)	0.49
14 (66.7)	11 (57.9)		4 (36.4)	9 (75.0)		19 (63.3)	21 (67.7)	
2 (9.5)	4 (21.1)		3 (27.3)	0 (0.0.)		6 (20.0)	3 (9.7)	

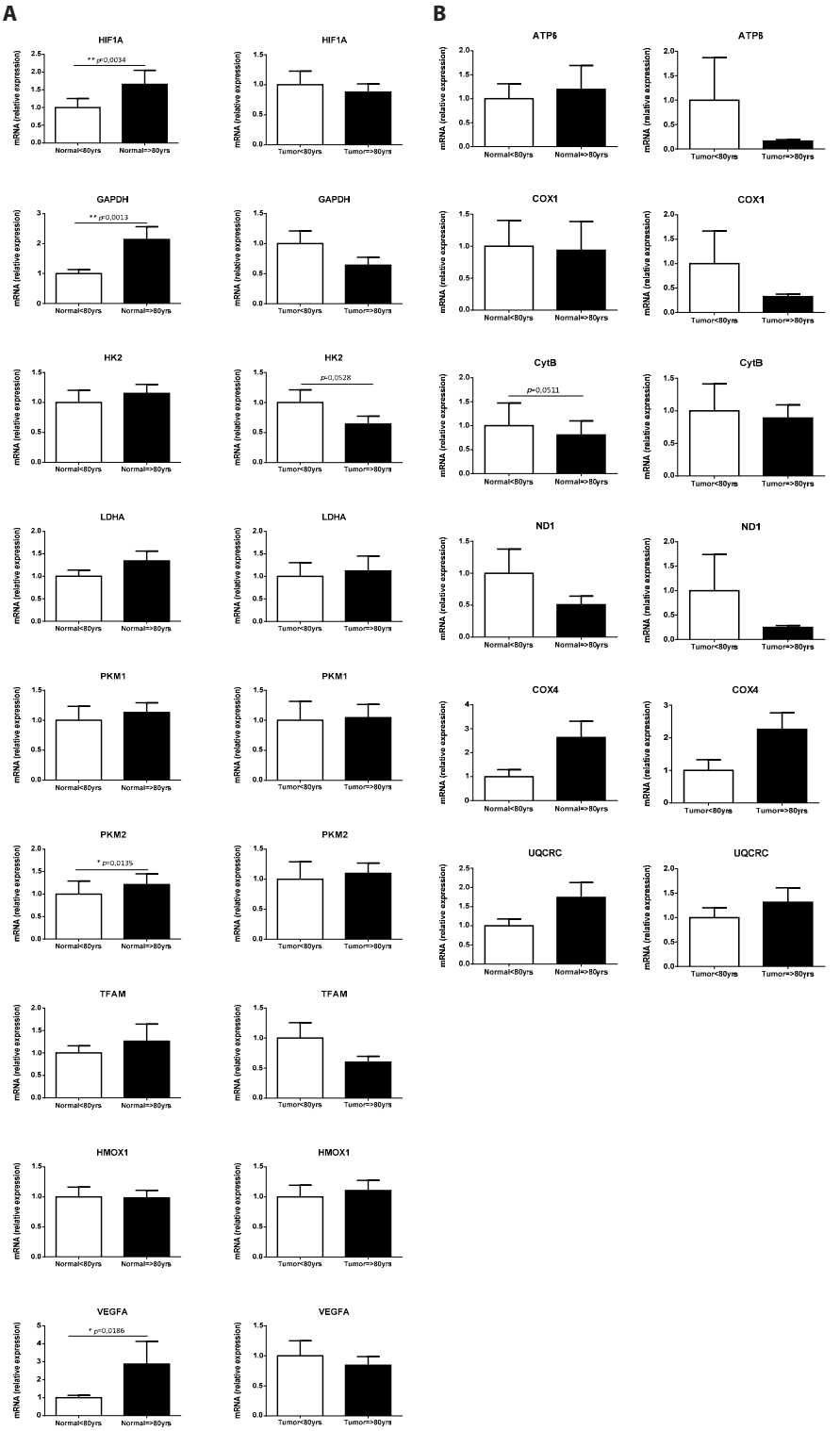

Figure 1A: mRNA expression per primer comparing middle-aged (65-79yrs) and old patients (≥80yrs) in normal breast tissue and breast tumor tissue.

Figure 1B: mRNA expression per mitochondrial gene primer comparing middle-aged (65-79yrs) and old patients (≥80yrs) in normal breast tissue and breast tumor tissue.

mRNA expression per age group

Compared to normal breast tissue, the breast tumor tissue of patients between the ages of 65 and 80 years old, showed a significant increase of HIF1-α (p=0.0011), GAPDH (p=0.0260) and TFAM (p=0.0171) mRNA expression (Figure 2A). This significant increase in the tumor tissue was also seen in patients older than 80 years for HIF1-α (p=0.0242) and TFAM (p=0.0041). A significant decrease was seen in the tumor tissue compared to the healthy breast tissue for the mitochondrially encoded OXPHOS mRNAs, ATP6 (p<0.0001 for age 65-80 years and p<0.0001 for age ≥80 years), COX1 (p=0.0067for age 65-80 years and p=0.0002 for age ≥80 years), and CytB (p=0.0466 for age 65-80 years and p=0.0291 for age ≥80 years)) in both age groups (Figure 2B). For ND1 and both nuclear encoded OXPHOS genes (COX4 and UQCRC) a non-significant decrease was seen.

Clinical outcome

PKM2 expression in the normal breast tissue showed significant association with the OS in the univariate analysis (high vs. low: HR1.71, 95%CI:1.03-2.84, p=0.038) (Table 5A). In the adjusted analysis, the significance was lost (p=0.17) when we included age as an important clinical outcome predictor. However, given the fact that the primary aim of this study was to identify aging markers with clinical value regardless of biological age, given the fact that biological age is often not one-to-one related with physical wellbeing and also given the fact that all of the patients in this cohort are already old, we performed the same adjustment but omitting age as an outcome predictor. Now, a statistical trend was seen in the favour of low PKM2 mRNA expression (HR1.69, 95CI:0.95-3.03, p=0.08, Table 5A). A statistical trend was also seen for high HIF1-α mRNA expression in the normal breast tissue, ignoring age as an outcome predictor (HR1.65, 95%CI:0.77-12.08, p=0.06, Table 5A), and for HMOX1 mRNA expression in the healthy breast tissue, maintaining significance in both forms of adjusted analyses (HR1.95, 95%CI:0.99-3.83, p=0.06 (without age adjustment) and HR1.95, 95%CI:0.98-3.89, p=0.06 (with age adjustment)) (Table 5A).

High PKM2 (HR1.72, 95%CI:0.92-3.22, p=0.087) and VEGFA (HR2.07, 95%CI:1.01-4.14, p=0.039) mRNA expression in the breast tumor tissue were significantly associated with OS in univariate analyses (Table 5B) but lost their significance in the adjusted analyses.

HIF-1α immunohistochemical staining

In normal breast tissue or the breast tumor tissue there was no significant association between HIF1-α and both patient and classical tumor characteristics. In contrast to the qPCR expression data, no significant association was seen for HIF1-α protein expression in the healthy tissue with the OS or in the breast tumor tissue for the OS, DFS and RFP (Table 6).

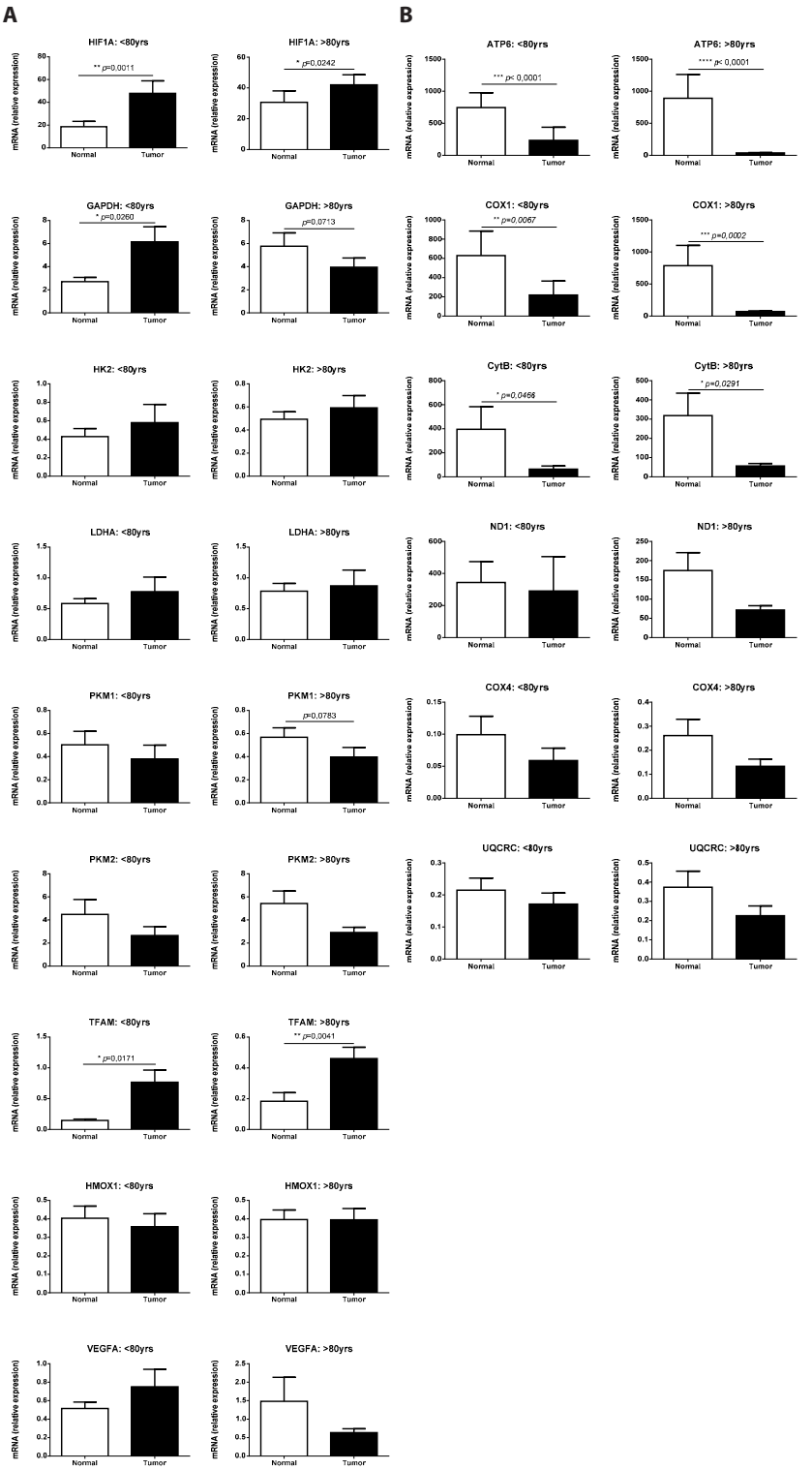

Figure 2A: mRNA expression per primer comparing normal breast tissue and breast tumor tissue in the middle-aged (65-79yrs) and old patients (≥80yrs).

Figure 2B: mRNA expression per mitochondrial gene primer comparing normal breast tissue and breast tumor tissue in the middle-aged (65-79yrs) and old patients (≥80yrs).

Table 5A: Overall Survival (OS) for normal breast tissue

	Univariate survival analyses			Adjusted analyses**			Adjusted analyses*		
	HR	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***
Normal tissue									
VEGFA									
Low	Ref			Ref			Ref		
High	1.49	(0.86-2.57)	0.16	1.27	(0.70-2.28)	0.43	1.17	(0.65-2.13)	0.60
PKM2									
Low	Ref			Ref			Ref		
High	1.71	(1.03-2.84)	**0.04**	1.69	(0.95-3.03)	0.08	2,76	(0.84-2.71)	0.17
PKM1									
Low	Ref			Ref			Ref		
High	1.59	(0.90-2.81)	0.11	1.63	(0.84-3.19)	0.15	1.52	(0.78-2.94)	0.22
LDHA									
Low	Ref			Ref			Ref		
High	1.47	(0.84-2.59)	0.18	1.13	(0.59-2.17)	0.71	1.14	(0.59-2.20)	0.69
HMOX1									
Low	Ref			Ref			Ref		
High	1.44	(0.77-2.68)	0.25	1.95	(0.99-3.83)	0.06	1.95	(0.98-3.89)	0.06
ND1									
Low	Ref			Ref			Ref		
High	1.35	(0.84-2.18)	0.22	1.81	(1.07-3.08)	**0.03**	2.2	(1.26-3.85)	**0.01**
CYTB									
Low	Ref			Ref			Ref		
High	1.50	(0.93-2.43)	0.10	1.84	(1.10-3.10)	**0.02**	1.62	(0.95-2.75)	0.08
COX1									
Low	Ref			Ref			Ref		
High	1.54	(0.95-2.49)	0.08	1.64	(0.98-2.74)	0.06	1.59	(0.95-2.69)	0.08
ATP6									
Low	Ref			Ref			Ref		
High	1.41	(0.88-2.27)	0.15	1.69	(1.01-2.83)	**0.05**	1.64	(0.97-2.77)	0.06
GAPDH									
Low	Ref			Ref			Ref		
High	1.46	(0.89-2.38)	0.13	1.28	(0.75-2.19)	0.36	0.89	(0.49-1.62)	0.72
HIF1A									
Low	Ref			Ref			Ref		
High	1.30	(0.81-2.09)	0.28	1.65	(0.77-12.08)	0.06	1.31	(0.77-2.25)	0.32
TFAM									
Low	Ref			Ref			Ref		
High	0.53	0.26-1.09	0.09	0.50	(0.21-1.23)	0.13	0.52	(0.21-1.27)	0.15
COX4									
Low	Ref			Ref			Ref		
High	1.77	(0.67-4.67)	0.25	1.39	(0.28-7.05)	0.69	0.84	(0.13-5.59)	0.86
UQCRC									
Low	Ref			Ref			Ref		
High	0.91	(0.49-1.69)	0.77	0.76	(0.38-1.54)	0.45	0.62	(0.29-1.32)	0.22

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age
**Not adjusted for age

Table 5B: Overall Survival (OS) for breast tumor tissue

	Univariate survival analyses			Adjusted analyses**			Adjusted analyses*		
	HR	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***
Tumor tissue									
ATP6									
Low	Ref			Ref			Ref		
High	1.52	(0.83-2.77)	0.18	1.15	(0.58-2.29)	0.69	0.78	(0.37-1.66)	0.52
COX1									
Low	Ref			Ref			Ref		
High	1.48	(0.80-2.73)	0.21	0.89	(0.42-1.91)	0.77	0.73	(0.33-1.58)	0.42
CYTB1									
Low	Ref			Ref			Ref		
High	2.06	(1.09-3.87)	**0.03**	1.92	(0.84-4.38)	0.12	1.99	(0.83-4.76)	0.12
ND1									
Low	Ref			Ref			Ref		
High	1.31	(0.71-2.41)	0.38	0.87	(0.42-1.84)	0.72	0.79	(0.36-1.72)	0.55
GAPDH									
Low	Ref			Ref			Ref		
High	1.14	(0.62-2.08)	0.68	1.27	(0.61-2.63)	0.53	1.55	(0.70-3.43)	0.28
LDHA									
Low	Ref			Ref			Ref		
High	1.72	(0.89-3.32)	0.11	1.54	(0.70-3.37)	0.28	2.34	(1.01-5.41)	**0.05**
HMOX1									
Low	Ref			Ref			Ref		
High	0.57	(0.27-1.20)	0.14	0.89	(0.35-2.29)	0.81	0.88	(0.34-2.27)	0.79
PKM1									
Low	Ref			Ref			Ref		
High	1.10	(0.57-2.14)	0.77	1.25	(0.53-2.96)	0.61	1.06	(0.45-2.52)	0.90
PKM2									
Low	Ref			Ref			Ref		
High	1.72	(0.92-3.22)	0.09	1.62	(0.76-3.48)	0.21	1.44	(0.65-3.20)	0.37
VEGFA									
Low	Ref			Ref			Ref		
High	2.07	(1.04-4.14)	**0.04**	2.05	(0.70-6.07)	0.19	1.53	(0.47-5.00)	0.49
HIF1A									
Low	Ref			Ref			Ref		
High	1.25	(0.67-2.29)	0.48	1.26	(0.59-2.72)	0.55	1.21	(0.54-2.71)	0.65
TFAM									
Low	Ref			Ref			Ref		
High	1.19	0.63-2.29	0.59	1.19	(0.48-2.94)	0.70	1.03	(0.42-2.54)	0.95
COX4									
Low	Ref			Ref	-		Ref	-	
High	1.58	(0.27-9.12)	0.61	-			-		
UQCRC									
Low	Ref			Ref			Ref		
High	1.46	(0.68-3.12)	0.33	0.95	(0.31-2.91)	0.92	0.87	(0.29-2.62)	0.81

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age,
**Not adjusted for age

Table 6: OS, DFS and RFP for HIF1α and PKM2

	Overall Survival								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
HIF1α Breast tumor tissue									
Low	Ref			Ref			Ref		
High	0.80	(0.48-1.36)	0.42	0.85	(0.44-1.62)	0.61	0.88	(0.44-1.76)	0.72
HIF1α Normal breast tissue									
Low	Ref			Ref			Ref		
High	0.76	(0.47-1.23)	0.27	0.78	(0.47-1.30)	0.33	0.78	(0.46-1.30)	0.33
PKM2 Breast tumor tissue									
Low	Ref			Ref			Ref		
High	1.02	(0.56-1.86)	0.95	1.11	(0.54-2.28)	0.78	0.82	(0.39-1.74)	0.61
PKM2 Normal breast tissue									
Low	Ref			Ref			Ref		
High	1.09	(0.26-4.66)	0.90	57939	(0-∞)	0.97	50155	(0-∞)	0.97

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age (+ tumor grade and HR status in tumor tissue), ** Not adjusted for age

	Disease Free Survival								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
Ref			Ref			Ref		
0.55	(0.18-1.69)	0.30	0.41	(0.11-1.57)	0.19	0.52	(0.13-2.04)	0.35
Ref			Ref			Ref		
1.25	(0.48-3.26)	0.64	1.99	(0.72-5.56)	0.19	2.04	(0.74-5.65)	0.17
Ref			Ref			Ref		
0.19	(0.02-1.46)	0.11	0.10	(0.01-1.17)	***0.07***	0.07	(0.007-0.67)	***0.02***
Ref			Ref			Ref		
22.76	(0-∞)	0.64	64530	(0-∞)	0.98	66055	(0-∞)	0.94

	Relapse Free Period								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
Ref			Ref			Ref		
0.73	(0.23-2.30)	0.59	0.54	(0.13-2.29)	0.41	0.59	(0.14-2.52)	0.48
Ref			Ref			Ref		
1.15	(0.43-3.08)	0.77	1.37	(0.49-3.85)	0.55	1.30	(0.46-3.67)	0.62
Ref			Ref			Ref		
0.20	(0.03-1.56)	0.12	0.12	(0.01-1.80)	0.12	0.11	(0.01-1.36)	0.09
Ref			Ref			Ref		
22.82	(0-∞)	0.64	60552	(0-∞)	0.98	53578	(0-∞)	0.98

PKM2 immunohistochemical staining

For normal breast tissue and the breast tumor tissue, PKM2 staining showed a significant association with patient and classical tumor characteristics. Tumor tissue showed a statistical trend for DFS, in the favor of high PKM2 staining (HR0.07, 95%CI: 0.007-0.67, p=0.02) (Table 6). A statistical trend, again in the favor of high PKM2 staining in the tumor tissue was seen for RFP (HR0.11, 95%CI: 0.01-1.36, p=0.09). No statistical significance was seen for PKM2 staining in the tumor tissue for OS. No association was seen for PKM2 staining in relation with clinical outcome in the normal breast tissue.

DISCUSSION

This study shows that HIF1-α and its gene targets are upregulated in the healthy breast tissue of older breast cancer patients, not merely closely associated with increased age, but also with surrogate markers of deteriorating clinical functionality of the patient. These include polypharmacy, residing in a nursing home and difficulty walking. Of the investigated markers, PKM2 had the most frequent association with functional surrogate markers, showing a higher expression in the normal breast tissue of the elderly breast cancer population, with a potential negative effect on survival. Furthermore, our results show that HIF1-α expression is significantly higher in the normal breast tissue of the older patient and that HIF1-α expression in the normal breast tissue is associated with a higher tumor grade in the patient. These observations strengthen the hypothesis that dysregulation of the HIF1-α metabolic pathway, presumably leading to an increase in ROS, is closely related with, and maybe even an important driving force of the high cancer incidence in the older population [4].

Given the fact that the adjusted OS analyses for HIF1-α and PKM2 expression in the normal tissue lose their significance when age is considered a confounder (but remains of significant value when age is not taken into account as an outcome predictor) strengthens the observation that these two markers are closely related at an advanced age. Thus, HIF1-α and PKM2 are promising age-related markers, showing a strong association with the patient's clinical condition.

It is already known that cancer cells evolve complex regulatory mechanisms that adapt their metabolism to match physiological states, such as sustained proliferation [18]. Differences in metabolism represent some of the first known variations identified between normal and cancerous cells. A recent study has shown that aging is associated with a decline in nuclear NAD^+ levels, leading to accumulation of HIF1-α under normoxic conditions, paralleling the Warburg effect [12]. It was shown that deletion of the NAD-dependent deacetylase SIRT1 accelerates this process, whereas raising NAD^+ levels in 2

year-old mice restores mitochondrial function to that of a young mouse (3-6 months of age) [12].

Consistent with this, PKM2, an important downstream target of HIF1-α, catalyzing the last step of glycolysis, is upregulated with age and may have important clinical value as an aging marker.

A recent study showed that switching from PKM1 to PKM2 is generated through alternative splicing of two mutually exclusive exons, which is controlled by hnRNPA1, hnRNPA2, and PTB [10]. Given their role in tumorigenesis, hnRNPA1, hnRNPA2 and PTB have potential as therapeutic targets. Promising results are seen with reduction of these proteins using small interfering RNA (siRNA) in cancer cells, leading to cancer tissue specific apoptosis induction [19]. Furthermore, it is proposed that hnRNPA1, A2 and PTB are involved in the early transforming events of tumorigenesis, suggesting that they play an important role in the initial stages of neoplastic transformation [20]. The results of this current study strengthen these previous observations; showing high PKM2 expression in the tissue of the older population, who, based on epidemiological findings, have an increased risk of carcinogenesis and a higher chance of death under these conditions. On the other hand, high PKM2 protein expression in the breast tumor was associated with a significantly better DFS and a trend toward better RFP compared to patients with low PKM2 protein expression in their tumor.

These observations match with the findings of Anastasiou *et al.*, who showed that activation of PKM2 altered cancer metabolism in vitro and reduced xenograft tumor growth [21]. A possible explanation for this finding lies in the fact that highly proliferating cells strongly depend on building blocks, favored by the less active dimeric PKM2. Thus, activation of PKM2 in the active tetrameric form may inhibit cell proliferation due to a deficiency of precursors for the synthesis of cell building blocks [15;21], ultimately leading to less cancer development and spread. PKM2 activation is thus considered a promising adjuvant treatment modality.

The presence of increased HIF1-α and its downstream marker PKM2 in healthy breast tissue are significantly associated with the functional condition of a patient, tumor aggressiveness and clinical outcome. If metabolic changes are important drivers of aging and corresponding tumorigenesis, molecules that prevent, halt or reverse metabolic aging may be useful anti-aging and anti-cancer therapies. Recent promising advances have been made with regard to HIF1-α inhibitors, SIRT activators, PKM2 modulators and NAD-boosting molecules [15;22-24].

A major strength of this study is that, to the best of our knowledge, this is the first study to investigate the hypothesis that metabolic reprogramming in normal tissue during aging correlates with patient and tumor characteristics. However, limitations were the relatively small patient sample size, increasing the chances of underpowered analyses. Second, all study material was formalin fixed paraffin embedded, leading to fragmentation of the RNA. Therefore, primers were designed to obtain small amplicon sizes. Although this did not interfere with our results since qPCR data was always reproduced in a duplicate plate and melting curves were checked for each primer set. Third, considering the hypothesis of this study, it would have been desirable to have younger patients (<65 years), in order to make a clear distinction between the difference in metabolic reprogramming in young versus old. Furthermore, it would have been of value to determine the clinical significance of HIF1-α-induced metabolic reprogramming in healthy tissue of a patient cohort in which survival was not influenced by cancer.

Although, more research is needed to elucidate the potential contribution of age-related changes in HIF1-α and PKM2, the current research supports the hypothesis that reversing or halting metabolic changes during aging could provide considerable benefits to individuals as they reach an age where the chances of tumorigenesis increase exponentially.

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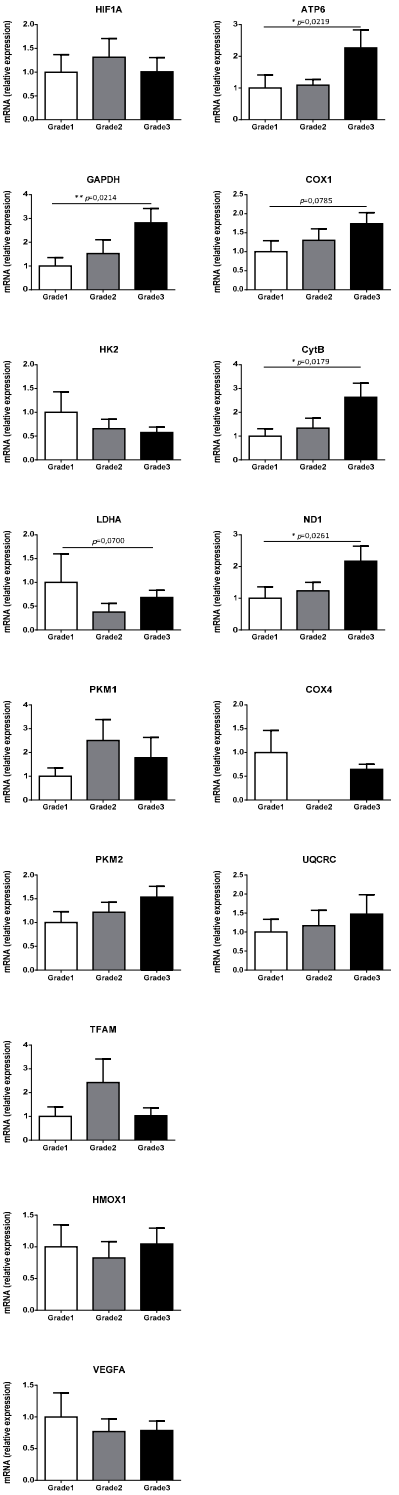
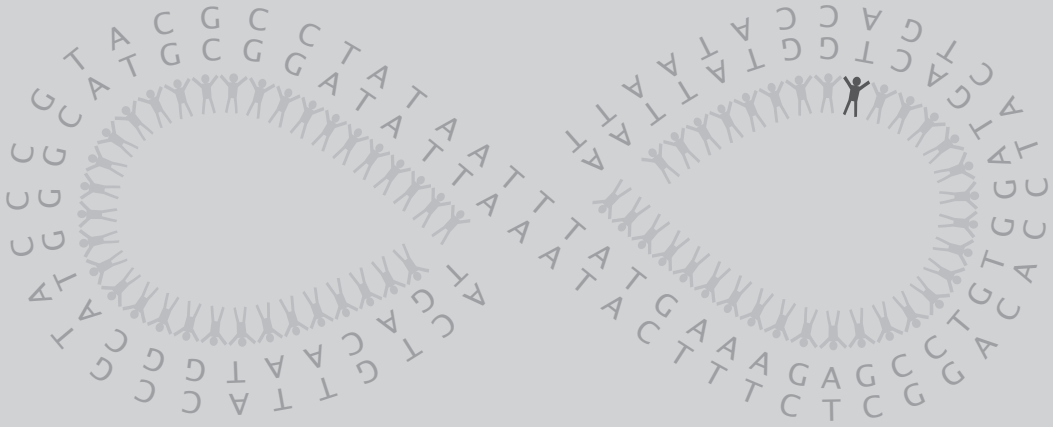
SUPPLEMENTARY TABLE

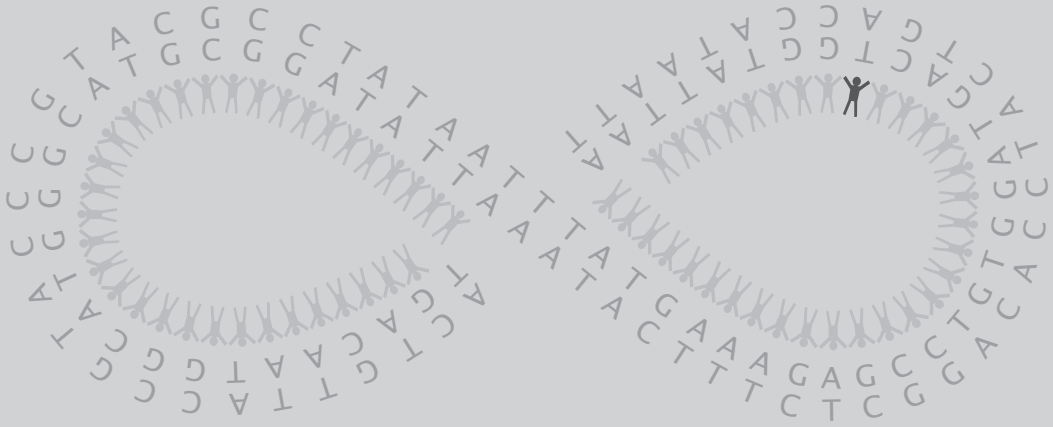


Extra figure: mRNA expression per primer in the breast tumor tissue per tumor grade.



Part IV

Precision medicine in the (older) breast cancer patient



Chapter 11

How does genome sequencing impact surgery?

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ABSTRACT

Cancer is a leading cause of death worldwide. Great efforts are dedicated to the development of prognostic and predictive biomarkers to improve diagnosis and achieve optimal treatment selection, thereby, introducing precision medicine in the multimodality treatment of cancer. Genomic aberrations are at the basis of tumor development, representing excellent candidates for the development of promising clinical biomarkers. Over the last decade, single-gene mutations and genomic profiling have been increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning for patients with cancer. We discuss the impact of such genetic-based information on surgical decision-making. Single-gene mutations have already influenced surgical decision-making in breast, colorectal and thyroid cancer. However, the direct impact of genomic profiling on surgical care has not yet been fully established. We discuss the direct and indirect influences of genomic profiling on surgery, and analyse the limitations and unresolved issues of a genotypic-approach to the surgical management of cancer.

INTRODUCTION

Despite early detection of cancer through screening programs and the development of new treatment modalities, the overall mortality as a consequence of this disease remains high [1]. The development of prognostic and predictive biomarkers for use in clinical practice has become a crucial part of cancer research. Single-gene mutations, which can be linked to cancer, have demonstrated promising prognostic and predictive value and have become increasingly used in multidisciplinary consultations for risk-assessment and subsequent individual treatment planning of patients with cancer [2-8]. Great examples are mutations within the *BRCA1* or *BRCA2* genes that are associated with a significantly increased risk of breast and ovarian cancer [9], and mutations in *KRAS,* which are extensively used for adjuvant treatment allocation in patients with colon cancer [2].

However, single-gene mutation analyses alone are unable to completely unravel the complexity of cancer. A more-global approach looking at changes in DNA, RNA or proteins that contribute to tumor growth and progression, is needed to capture the simultaneous interaction of many different mutated genes within malignant cells and their surrounding tissues. Genomic profiling, which enables gene expression profiles at a genome-wide level to be obtained, has already proven to have an impact on the diagnosis and prognostic classification of tumors, as well as on the prediction of response of individual patients to specific therapeutic regimens [10-12].

The promise of delivering precision medicine has been an incredibly strong driving force for the vast and rapid development of high-throughput genomic technologies. By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and outcomes, with the aim of delivering a treatment targeted to the specific disease characteristics of an individual patient. Genomic, epigenomic, and environmental data are studied together with specific patient information to understand individual disease patterns and to design personalized preventive, diagnostic, and/or therapeutic solutions. Current regimens of cancer treatment are effective in a minority of patients, whereas adverse effects occur in many of the treated patients. Genome wide approaches may contribute to increase therapy benefit and decreasing adverse events by tailoring treatment decisions [13].

From a clinical perspective, the added value of genetic and genomic approaches is clear. However, their impact on surgery, which is still the cornerstone of cancer treatment, is less obvious. This Perspectives article discusses the effect and associated limitations of introducing single-gene mutations and genomic profiling in the surgical decision-making process in terms of timing, extent and subsequent treatment of the patient.

SINGLE-GENE MUTATIONS AND SURGERY

There are several examples of how single-gene mutations can guide surgical management, including mutations in *BRCA*1 and *BRCA2* in breast cancer, adenomatous polyposis coli (*APC*) in colorectal cancer (CRC), the mismatch repair genes (*MMR*) in hereditary colon cancer and other cancers, and *RET* in multiple encocrine-relared tumors [3;14-17].

BRCA mutations

Specifically, women carrying mutations in the tumor suppressor genes *BRCA1 or BRCA2* have a high (cumulative risk of 60–80%) lifetime risk of breast cancer [18]. The *BRCA* genes are normally expressed in breast cells and other tissues, where they have a crucial role in DNA damage repair. If a mutation occurs in one of these genes, DNA damage is not repaired properly, resulting in an increased risk of breast and ovarian cancer [19;20]. Nowadays, bilateral prophylactic mastectomy and oophorectomy are the most effective strategy available for risk reduction of breast and ovarian cancer in mutation carriers [15;20-22]. In a recent study, Neuburger *et al.* [23], showed that in the UK the number of women who had a bilateral mastectomy nearly doubled over the last decade, and more than tripled among women without breast cancer. Of note, bilateral prophylactic mastectomy has been shown to reduce breast cancer risk by 90% in *BRCA1* or *BRCA2* mutation carriers [24]. Despite this great risk reduction, nearly 64% of *BRCA1* or *BRCA2* carriers in the USA choose to avoid surgery as a result of the high sensitivity of MRI that allows early tumor detection [25]. Since ovarian cancer screening methods are largely ineffective, bilateral prophylactic salpingo-oophorectomy remains the standard of care in all *BRCA1* or *BRCA2* mutation carriers, leading to a risk reduction of 80-96% in women with *BRCA* associated gynaecologic cancers [26;27].

APC mutations

In CRC, familial adenomatous polyposis (FAP) is a syndrome in which the inherited defect in the gate-keeper tumor-suppressor *APC* gene leads to the development of multiple premalignant polyps throughout the colon as a result of uncontrolled growth, and subsequent malignant progression before the age of 40 years [28]. Therefore, a colectomy is advised after detection of a germ line mutation *APC*. Depending on the clinical features (such as patient age, the number, nature and location of polyps), a rectal or pouch-anal anastomosis is recommended [29]. Various aspects of surgical decision-making are influenced by both surgeons and patients, whose preferences should be taken into account with regard to optimal time for surgical intervention, extent of surgery and the type of anastomosis performed. Independent of mutation type, surgery will be recommended as soon as FAP syndrome is diagnosed because this is associated with an almost 100% risk of CRC [30]. However, since cancer is rare before the age of 20, surgery is often

deferred to the late teen years or in between major life changes, such as in academic transitions or between jobs [29]. The amount of polyps in the rectum are correlated with disease severity and are of crucial importance for deciding on the type of anastomosis [31]. When fewer than five rectal polyps are observed, an ileorectal anastomosis is advised as this correlated with mild disease. Conversely, if 20 or more rectal polyps are identified, indicating severe disease, an ileal pouch anal anastomosis will be recommended. Furthermore, morbidity quality of life and desired subsequent bowel function should be taken into account. Although pouch-anal anastomosis nearly eliminates CRC risk, it is associated with worse functional outcome, including an increased daily stool frequency, 24-hour incontinence, sexual dysfunction, decreased fecundity in females, impotence in men and decreased quality of life when compared to preservation of the rectum [32-35].

MMR mutations

Germline mutations in DNA *MMR* genes, *hMLH1*, *hMSH2*, *PMS2* or *hMSH6,* are responsible for another form of hereditary colon cancer, namely non-polyposis CRC (or Lynch Syndrome) [36]. MMR genes are involved in numerous cellular functions including DNA repair, apoptosis, anti-recombination and destabilization of DNA [37]. Lynch Syndrome is also associated with an increased risk of cancers of the stomach, small intestine, liver, bile ducts, upper urinary tract, brain, and skin [38;39]. Additionally, women with this disorder have a high risk of cancer of the ovaries and the endometrium [39]. Although the need for prophylactic surgery is less evident in Lynch syndrome patients than in FAP syndrome patients, those with Lynch syndrome who are diagnosed with CRC should consider total colectomy rather than a segmental colon resection due to the increased risk of metachronous neoplasia associated with this condition. A large observational study of 382 *MMR* gene mutation carriers (172 *MLH1*, 167 *MSH2*, 23 *MSH6* and 20 *PMS2*) followed for 9 years confirmed a high cumulative risk of metachronous CRC for 332 carriers treated by segmental resection for their primary CRC. In contrast, there were no diagnoses of metachronous CRC for the other 50 *MMR* gene mutation carriers treated by extensive colon resection [16].

RET mutations

Multiple endocrine neoplasia (MEN) are clinical inherited syndromes affecting different endocrine glands. The different patterns of MEN syndromes includes MEN1, MEN2A, MEN2B and medullary thyroid cancer (MTC) [17], which is commonly associated with pheochromocytoma (PHEO) and/or multiple adenomatosis of parathyroid glands with hyperparathyroidism (PHPT). These syndromes have very different clinical courses: MEN2B is very aggressive, MTC is almost indolent in most patients, and MEN2A is associated with variable degrees of aggressiveness [17]. Activating germline point mutations of the *RET* protooncogene—a 21-exon gene encoding for a tyrosine kinase transmem-

brane receptor involved in the transduction of signals for cell growth and differentiation—are present in 95% and 98% of families with MEN2A and MEN2B respectively, and in approximately 95% of families with MTC [17]. A presymptomatic gene diagnosis aimed at detecting the presence of *RET* mutations in patients with MEN2 syndrome has been established to improve morbidity and mortality for patients with this disease. The treatment of choice for primary MTC is total thyroidectomy with central neck lymph nodes dissection. However, even after radical surgery for MTC, there is a 30 percent chance of recurrence. Therefore, a prophylactic thyroidectomy is advised in patients with MEN2 carrying mutations in *RET* in order to guarantee a definitive cure and avoid morbidity of a central neck lymph node dissection [17].

The American Thyroid Association task force has suggested four different risk levels—from A (the lowest) to D (the highest)— for *RET* mutations , which are incorporated in their most recent management guidelines [40]. Specifically, children from families with MEN or MTC that carry *RET* mutations associated with a risk level D-(such as Met918Thr) should be surgically treated as soon as possible in the first year of life; whereas patients with level B and C risk levels (with *RET* mutations located in exons 10, 11, 13, 14, and 15) should be operated with a total thyroidectomy before 5 years of age; total thyroidectomy can be delayed till after the age of 5 or until the calcitonin positivity only for patients with a level A risk level (with *RET* mutations mapping to exon 5 and 8) [41]. Removing the thyroid in young children has a great impact on the child's life, as lifetime levothyroxine supplementation is required [42].

Recent data have shown that *RET* mutations carriers with undetectable levels of basal calcitonin have an almost no risk of developing MTC [43]. Moreover, serum levels of calcitonin <30–40 pg/ml are always associated with intrathyroidal micro-MTC without any evidence of lymph node metastases [43]. Elisei *et al.* [43] designed a study in which they operated on only *RET* mutation gene carriers depending on their basal and stimulated level of calcitonin. Total thyroidectomy was strongly indicated in patients when their basal or stimulated calcitonin levels were above 10 pg/mL. Importantly, this study showed that the time of surgical treatment could be personalized and safely planned once the positivity to calcitonin is detected at the annual assessment, independent of the type of *RET* mutation and its associated level of risk. This strategy obviously implies a high compliance of carriers of *RET* mutations to the scheduled follow-up if surgery is postponed as long as possible. The detection of mutations in the proto-oncogene *RET* has, therefore, become standard practice with surgical implications in MTC, that have crucially influenced the timing of surgery [41]. Furthermore, Xing *et al.* [44] have recently published an algorithm that incorporates cytology and molecular *(RET)* testing for the management of patients with thyroid nodules presenting with atypia of undetermined clinical significance, with the aim of limiting unnecessary and/or extensive surgery. This study suggests that in these patients, fine needle aspiration biopsy molecular analysis

should be performed for malignancy risk stratification. For example, a *BRAF* mutation in thyroid nodules from this specific patient group tends to be associated with increased risk of thyroid cancer and thus need for surgical intervention [44].

GENOMIC PROFILING

In the past decades, the technology for DNA and RNA analysis has evolved rapidly, shifting from single-gene mutation analysis to a genome wide, system-biology approach, well placed to assist in unravelling the complexity of cancer [5]. Since then, genomic profiling has been increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning for cancer patients. In the first part of this section the influence of these established RNA-based gene profiles on cancer management are discussed. The second part of this section focuses on the impact of genomic profiling on surgical decision-making in terms of timing and surgical extent.

Genome sequencing in cancer care

The first genome-wide approaches used to predict clinical outcome in patients with cancer were based on RNA microarray analyses [45]. In one study that used microarray analysis, a panel of 50 genes identified low-risk and high-risk lung cancer patients with significantly different survival outcomes. Since then, many RNA expression profiles have been published with varying clinical value (Table 1).

Specifically, the Onco*type* DX® profile (Genomic Health Inc., Redwood City, CA) showed a promising prognostic value and also proved beneficial for adjuvant treatment allocation for patients with breast cancer [46]. In this assay, the recurrence score is calculated using a 21-gene assay, which includes 16 cancer-related genes and five reference genes for standardization, and determined a recurrence risk estimate (low, intermediate, or high) for each patient [46]. In breast cancer, the recurrence score proved to be an independent predictor of distant recurrence in patients with node-negative, estrogen receptor (ER)-positive breast cancer treated with tamoxifen. The recurrence score was also shown to be a predictor of the magnitude of chemotherapy benefit, with patients with high recurrence score showing the greatest benefit from chemotherapy [46;47]. The recurrence score was also found to be prognostic and predictive for postmenopausal patients with hormone receptor-positive disease and with positive nodes who were treated with tamoxifen. However, these studies showed no benefit from chemotherapy in patients with low recurrence scores [10;47].

These results were validated in a separate study, in which the prognostic value of the recurrence score for postmenopausal hormone receptor-positive, node-negative and –positive patients with breast cancer treated with aromatase inhibitors was also

Table 1: Established RNA based prognostic and predictive profiles for breast and colorectal cancer

Breast Cancer Profiles								
Test	**Company**	**Technique**	**Proven value**	**Tissue requirements**	**Output**	**Results**	**Validation**	**References**
Oncotype DX	Genomic Health, Inc. (Redwood City CA, USA)	qRT-PCR (21 genes)	Prognostic	Fresh frozen or FFPE	**RS**: *Low: <18* *Intermediate: 18-31* *High: ≥31* 10-years distant recurrence risk for ER+ve, LN- BC patients	*Low risk:* 6.8% chance of distant recurrence (95% CI 4.0-9.6) *Intermediate risk:* 14.3% (95% CI 8.3-20.3) *High risk:* 30.5% (95% CI 23.6-37.4) *High risk* & LN-: significant benefit from CT (HR 0.26 (95%CI 0.13-0.53)). Same is seen for LN+ (HR 0.59). Not seen in *low risk* patients	Yes Current Prospective trials: TAILORx: LN- patients RxPONDER: LN+ patients	46, 47
Mamma-Print	Agendia BV (Amsterdam, Netherlands)	Micro-array based gene expression profiling (70 genes)	Prognostic	RNA of fresh tissue cores or frozen material or FFPE	**Mammaprint risk score:** *Low & high risk* to develop metastasis in five years follow-up in BC patients	*Low vs. High*: HR 4.6 (95% CI 2.3-9.2) Sensitivity>90%	Yes Current prospective trial: MINDACT: LN-/LN+ patients	12, 50, 51
Colorectal Cancer Profiles								
Oncotype DX	Genomic Health Inc. (Redwood City CA, USA)	qRT-PCR (12 genes)	Prognostic	Fresh frozen or FFPE	**RS**: *Low: <18* *Intermediate: 18-31* *High: ≥31* 10-years distant recurrence risk in stage II colon cancer patients	Chance of distant recurrence in 3 years: *Low risk:* 12% *Intermediate:* 18% *High risk:* 22% High vs. Low risk: HR 1.47 (95% CI 1.01-2.14)	Yes	11, 54
ColoPrint	Agendia BV (Amsterdam, the Netherlands)	Micro-array based gene expression profiling (18 genes)	Prognostic	Fresh frozen material	**Coloprint risk score:** *low & high risk* to develop metastasis in five years follow-up for stage II and III colon cancer patients	Five years distant metastasis free survival: *Low:* 94.9% *High:* 80.6% High vs. Low risk: HR 4.28 (95% CI 1.36-13.5)	Yes	55, 56

Abbreviations: FFPE: formalin fixed paraffin embedded; qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction; RS: recurrence score; ET: endocrine therapy; LN-: lymph node negative; LN+: lymph node positive; CT: chemotherapy; ER+ve: estrogen receptor positive; HR: hazard ratio; 95% CI: 95% confidence interval

demonstrated [48]. Furthermore, recent findings have also suggested that the recurrence score is able to predict locoregional recurrence (LRR) in patients with node-negative ER-positive breast cancer treated with tamoxifen [49]. This same study further showed that patients who underwent a mastectomy had significantly less LRR compared with patients who received lumpectomy followed by breast radiotherapy. When subdivided by age categories (<50 or ≥50 years), patients aged <50 years with high recurrence score seemed to have better clinical benefit from mastectomy than from lumpectomy and radiotherapy. On the basis of these results, patients with breast cancer, aged <50 years, featuring a high recurrence score should be advised to undergo a mastectomy.

In addition to the Onco*type* DX® profile, the MammaPrint® (Agendia Inc. , Amsterdam, The Netherlands) RNA mini-array was developed for use in the high-throughput clinical setting for the diagnosis of breast cancer [12;50;51]. Using a supervised classification method, the correlation coefficient of the expression for approximately 5,000 genes was correlated with disease outcome in a retrospective cohort of 78 patients [12]. Classification was made on the basis of the correlations of the expression profile of the 'leave-one-out'sample with the mean expression levels of the remaining samples from the good and the poor prognosis patients, respectively. The accuracy improved until the optimal number of marker genes was reached (70 genes). In a validation study, this prognostic profile was tested in 295 consecutive patients. The estimated HR for distant metastases in the group with a poor-prognosis signature, was 5.1 (95% CI, 2.9-9.0; $p<0.001$) [51]. MammaPrint® is a 70-gene prognosis profile that was reported to be superior to standard clinical parameters, such as nodal status and grade, in predicting the occurrence of distant metastasis in patients with breast cancer [51]. Moreover, the MammaPrint® profile also showed predictive value in patients assigned to the 'high-risk' subgroup, who had a significant benefit of 12% for combined (chemotherapy and hormone therapy) treatment when compared with patients in the low risk subgroup [52]. Once available, the results of the randomized controlled trial MINDACT (Microarray in Node-negative Disease may Avoid ChemoTherapy) will contribute to the validation of the predictive role of MammaPrint® [53].

As in breast cancer, one of the clinically established RNA profiles for colon cancer is the Onco*type* DX® profile. This profile was established from four studies performed in over 1,800 patients with stage II or stage III colon cancer [54]. Genomic profiling in these studies allowed the identification of seven genes associated with tumor recurrence risk, six genes associated with chemotherapy benefit and five reference genes, that were predictive of recurrence in patients with resected colon cancer who were treated with surgery alone or surgery followed by 5-Fluorouracil and Leucovorin chemotherapy. This analysis led to the design of a 12-gene colon cancer recurrence score, which was validated in the QUASAR clinical trial [11]. According to this 12-gene score, predefined risk groups are categorized as low, intermediate or high risk for tumor recurrence, which

gives the possibility to allocate high-risk stage II colon cancer patients to adjuvant treatment, ultimately protecting patients from costly overtreatment. Of note, currently the Onco*type* DX® assay has prognostic value regarding outcome in colon cancer, however, no predictive value has been established for adjuvant treatment so far.

In addition, the ColoPrint® (Agendia,Amsterdam, The Netherlands), a prognostic 18-gene signature that was identified through unsupervised hierarchical clustering of a whole-genome oligonucleotide high-density microarray leading to unbiased gene selection, also showed promising results in patients with colon cancer [55]. The signature was validated in an independent set of patients with stage II colon cancer and identified a 5-year distant metastasis-free survival of 94.9 ± 2.2% for low-risk patients and 80.6 ± 6.6% for high-risk patients, (p=0.009) [56]. These results support the prognostic value of RNA profiling in patients with stage II colon cancer and herewith facilitate the identification of patients who may benefit from chemotherapy. Nevertheless, surgical treatment will not change at all, using this type of prognostication.

High-throughput genomic analysis have led to the identification of different genomic signatures (or profiles) that can be used for cancer management and can contribute to the multidisciplenary decision making process for cancer treatment. However, as described in the following section, the direct impact of genomic profiling on surgery, timing and/or extent of the procedure, is currently less clear.

Impact of genomic profiles on surgery

Breast cancer

Several studies have shown that gene expression profiling of biopsies is a succesful tool that can predict response to neo-adjuvant treatment [57;58]. Specifically, Ayers *et al.* [57] suggested that transcriptional profiling had the potential to identify a 74-gene expression pattern on biopsies of breast cancer that might lead to clinically useful predictors of pathological complete response (pCR) to the neo-adjuvant treatment regimen of sequential weekly paclitaxel in combination with 5-fluorouracil, doxorubicin and cyclophosphamide. However, this small sample study still needs further validation. Chang *et al.* [58] analysed core biopsy samples from 24 patients with breast cancer and found an association of a 92-gene signature with treatment response to neo-adjuvant monotherapy with docetaxel. These studies suggest that genomic-profiling on biopsies represents a clinically relevant progress in cancer management. It can be argued that current practice should focus on genomic profiling of the tumor biopsy, before assignment of a targeted neo-adjuvant treatment. Although this aspect does not have a direct impact on surgery, it could influence the extent and timing of surgery indirectly (Figure 1). Targeted neo-adjuvant treatment could potentially lead to downsizing of the tumor, with consequently less-extensive surgery or even a delay in surgery in case of a clinical

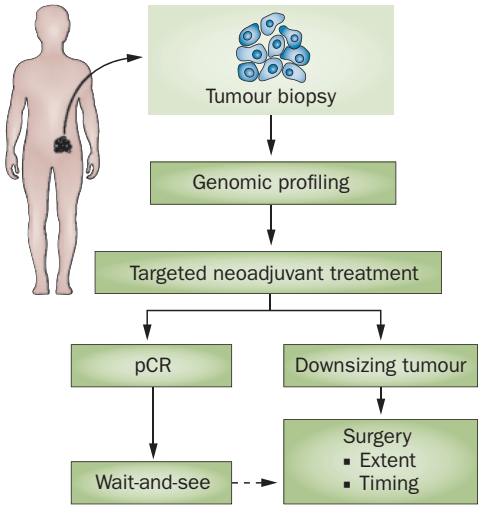

Figure 1: Impact of genomic profiling on surgery
This figure shows two ways by which genomic profiling might impact surgical intervention. Through genomic profiling of a tumor biopsy targeted neo-adjuvant treatment can be administered to a patient, possibly resulting in pathological complete response (pCR) or downsizing of the tumor. Downsizing of the tumor might influence surgery with regards to extent or timing of surgery. In case of pCR a wait-and-see approach can be followed, where surgery is no longer necessary and a strict follow-up is advised.

complete response (cCR). By using genomic profiling to tailor neo-adjuvant treatment, response rates may increase. This will result in lower mastectomy rates.

In breast cancer, there is already a shift from mastectomy to breast-conserving surgery after tumor shrinkage by neo-adjuvant chemotherapy, which proved to be oncologically safe in terms of survival outcomes [59;60]. This decrease of mastectomy rates is a result of response to chemotherapy. Although this response can be predicted by molecular profiling of the tumor, the surgical planning in itself is not directly influenced by any gene expression signature. For local control, the studies by Cho *et al.* [59] and Shin *et al.* [60], investigating the oncologic safety of conservative surgery versus mastectomy after neo-adjuvant chemotherapy also improved outcome in terms of local recurrence. However, the number of patients included and the number of local events were too small to draw a significant conclusion in terms of therapeutic safety. These studies imply that through targeted neo-adjuvant treatment, based on biopsy profiling, further downsizing of the tumor could occur and result in less invasive surgery. Today there are no known genomic profiles that guide surgical planning directly for breast cancer. Perhaps in the future, the risk of local regional recurrences can be predicted on the basis of genomic profiling in such a way that even after excellent response to neo-adjuvant therapy, a mastectomy is advised.

Pancreatic cancer

An other example of the potential impact of genomic profiling of biopsies is pancreatic cancer. Neo-adjuvant chemotherapy with gemcitabine and docetaxel in patients with borderline resectable cancer of the pancreatic head showed that operative exploration was associated with curative intent in 48% of the patients investigated [61]. Of the patients that underwent surgery, 87% had a R0 resection and 10% had a complete pathological

response. This treatment was associated with a low perioperative morbidity and favourable survival: 81% of patients with resected cancers were alive at a median follow-up of 21.6 months [61]. Although this result was not directly based on genomic profiling, it is expected that genomic analysis of these tumors (both mutation analysis and expression profiling) will better identify 'treatment sensitive'tumor characteristics, which may lead to optimization of allocation of directed neoadjuvant treatment per individual patient.

In the future, a more curative surgical intervention could be achieved for patient groups with limited resection options, as a result of genomic profiling of the tumor biopsy, when therapeutic regimens are further optimized by targeted neo-adjuvant treatments.

Rectal cancer

As described above, neo-adjuvant treatment sometimes leads to downstaging of the primary tumor or even a complete clinical or pathological response. Therefore, more R0 resections and less-extensive surgeries can be achieved. With the use of genomic profiling on biopsy samples, followed by targeted neo-adjuvant treatement, the impact on surgical intervention can be striking, possibly leading to the omission of surgery. One can argue that based on specific genomic profiles from tumor biopsies, a wait-and-see approach might be indicated following complete clinical response after tailored neo-adjuvant therapy [62]. With this wait-and-see approach surgery can be delayed or even omitted. In patients with rectal cancer, this wait-and-see approach, however, is under debate. Curative total mesorectal excision after preoperative chemoradiation is the current standard of care in rectal cancer, in which pCR is observed in nearly 14% of these patients [63]. This example highlighted the rationale of a wait-and-see policy, which was further suggested by the results from a series of retrospective studies from Brazil. The Brazilian studies reported similar survival rates in patients that after complete clinical response following neo-adjuvant treatment underwent radical resection or observation only [64-68]. Furthermore, Maas *et al.* [69] showed that a wait-and-see policy with strict selection criteria, up-to-date imaging techniques and follow-up is feasible with promising rates of 89% and 100% for cumulative probabilities of 2-year disease-free survival and overall survival, respectively, in patients with rectal cancer showing a complete clinical response. However, this study was small with a low local event rate, making clinical significance debatable. Recently, a study investigating criteria for determination of residual disease after neo-adjuvant chemotherapy showed that the majority of patients with a complete clinical response still had pathological residual disease [70]. For maximal benefit from a wait-and-see approach in rectal cancer, we should aim for better identification of patients with pathological complete response.

Oesophageal cancer

In oesophageal cancer, neo-adjuvant treatment can downstage tumors, thereby increasing R0 resections [71]. In one study, patients were randomly assigned to surgery alone or to chemoradiotherapy with carboplatin and paclitaxel followed by surgery [71]. Complete resection with no tumor within 1 mm of the resection margins (R0) was achieved in 92% of patients in the chemoradiotherapy-surgery group versus 69% in the surgery group ($p<0.001$). A pCR was achieved in 47 of 161 patients (29%) who underwent resection after chemoradiotherapy. In this scenario, targeted neo-adjuvant therapy based on the genomic profile of a biopsy was shown to influence surgery by improving the R0 resections and pCR rates.

In patients with locally advanced oesophageal cancer, the benefit from neo-adjuvant chemoradiotion is clear, but the benefit from surgery afterwards is less obvious [72]. Some patients with oesophageal cancer will have a pCR after neo-adjuvant chemoradiation and some of these patients would be able to forego surgery, but unfortunately evidence to guide treatment is scarce. For patients with squamous cell oesophageal cancer, those with a good clinical response after neo-adjuvant chemoradiation do not have a worse survival when undergoing observation only compared to surgery after chemoradiation [73]. The absolute benefit from surgery after neo-adjuvant chemoradiation seems to be relatively modest for patients with a good clinical response [72]. In selected patients with a complete clinical response following neo-adjuvant treatment, 3-year survival rates of 50% are seen irrespective of subsequent surgical intervention [74]. The accurate prediction of response to neo-adjuvant therapy can, therefore, have a direct influence on the surgical management of cancer. As treatment regimens improve and detection of earlier-stage disease increases (resulting in higher percentages of pCR), alternative approaches for patients at high risk of morbidity from surgery should be sought [75]. Even though evidence is not derived from randomized controlled trials, it might be reasonable to forego surgical intervention in patients with a complete clinical response, especially in elderly with comorbidities who are less fit to undergo surgery and more likely to experience adverse events. On the basis of these results, one can imagine that genomic-profiling could have an additional role in targeting the tumor with the most optimal neo-adjuvant treatment, possibly leading to an even better local control and survival outcome. However, in current clinical practice, this approach has not been routinely established yet.

FUTURE DEVELOPMENTS

Genomic profiling is gaining importance in the multidisciplinary treatment of cancer. A direct impact on surgical oncology, however, cannot yet be claimed. Genomic testing

on biopsies could potentially affect surgical management, but some important issues still remain unresolved and warrant further investigation before genomic profiling on biopsies can truly influence surgical decision-making.

First, several studies in different types of cancer have shown that in most cases sufficient tissue can be obtained from biopsies for performing genomic profiling [76;77]. However, in 20% of the cases limited tissue quantity is available from a biopsy, precluding further analysis [76]. Furthermore, low tumor content may need more in-depth sequencing or even a repeated biopsy to obtain more material for analysis, which is undesirable from the patient perspective. Therefore, improvement of profiling techniques is necessary to allow the identification of a valid profile in these more complicated circumstances.

Second, the risk of tumor seeding while performing the biopsy should not be underestimated. Case reports of malignant seeding following needle-biopsy have in fact been described in several tumors [78-80]. However, the clinical significance of this seeding is not known. In breast cancer, although data are limited, no increased morbidity has been observed as a consequence of tumor seeding [81].

Third, the heterogeneous nature of the tumor could contribute to unreliable prognostication and prediction. Genomic and epigenomic factors, among others, contribute to this heterogeneity and, consequently, newly developed targeted anti-cancer drugs will only be effective in a subset of patients, and perhaps only at a specific stage of their disease. A biopsy represents only a small fraction of the primary tumor, and owing to the heterogeneity of the tumor, important information could be missed, possibly resulting in a misleading phenotype. A solution for this issue is to obtain multiple biopsy samples from several locations throughout the tumor, although a higher risk of tumor seeding may be a consequence of this increased sampling.

Finally, the interactions of the tumor with the micro-environment influence tumor development and maintenance [82]. These patient-specific factors challenge adequate tumor sampling for biomarker discovery, warranting the use of techniques such as laser capture microdissection for separate analysis of tumor and normal tissue for biomarker profiling. Some profiles, such as MammaPrint®, were derived from the analysis of tissue sections containing both the tumor and its closely surrounding micro-environment, whereas others, such as Onco*type* DX® , analysed only cancer cells. The different gene signatures identified from these approaches reveal a great variety of differentially expressed genes, with minimal overlap between the signatures identified. For example, Varga *et al.* [83] showed that nearly 18% of breast cancer patients showed major-discrepancy between Endopredict and Onco*type* DX® assay. In current clinical practice, the use of these techniques would require highly trained personnel and are associated with high costs and, therefore, is not advisable. It is important to implement sample handling, processing and data analysis into a routine standardized practice, thereby increasing quality of the array and decreasing costs and inter-laboratory variability [84].

Lack of clarity regarding how to assess a pCR, the ideal timing for a clinical, radiological and pathological assessment of response, the uncertainty of the long-term efficacy of this strategy and new follow-up protocols are all factors that currently influence the surgical implication of genomic profiling [85]. Of note, the decision of when to have surgery after chemoradiation is still an important issue. Patients should be given adequate time to recover from chemoradiation-associated toxic effects and sufficient time should be allowed for the tumor to respond to treatment. The optimal time-frame between neo-adjuvant treatment and surgery remains unclear and is most probably dependent on the specific tumor as well as on the individual patient. However, retrospective data in patients with rectal cancer and oesophageal cancer indicate that, in general, delaying surgery after neo-adjuvant therapy improves neo-adjuvant treatment response and decreases surgical complications [86;87]. These studies reported an increased pCR rate among patients who had a greater time frame between neo-adjuvant treatment and surgery [86;87], and an improved 5-year survival and a lower recurrence rate [88].

Finally, an important issue is that if genomic profiling is performed on tumor biopsies prior to the targeted neo-adjuvant treatment, the genomic signature identified might not be factual as the treatment could alter the genomic profile of the remaining tumor, possibly resulting in unreliable prognostication and prediction of adjuvant treatment benefit owing to this prespecified genomic profile [62]. Hannemann *et al.* [62] analyzed changes in gene expression patterns of breast tumors induced by chemotherapy, and compared the profiles of the pretreatment tumor-biopsy with the profiles of the remaining tumors after treatment. The researchers found that major changes in gene expression in locally advanced breast cancer were observed in responders to neo-adjuvant treatment, defined as patients with a tumor shrinkage >50%, but not in patients with resistant tumors [62]. Furthermore, Buchholz *et al.* [89] showed that genomic profiles of biopsies obtained from one patient before treatment or 24h and 48h after initiation of treatment clustered together more than samples obtained from different patients with comparable tumor stage [89]. The fact that no differences were observed before and after treatment in the study from Buchholz *et al.* [89] might be due to the time-points chosen for the biopsies. In fact, changes in gene expression might only occur at later time points (after 48 h). From a surgeon's perspective, neo-adjuvant-induced tumor shrinkage is desirable as it leads to less extensive surgery with a higher chance of free surgical margins. However, not knowing the blueprint of the tumor left behind when radical surgery is avoided still leaves us in the dark. Overall, the value of this prespecified genomic tumor biopsy profile before neo-adjuvant treatment is largely unknown, owing to the fact that redetermination of the genomic profile of the remaining tumor after neo-adjuvant treatment cannot be ruled out.

CONCLUSION

The multimodality treatment of cancer has witnessed an increasing influence of genomic profiling in clinical decision-making. The complex interplay of genetic and epigenetic alterations in our genomes leads to disrupted biochemical interactions in multiple pathways, which are responsible for tumor development (Box 1). Ultimately, identifying these genomic abnormalities will lead to accurate prediction of tumor recurrence or to cancer-related death, non-responsiveness to therapy, and might even provide potential new targets for cancer therapy.

BOX 1: IMPACT OF EPIGENETIC CHANGES ON SURGERY

Epigenetics, including DNA methylation and histone modifications, is defined as the study of inherited changes in gene expression or cellular phenotype, caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic changes have shown to be critical for the development and progression of all cancer types [93-95]. Of note, these changes are intrinsically reversible and are therefore attractive targets for therapeutic intervention [93;96-98]. Drugs for both DNA methyl transferases (DNMTs) and histone deacetylases (HDACs), involved in addition of methyl-groups to DNA and removal of acetyl groups on histone tails, are available [99;100]. DNMT inhibitors have shown promising results in cancer therapy, but unfortunately their activity is genome-wide rather than targeting specific genes [101]. A number of HDAC inhibitors have been designed to drive re-expression of aberrantly silenced genes, leading to inhibition of cell proliferation, hormone receptor reactivation and/or apoptosis [102]. In the future, these directed epigenetic treatments could potentially have the same impact on surgery as seen with targeted neo-adjuvant chemotherapy after biopsy profiling. Furthermore, epigenetic changes can be detected in tumor-derived DNA in stool, tissues or blood [103-105], allowing the use of epigenetic markers in a clinical setting. This advance could lead to earlier tumor detection with an indirect impact on surgical care, influencing extent and timing of surgery with less delay in surgical intervention [106].

In prostate cancer, DNA hypermethylation of glutathione *S*-transferase pi 1 (*GSTP1*) [107] can be detected in urine, serum and ejaculate [108], which was able to increase sensitivity of prostate cancer diagnosis [109] and distinguish between primary cancer tissue and benign tissue [110].

In CRC, identification of hypermethylation of *P16* [111] , *DAPK* (death associated protein kinase)[112], *RUNX3* [113] and *ALX4* (aristaless like homeobox-4) [114] in blood or stool also served as a screening tool. Recently, a panel of highly sensitive and specific biomarkers for methylated DNA in plasma was identified, which resulted in three genes (*TMEFF2*, *NGR2* and *SEPT9*) specific in discriminating healthy subjects from patients with colorectal neoplasia [115].

It is hoped that these screening methods will lead to earlier tumor detection, however, this will not necessarily translate to increased survival and reduced mortality. Future studies, especially randomized controlled trials are warranted to tackle these issues and increase sensitivity of this exciting diagnostic field.

In current clinical practice, surgery still is the cornerstone of cancer treatment and the most valuable outcome predictor. Whereas some single-gene mutations described

here have successfully impacted on cancer surgery, genomic tumor profiling has no direct impact on surgical decision-making, thus far. Today's research, however, is showing promising results, in particular genomic profiling of tumor biopsies, before and/or after targeted neo-adjuvant treatment, may result in less-extensive surgical techniques owing to optimal tumor shrinkage, or even lead to a wait-and-see approach.

The data disscussed in this Perspectives article are mainly derived from retrospective analyses in prospectively designed studies. These studies were not conducted in a randomized setting; therefore, confounding may be present. Furthermore, patient numbers were often limited, thereby decreasing statitiscal power and clinical significance. Currently, two large randomized controlled trials in the adjuvant setting are ongoing, where according to risk stratification using Onco*type* DX® or MammaPrint®, patients are randomly assigned for adjuvant chemotherapy in the TailorX or Mindact Trial, respectively [53;90]. The results of these trials will help define the true surgical implication of genomic profiling.

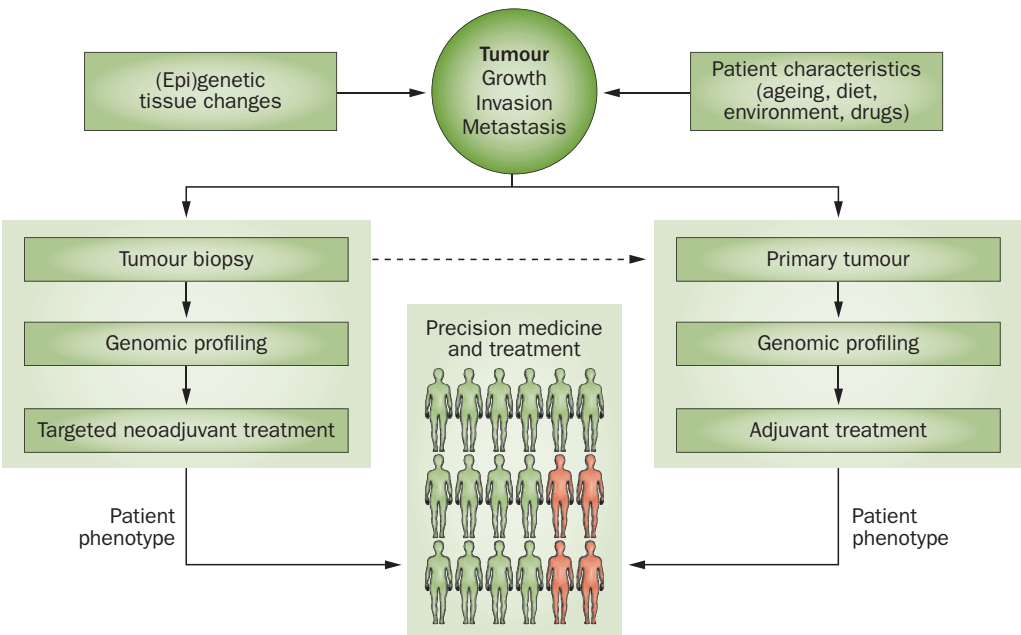

Figure 2: Global overview of the effect of genomic profiling on precision medicine
This figure shows the effect of genomic profiling on precision medicine. (Epi)genetic tissue changes and patient characteristics influence tumor growth, invasion and metastasis. Genomic profiling can result in targeted neo-adjuvant treatment and adjuvant treatment through profiling of tumor biopsies or primary tumors consecutively, with as main goal targeted treatment of the individual patient, better known as precision medicine. However, a patient's phenotype, e.g. comorbidities, frailty and poly-pharmacy, must be taken into account for optimal targeted treatment and to reduce therapeutic morbidity, as written in the discussion session.

More comparable trials, for example, in the neo-adjuvant setting, are needed with the aim of limiting the extent of surgery.

Molecular targeted therapy might radically alter cancer treatment in the future and have the potential to greatly improve cancer survival by delivering the most effective drugs to the right patients [91]. Nevertheless, the treatment of cancer, especially in older patients or in patients with multiple comorbidities, should also take into account these comorbid conditions, quality of life, patient resilience, and preferences. Despite the great contribution of genetics and genome profile to cancer therapy, considering only the sum of genetic aberrations in cancer is insufficient for developing and deciding adequate cancer treatment, especially in elderly patients. In the USA, the estimated number of cancer patients older then 65 years of age will rise from 850,000 cases in 2012 to 1.3 million in 2025 [92]. This population is characterized by a great heterogeneity in terms of comorbidities, quality of life and patient preferences. These factors are as crucial as the molecular signature of the tumor in the multidisciplinary approach to cancer. Thus, phenotypic profiling must be part of the vanguard of cancer research (Figure 2).

In conclusion, genomic profile-directed cancer therapy is still in its infancy. Much more is expected from this field of research, which might contribute to precision medicine in the future of cancer treatment. Currently, it is not clear if genomic profiling will ever gain full ground in direct surgical decision-making. It might contribute to improved informed decision and better outcome, however, surgery still is, and will remain the most important cornerstone in cancer management.

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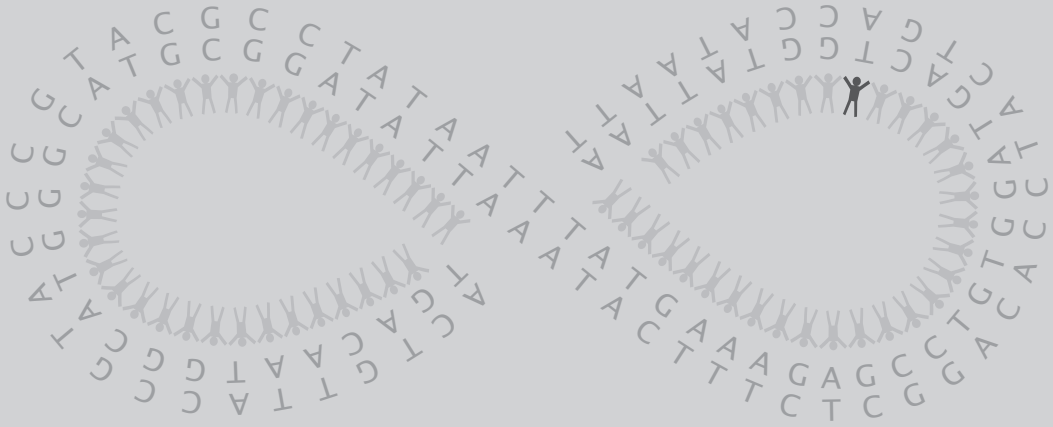

Chapter 12

Summary and General discussion

Over the past decades, major paradigm shifts have occurred in the treatment of breast cancer. The introduction of population screening, new surgical techniques and (neo)adjuvant therapies greatly improved clinical outcome of breast cancer patients. Examples hereof are the introduction of the sentinel node procedure, neo-adjuvant systemic therapy in patients with locally advanced breast cancer aiming for down-staging of the tumor, thus enabling breast conserving surgery, and adjuvant endocrine treatment [1-3]. Even though major advances in the treatment of breast cancer have been made, mortality remains high. In the Netherlands, each year approximately 14000 patients are diagnosed with breast cancer and 3200 deaths occur as a consequence of this disease [4].

Morbidity associated with current therapy regimens should not be underestimated. Apart from the well-known adverse events of chemotherapy, such as nausea, neutropenia and alopecia, anti-HER-2 and endocrine treatment also harbor a vast array of unpleasant side-effects. For example, anti-HER-2 treatment, Herceptin, is associated with increased cardiotoxicity and hormonal treatment is notorious for life threatening adverse events such as pulmonary embolisms and endometrial cancer, but also less severe incidentals such as hot flashes, vaginal dryness, osteoporosis and musculoskeletal adverse events such as arthritis, arthrosis, arthralgia and myalgia [4-7]. Therefore, prescription of (neo) adjuvant systemic treatment should not be done without careful consideration of the tumor- and patient characteristics.

With the increasing pathological knowledge, tumor classification has become more complex over the last years. Currently, prognostication and treatment allocation are still majorly influenced by tumor stage (TNM) [8;9]. Retention thereto leads to under-treatment and over-treatment [10;11]. Therefore, the use of merely TNM tumor stage for treatment allocation in daily medical practice falls short, and needs to be supplemented with additional tumor biomarkers and patients characteristics that can improve current staging and treatment allocation criteria substantially. Predicting the clinical behavior of a tumor and ultimate clinical outcome of a patient through a combination of clinical, pathological, and biological characteristics will lead to well-targeted treatment of individual patients, hereby increasing treatment benefit and limiting unnecessary adverse-events.

In this thesis we contributed to the foundation for the introduction of precision medicine by evaluating prognostic and predictive biomarkers in breast cancer. The ultimate goal is to improve risk stratification and thus treatment benefit in the individual patient.

This thesis is divided into four parts. In ***part I*** we investigated biomarkers related to important hallmarks of cancer, which were able to adequately assess clinical prognosis in breast cancer patients. In ***part II*** we established the importance of predictive biomarkers, such as human epidermal growth factor receptor-2 (HER-2) and insulin growth factor

1-receptor (IGF-1R), in order to predict who could benefit from directed treatment after breast cancer diagnosis. The potential implication of these (neo)adjuvant therapies on breast cancer in the older is also discussed. In ***part III*** the effect of aging, the most potent risk-factor for oncogenesis, and the entailed metabolic reprogramming, are studied in both healthy and cancer tissue and correlated with the clinical characteristics and clinical outcome of the patients. Finally, in ***part IV*** we discussed the use of prognostic and predictive biomarkers in clinical practice, its utility and the road to precision medicine. Lastly, in ***part V*** the future perspective is discussed.

PART I. PROGNOSTIC BIOMARKERS IN BREAST CANCER

In 2000, Hanahan and Weinberg published an important paper titled: 'the hallmarks of cancer', which initially were six biological capabilities a cell needs to acquire during the multistep developmental process to become a cell with malignant characteristics, ultimately resulting in a full-blown tumor cell. The hallmarks Hanahan and Weinberg proposed are: 1. Sustaining proliferative signaling, 2. Evading growth suppressors, 3. Activating invasion and metastasis, 4. Enabling replicative immortality, 5. Induction of angiogenesis, and 6. Resisting cell death [12]. Eleven years later, in 2011, reprogramming of energy metabolism and evasion of immune recognition were added to the already existing hallmarks [13]. With the addition of the latter two hallmarks, the importance of the tumor-microenvironment in tumor development was taken into account. These cancer hallmarks constitute an organizing principle for rationalizing the complexity of neoplastic disease. Validation and recognition of these much discussed cancer hallmarks will increasingly effect prognostication and effect new means to tackle human cancer [12]. In the first part of this thesis we investigated biomarkers related to these cancer hallmarks, such as sustained proliferative signaling, apoptotic resistance and evasion of immune recognition.

In **chapter 2**, we performed a combined analysis of a proliferative biomarker, Ki67 and apoptotic biomarkers *p*53 and cleaved caspase-3. The inability to undergo apoptosis and the presence of continued proliferation are thought to contribute to tumorigenesis and tumor progression [14-16]. Over the course of years, research performed in this field often showed contradictory results [17;18]. We believe that these contradictory results could be attributed to the fact that a key factor in tissue homeostasis is the balance between the level of cell proliferation and cell death, and that disturbance of this balance could contribute to initiation and maintenance of oncogenesis and tumor growth [12;13]. In our study we circumvented the shortcoming of previous studies by combining the dual markers and constructing an apoptotic-proliferative subtype model. Our study showed

that patients with a high apoptotic marker rate, cleaved-caspase-3, counterintuitively showed worse clinical outcome. However, in the combined analyses, high apoptosis was significantly associated with worse outcome in the presence of a high proliferation rate, indicating that the high proliferation rate outplays the high apoptotic rate, ultimately leading to worse clinical outcome. These data stress the importance of combined analyses for such finely balanced markers, both in immunohistochemical as well as biochemical assays, as is indicated in our study. Furthermore, our study showed that the combination of the two apoptotic (cleaved-caspase-3 and *p*53 status) and proliferative marker (Ki67) into an apoptotic-proliferative subtype model, was also significantly associated with clinical outcome in 488 stage I-III breast cancer patients with respect to overall survival and relapse-free-period. Patients with high proliferation and cellular apoptosis and a mutated *p*53 status had the worst survival and relapse rate outcome. However, only in stage I breast tumor patients this clinical association remained statistically significant in the adjusted analyses. This observation leads to assume that the apoptotic-proliferative subtype model could be of crucial importance in identifying patients with a low tumor grade with an increased risk of poor prognosis, being those containing the most detrimental apoptotic-proliferative marker combination. With the current tendency of earlier breast cancer diagnosis, partly due to better breast cancer awareness and the introduction of population based screening, a shift is seen in the advantage of more early stage detection [19]. Clinical introduction of the apoptotic-proliferative tumor subtype model could lead to targeted selection of the grade I breast cancer patients that would truly benefit of an aggressive therapeutic regime due to an adverse apoptotic-proliferative balance. Especially in current medical practice, which hosts considerable debate on under and overtreatment, identification of patients for the implementation of targeted therapy will continue to conquer ground. Therefore, further research is needed to elucidate the importance of these two hallmarks in light of today's breast cancer related therapeutic standards.

The last decades, research has proposed a substantial influence of the immune system on tumor growth, which showed to be both tumor suppressing and promoting [20]. In **chapter 3,4 and 5** we investigated the prognostic value of important immune recognition evading mechanisms in breast cancer by analyzing classical HLA class I expression on tumor cells; tumor expression of non-classical HLA class I; HLA-E and HLA-G; cytotoxic T-cell tumor infiltration; natural killer cells (NK cells) tumor infiltration and infiltration of immunosuppressive regulatory T cells (Tregs) in the tumor. The goal of these studies was to determine a tumor immune profile based on biomarkers reflecting a tumor's immune susceptibility status and to correlate this with the clinical outcome of each patient.

Previous research has briefly touched on the importance and the complexity of the interaction between breast tumor cells and cells from the immune system [21]. Evidence is accumulating stating that such interactions should be accounted for; therefore we defined tumor immune subtypes, based on tumor expression of immunogenic and immune evasive cellular immune markers.

For 293 breast cancer patients in the training cohort and 219 breast cancer patients in the validation cohort (**chapter 3**), a significant association was found with relapse-free-period and relative survival. Both, relapse rate and relative survival showed worse outcomes in the low immune susceptible tumor types, compared to intermediate and high tumor immune susceptibility. High tumor immune susceptibility was characterized by cytotoxic T-cells being able to recognize tumor-associated antigens presented by classical HLA class I and absence of Tregs (presence of HLA class I, CD8+ cytotoxic T-cells, without Tregs) or, in case of lack of classical HLA class I expression on the tumor surface, resulting in escape of cytotoxic T-cell recognition, natural killer cells come into play and recognize and destroy the diseased cells (loss of classical HLA class I, no expression of HLA-EG, present infiltration of NK cells without infiltration of Treg). Intermediate immune recognition is identified by classical HLA class I expression in the tumor surface, with a lack of cytotoxic T-cell presence or the abundant presence of immune suppressive Tregs, resulting in limited anti-tumor immune reaction. Finally, low immune susceptibility is characterized by the lack of classical HLA class I expression on the tumor cell surface, either in combination with lack of natural killer cells presence, or Treg presence, which results in diminished natural killer cell recognition due to its immune suppressive effect, or lastly, HLA-E and HLA-G presence, resulting in diminished natural killer cell ability of tumor attack. In summary, this study showed a complex and multifaceted interplay between immune cells and tumor cells, resulting in different immune escape mechanisms, highlighting the need for combined immune marker analysis to better reflect patient outcome. In this study we were able to determine three distinct survival patterns in breast cancer based on immune surveillance and escape, which represented significant independent clinical prognostic value in breast cancer patients. Furthermore, evidence is emerging that treatment response is in part regulated by the tumor immune microenvironment [22]. If this holds true, the value of comprehensive determination of the tumor immune status is unthinkably important. Future research should therefore focus on the association between tumor immune susceptibility, preferably taking into account the interplay between immune surveillance and escape, and treatment response.

In **chapter 4**, we investigated the prognostic relevance of the same immune markers as in chapter 3, and the molecular intrinsic breast tumor subtypes in invasive ductal carcinomas and invasive lobular carcinomas separately.

Research has consistently shown that compared to invasive ductal carcinomas, invasive lobular breast tumor tend to have a single-file growth pattern, are larger, more often hormone receptor positive, and harbor a less aggressive character [23;24]. Nevertheless, these two types of breast cancer are still treated similarly, which is largely driven by known classical tumor characteristics such as tumor size, histological grade, hormone receptor status and HER2 status.

Gene expression studies have identified at least four distinct molecular breast cancer subtypes with marked differences in patient prognosis: Luminal A and B, basal-like tumors and tumors overexpressing HER-2. As described above, there is also strong evidence that the breast cancer host's adaptive immune system and the tumors ability to circumvent immune recognition, play a crucial role in the control of tumor growth and progression [25;26]. The aim of this study was therefore to investigate the relevance of the host immune response, the apoptotic-proliferative interaction and molecular tumor types in the two major histological subtypes of breast cancer.

Our results showed no significant difference between invasive ductal and invasive lobular breast tumors with regard to their association with tumor immune subtypes and molecular intrinsic tumor subtypes. Suspicion of the influence of tumor histology on the prognostic value of immune and molecular subtypes was confirmed by a significant effect modifications in the interaction term for immune subtype, the combined cleaved caspase-3- proliferative Ki67 marker and the molecular intrinsic tumor subtypes in relation to relapse rate. In invasive ductal tumors, low tumor immune susceptibility, high cleaved caspase-3 and high proliferative Ki67 expression were associated with a worse relapse free period. This was not seen for invasive lobular tumors, suggesting that neither the apoptotic or proliferative marker, nor immune profiling applies to invasive lobular carcinomas.

Immune profiles were strong prognostic indicators in Luminal A tumors only. This confirms that tumor aggressiveness, as established by the molecular intrinsic subtype of breast cancer, is not dependent on a tumor's immunological profile. Luminal A tumors make up the largest group of invasive ductal breast tumors. Therefore it is not surprising that these results show a similar prognostic association within the immune profiles. Proposed is that invasive lobular tumors harbor characteristics, such as having a high probability of being hormone receptor positive, HER-2 negative, and with a low cellular proliferation rate, making them very probable to be characterized as a Luminal A molecular breast tumor subtype [27]. However, the assumption that therefore molecular and histological subtypes are similar, was not confirmed in our study, implying that a simple extrapolation cannot be made and that breast tumor(s) (subtypes) are presumably far more complex.

Although frequently treated as similar entities, there are obvious differences in tumor-biological and prognostic characteristics for the two major histological subtypes.

Therefore, in order to provide breast cancer patients with the best, targeted treatment it should be stressed that the urgent call for differentiation, especially in therapeutic sense, between these two major histological breast tumor subtypes should be answered. It is of utmost importance that research is performed focusing on the therapeutic sensitivity of these histological breast tumor subtypes, in which, next to classical tumor characteristics, the immune and molecular tumor characteristics should also be accounted for.

In **chapter 5** the difference in prognostic value of tumor immune subtypes in relation with type of hormonal treatment received in hormone receptor positive, postmenopausal breast cancer patients was investigated. Patients of the TEAM trial, consisting of treatment with either exemestane, 25mg daily for five years, or sequential therapy consisting of tamoxifen 20mg daily for 2.5 years followed by exemestane 25mg daily for another 2.5 years [3], allocated in a 1:1 ratio were included in this study. Elaborating on the fact that tumor-associated lymphocytes act as an independent predictor of response to chemotherapy treatment [28;29], evidence also exists for an immunomodulatory effect of the estrogen receptor blocker tamoxifen, inducing a shift from cellular (T-helper 1) to humoral (T-helper 2) immunity [30]. One could speculate on the importance of T-helper 1 immunity for anti-tumor immune response. A shift away from cellular immunity may represent a significant step in tumor development, which could explain the differential effect of aromatase inhibitors versus tamoxifen on clinical outcome [30;31]. Patients assigned to sequential hormonal therapy showed a significant preferential outcome in the adjusted analysis for high FoxP3+ presence in the overall survival. This was not seen for patients in the exemestane only treated arm. This outcome was supported by a significant interaction term for endocrine treatment and FoxP3+ presence in the tumor. This outcome is in stark contrast with the previous studies we performed on tumor immune modulation and cancer development. This result could be explained by the proposition that Tregs harbor a dual role in cancer: being 1. suppressing anti-tumor immune response, known as inducible Treg, and 2. suppressing inflammation which is known to promote carcinogenesis (natural Treg) [32]. It is thought that the clinical and prognostic significance of Tregs in cancer depends on its environmental factors. Given the fact that the TEAM patients are post-menopausal, known for its association with increased systemic inflammation, and are hormone receptor positive [33], herewith attracting higher estrogen levels in and around the tumor due to an increased tendency of estrogen binding, we propose that this milieu leads to more degradation of Adenosine (ADO), a potent anti-inflammatory agent [34;35]. Thus, this line of thought would assume a preference for natural Tregs and would also explain the loss of prognostic significance in solely exemestane treated patients, as aromatase inhibition leads to lower estrogen levels, diminishing ADO degradation. In addition, only for the sequentially endocrine treated TEAM patients the tumor immune subtypes were of significant prognostic value. However, merely a

statistical trend was seen for the interaction between endocrine treatment and tumor immune subtypes in the multivariable interaction model. Given this outcome, one could postulate that the immune profile of breast tumors in sequentially endocrine treated breast cancer patients could predict breast cancer death and overall death in this subset of breast cancer patients, on which additional adjuvant therapy could be allocated.

The result of this study cannot be explained by the previously proposed tamoxifen driven shift from Th1 to Th2 immunity [30]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors, by means of other immune interactions, have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift. Another possibility for the loss of prognostic value of the tumor immune subtypes in exemestane-treated patients could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published previously, proposing a significant increase in the CD8+/Treg ratio in estrogen receptor positive patients responding well to aromatase inhibiting therapy and an observed decrease in FoxP3+ after letrozole treatment [36;37]. One could hypothesize that exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor immune subtypes in the solely exemestane treated adjuvant treatment arm. If this proves true, one could speculate on the great importance of Tregs for inhibition of tumor development in post-menopausal, hormone receptor positive breast cancer patients.

In **chapter 6** of this thesis the prognostic value of the molecular intrinsic breast tumor subtypes in the older breast cancer patient was determined. With four described subtypes, molecular breast tumor classification shows promising prognostic results in modern-day molecular diagnostics [38-40]. Luminal A and B, which are mostly hormone receptor positive and express high amounts of genes related to the luminal epithelial cell layer [38-40], possess the most indolent characters. The basal like tumors, which are triple negative tumors combined with expression of genes characteristic of the basal epithelial layer such as cytokeratin 5 and 6; and the ERBB2 tumor subtype, which clusters near the basal-like subtypes, but expresses high HER-2 on the tumor surface, are both characterized by more aggressive phenotypes, leading to unfavorable outcome [39]. It should be noted that, unlike the HER-2 allocation group in chapter 8 of this thesis, all HER-2 2+ expressing tumors in this study were considered HER-2 negative, due to the lack of confirmatory in situ hybridization and the fact that these elderly patients did not show a significant difference in clinical outcome compared to patients harboring breast tumors with HER-2 scores of 0 and 1+ (chapter 8).

It was proposed that the molecular breast cancer subtypes have a different distribution in older breast cancer patients compared to their younger counterparts [41], and that its prognostic distinction is lost. However, given the fact that that study only included 189 breast cancer patients above the age of 65, leading to very limited discriminative power in the statistical analyses, we felt that validating the prognostic outcome of this previous study in a larger older breast cancer cohort is a valuable contribution. In our study the molecular intrinsic breast tumor subtypes were of significant prognostic value in the older (≥65years) breast cancer population. In accordance with current knowledge, our results were indicative of a higher relapse rate in the ERBB2 and basal molecular breast tumor subtypes and a poor relative survival for all the molecular breast tumor subtypes compared to the Luminal A subtypes. The distribution of the molecular-intrinsic breast tumor subtypes in this older breast cancer population showed a higher prevalence of the more indolent Luminal A tumor and a relatively low prevalence of the more aggressive molecular tumor subtypes compared to the numbers known for the younger breast cancer population [42]. Thus, the chance of getting a more aggressive molecular tumor subtype decreases with increasing age, which is in accordance with the observation of milder tumor characteristics in the older breast cancer population. Furthermore, our results prove the prognostic value of the more aggressive tumor subtypes in the older breast cancer population, reflected in higher relapse rates and worse relative survival.

This is the first study performed in a large older breast cancer cohort, showing significant prognostic value of the molecular breast tumor subtypes, even after taking the risk of competing mortality into account. Therefore, we support the use of molecular subtyping in the older breast cancer patients for prognostication and consequently therapy allocation. However, given the increasing age, we stress that the importance of the functional status of the older breast cancer patient, and the individual treatment wish should not be buried under the molecular force of modern day's diagnostics.

PART II. PREDICTIVE BIOMARKERS IN BREAST CANCER AND TARGETED TREATMENT

Signaling via the Insulin-like Growth Factor type 1 Receptor (IGF1R) plays a crucial role in the development of many cancers, including breast cancer [43;44]. It was shown that IGF1R expression is correlated with the expression of the estrogen receptor [45], and that 17β-Estradiol, although to a lesser extent than IGF1, can activate a linear pathway involving the activation of IGF1R, resulting in a boost of the mitogen-activated protein kinase (MAPK) [46;47]. We proposed that patients treated with an aromatase inhibitor could lose this additional tumor growth-stimulating pathway due to complete blockage of estrogen production, independent of IGF1 stimulation. Furthermore, metformin, which

has long been known for lowering plasma insulin and insulin growth factor levels by increasing insulin sensitivity [48], and thus leading to less IGF1R binding, has also been suggested beneficial in breast cancer treatment [49;50]. In **chapter 7** of this thesis, we performed a sub-study analysis in 2.446 Dutch patients of the TEAM cohort, investigating the clinical effect of exemestane and metformin treatment on IGF1R expression of the tumor in a hormone receptor positive breast cancer cohort. Results of this study showed a significant improvement in relapse free survival in patients treated with exemestane harboring breast tumors with high IGF1R expression their surface, compared to sequentially endocrine-treated patients. No association was seen in low IGF1R expressing tumors. Metformin use in addition to endocrine therapy, resulted in further improvement of the relapse free survival and overall survival in patients harboring high IGF1R expressing tumors treated with exemestane. These interesting findings are in contrast with the main results of the TEAM-trial, which showed no difference in OS, BCSS nor DFS for the two treatment arms [3]. There may be several explanations for the observed benefit of exemestane in patients with high IGF1R expression. Evidence is building for the potential of estrogen to, next to binding and activating its classic estrogen receptor, also phosphorylate and activate the IGF1R [47]. Our results lead to speculate that the interaction between the degree of IGF1R expression on the tumor surface and the efficacy of exemestane is mainly induced by suppressed estrogen production, resulting in reduced estrogen-induced activation of IGF1R and thus less activation of the mitogen-stimulating pathway. This theory also supports our finding that patients with high IGF1R expression who were treated with tamoxifen did not experience a clinical benefit, as these patients still have circulating estrogen in their system, which is able to activate the IGF1R, thereby stimulating breast cancer cell growth. The fact that no clinical benefit of exemestane treatment was observed in patients with tumors harboring low IGF1R expression is also in support of our hypothesis, as the effect of estrogen induced growth promoting signaling through IGF1R is too small in these tumors. We propose that the additive therapeutic effect of metformin is induced by direct lowering of the IGF concentration. In patients with high IGF1R expression on their tumor surface, treatment with exemestane and metformin leads to a dual blockage of the ER-IGF1R crosstalk, resulting in better clinical outcome. By stratifying patients according to IGF1R expression of the tumor, which is up-regulated in roughly two-thirds of the postmenopausal breast cancer population and thus widely applicable, it may become possible to identify a subgroup of patients who may benefit of these combined treatments, thereby further individualizing treatment and improving outcomes for particular subgroups within the heterogeneous BC population. Lastly, we feel that these findings may especially be of interest for the older breast cancer population. Since older patients are at increased risk of toxicity of chemotherapy [51], and around 80% of tumors in older patients are hormone receptor-positive, they are frequently treated with endocrine therapy only [52]. As breast

cancer mortality increases with age, which may be explained by both undertreatment and overtreatment [53], new treatment strategies for this group of patients are highly warranted, preferably with a low toxicity profile. In this study it is proposed that the effect of exemestane may be enhanced by adding metformin without causing additional toxicity, since metformin is a well-tolerated drug with few side effects, thus being a drug with immense potential in the treatment of older patients with breast tumors expressing high IGF1R on their surface.

In **chapter 8** we investigated the potential restoration of the clinical interest of anti-HER-2 treatment in the older breast cancer patients. It is known that HER-2 overexpression is associated with a more aggressive tumor phenotype [54], resulting in worse clinical outcome [55;56]. Treatment of HER-2 overexpression improves clinical outcome in both node negative and node positive breast cancer disease [57;58]. Aberrant activation of the Phosphatidylinositol 3-kinase (PI3K)/AKT pathway by PIK3CA mutations, which often co-occurs with HER-2, results in tumor growth promotion [59], and diminishes response to HER-2-directed therapies [60;61]. A well-known shortcoming in current clinical research of breast cancer patients is that the majority of the studies elucidating the value of biological tumor markers are mainly performed in the younger breast cancer population, impeding extrapolation of study outcomes to the elderly. Foregoing, in combination with increased incidence of cardiac adverse events due to anti-HER-2 treatment resulted in fear of the administration of this drug in the older population. However, evidence for the omission of anti-HER-2 therapy in this specific subset of breast cancer patients is lacking. Therefore, we believe that research is needed to confirm or refute this non-evidence based clinical practice.

This study showed, in 1698 breast cancer patients of 65 years or older (FOCUS cohort), that patients with a HER-2 score of 3+ had a significantly higher risk of recurrence at 5-years post-diagnosis and a worse 10-year relative survival, compared HER-2 negative patients, even when competing risk of mortality was taken into account. Interestingly, patients with HER-2 2+ tumors had a similar recurrence risk as patients *without* HER-2 overexpression. PIK3CA mutations were not of prognostic value for recurrence risk or relative survival in this specific breast cancer population, neither after stratifying for HER-2 status.

These results imply that older patients with HER-2 3+ tumors might benefit from anti-HER-2 treatment. Recent studies have shown that the often severely dreaded, mainly cardiac related adverse events, are in practice less severe and present with a lower incidence than initially thought [62;63]. In current medical practice, anti-HER-2 therapy is frequently omitted from treatment options in the older breast cancer patients due to these shuddered adverse-events, in combination with an often already limited life expectancy. Given the results of this study, it could be suggested that the fit-older breast cancer

patient, in good cardiac health, should be treated with the same adjuvant-regimen as the younger HER-2 positive breast cancer patients. Older patients with less desirable clinical conditions, or with a strong preference to omit chemotherapy, dual HER-2 blockage could be considered. One of the major characteristics of the older cancer population is the heterogeneity patients of the same chronological age. We believe that, if no clear distinction is made between fit and frail older patients, care tends to fall-short for the fit older population, resulting in unfair survival chances. Future research should point out whether it is possible to establish an effective anti-HER-2 regimen with minimal toxicity for the older breast cancer population. It is for this reason that, in this current study, we investigated the difference in expression of HIF-1α and its associated target genes in normal breast tissue and in breast tumor tissue of both young and old patients, and we hypothesize that HIF-1α and its related target genes will be highly expressed and involved in tumor development and maintenance in the older breast cancer population and less so in their younger counterparts.

PART III. AGING IN THE BREAST CANCER PATIENT

Of all the factors that contribute to cancer, aging is the most potent [64].

The multi-hit or Knudson hypothesis, states that cancer occurs more frequently as we age because time is necessary for genetic mutations to accumulate and push these cells over a certain mutagenic threshold [65]. What this hypothesis fails to explain is why cancer risk is greatly reduced by calorie restriction and physical exercise [66]. During aging, the decline in nuclear nicotinamide adenine dinucleotide (NAD^+) levels, leads to a reduction of Sirtuin 1 (SIRT1) activity in the nucleus, causing Von Hippel-Lindau (VHL) to decline and HIF-1α to be stabilized [67]. This age-induced stabilization of HIF-1α, leads to a so-called pseudo-hypoxic state that disrupts oxidative phosphorylation (OXPHOS), thus initiating a Warburg-like state. The subsequent increase of reactive oxygen species (ROS) may establish an environment for subsequent mutations leading to carcinogenesis, which helps to explain why cancer risk increases exponentially as we age [67;68]. The age-induced metabolic decline as a driver of tumorigenesis is also known as "geroncogenesis".

Although hypoxia is toxic for the cell, cancer cells can adapt by genetically modifying oneself to survive, and even proliferate in these stressful conditions. Known cell response to (pseudo) low tissue oxygen levels is through up-regulation of hypoxia-inducible factor-1 (HIF-1). In the (pseudo) absence of oxygen, HIF-1 binds to hypoxia-response elements (HREs), which activate the expression of numerous hypoxia response genes [69]. Known HIF-1 target genes are involved in cell proliferation, angiogenesis, inflammation, metabolism, apoptosis, immortalization, and migration [69;70]. In **chapter 9** we show an increase of HIF-1α mRNA expression and that of its target genes in breast

tumor compared to the normal breast tissue, however this was only seen in patients of 65 years or older, even though there was no significant difference in the pathological tumor stage, grade and tumor morphology for patients <65 years compared to patients of 65 years of age or older. It was noted that for HIF-1α and its targets, the same trend between normal and breast cancer tissue as that observed in the older patient group was also seen in the younger patients of this cohort, implying that HIF-1α and its targets undoubtedly play a role in tumor development of the younger breast cancer patients, but is less stringent then in patients above the age of 65 years. An explanation could be that healthy cells from an older patient are already primed with high HIF-1α expression due to the so-called age-induced HIF-1α stabilized pseudo-hypoxic state, as proposed by Gomes *et al.* [67]. Tumor development, known for its high HIF-1α expression [71], in an already HIF-1α primed environment, results in an exponential increase of HIF-1α in the tumor. Proposed is that the significantly higher HIF-1α expression in the breast tumor of the older breast cancer patients, plays an important role in the more aggressive, and less therapy sensitive character of breast cancer in the old [53]. Therapeutic blockage of HIF, by means of antisense HIF-1α [72], or up-regulation of the VHL gene [73], would result in a reduction of tumor growth, due to a disruption in neovascularization and metabolic reprogramming, which could lead to better clinical outcome. If proven successful, this very promising novel pharmacologic approach to cancer will, based on the expression profiles presented in our study, be of special interest for the older breast cancer patients.

The above-mentioned metabolic shift away from oxidative phosphorylation towards aerobic glycolysis is partly achieved and dependent on the glycolytic enzyme pyruvate kinase(PK) [74]. Normal cells express the pyruvate kinase M1 isoform (PKM1), tumor cells predominantly express the M2 isoform (PKM2). The latter catalyzes the last step of glycolysis and reprograms the glycolytic flux to feed the special metabolic demands of proliferating cells [74]. Over the last decades, PKM2 has identified itself as a promising therapeutic target for cancer treatment, but could potentially also contribute to anti-aging interventions.

In **chapter 10** we investigated the difference in expression of HIF-1α and its associated target genes, including PKM1 and 2, for patients between the ages of 65 and 80 years of age and older (≥80 years) patients in both normal breast tissue and in breast tumor tissue. Next, we investigated whether the degree of expression, or metabolic reprogramming is associated with clinical characteristics associated with aging and outcome. We showed that HIF1-α is significantly higher expressed in the normal breast tissue of the older patient, and that HIF1-α expression in the normal breast tissue is associated with a higher tumor grade of the adjacent tumor. PKM2 had significant association with functional surrogate markers like polypharmacy and difficulty walking, showing a

higher expression in the normal breast tissue of the older breast cancer population, with a potential negative effect on survival.

These observations strengthen the hypothesis that dysregulation of the HIF1-α metabolic pathway, leading to an increase in ROS, is closely related with the high cancer incidence seen in the older population.

On the other hand, our study also showed that high PKM2 *protein* expression in the breast tumor was associated with a significantly better disease free survival and a trend toward better relapse free period compared to patients with low PKM2 protein expression in their tumor, matching the findings of a previous study, showing that activation of PKM2 altered cancer metabolism *in vitro* and reduced xenograft tumor growth [75]. A possible explanation for this finding is the deficiency of precursors for the synthesis of building blocks, favored by dimeric PKM2, needed in high proliferative cellular states. Activation of PKM2 in the active tetrameric form thus inhibits cell proliferation [75;76], resulting in less cancer development and spread. Some advocate PKM2 activation a promising adjuvant treatment modality. However, presence of PKM2, and its importance in the aging process should not be underestimated and could limit its efficacy.

More research is needed to elucidate the potential contribution of HIF1-α and PKM2 on the aging process and the influence on tumorigenesis. If metabolic changes are indeed important drivers of aging and geroncogenesis, molecules that prevent, halt or reverse metabolic aging may be useful anti-aging and anti-cancer therapies. Promising advances have been made with regard to HIF1-α inhibitors, SIRT activators, and both inhibiting, targeting hnRNPA1, hnRNPA2 and PTB, and activating PKM2 treatment [76-79]. Based on current knowledge, it is highly likely that treatment leading to reversal or halting of aging and age-induced disease will experience a rapid development, with major clinical consequences in the coming years.

PART IV. PRECISION MEDICINE IN THE (OLDER) BREAST CANCER PATIENT

Over the course of years it is proven that the TNM stage of the tumor falls short in clinical practice and needs to be supplemented with additional biomarkers to substantially improve current staging and treatment allocation criteria. A lot of research has been dedicated to the discovery and development of clinical prognostic and predictive biomarkers, in order to improve diagnosis and to allocate optimal treatment modalities, introducing precision medicine in the multimodality treatment of cancer. By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and clinical outcome, and places the individual patient at the center of all elements. Genomic, epigenomic, patient and environmental data are studied altogether to understand individual disease patterns and to design

preventive, diagnostic, and therapeutic solutions. Over the last decades genomic profiling demonstrated its promising prognostic and predictive value in precision medicine, mainly in terms of systemic therapy. Therefore, it is increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning of the individual cancer patient. The added value of genomic profiling on surgical decision making is discussed in **chapter 11**. Apart from a handful of single-gene mutations, genomic tumor profiling in current clinical practice merely directly impacts surgical decision-making. Present-day, influence of genomic profiling on surgery is only seen in the context of profiling of the tumor biopsy, leading to a possible influence on timing, extent and type of surgery by means of optimal tumor shrinkage through targeted neo-adjuvant therapy. Possibly, this may also lead to a wait-and-see approach in case of pathological complete response. This possible influence is not without snags; important questions that need to be resolved are: what is the long-term efficacy of this strategy? Should new follow-up protocols be initiated, and when should the surgical intervention be planned?

To achieve optimum and swift introduction of precision medicine into clinical medical practice, some crucial steps should be warranted:

First, in order to increase clinical applicability, studies investigating biomarkers should focus on using standardized methods and comparable patient selection criteria in order to validate the results. Second, as current cancer research mainly focuses on the genotypical approach of cancer treatment, which is believed to alter cancer treatment radically in the near future, the phenotype of the patient is completely ignored. In the current greying society, it is not uncommon that cancer patients suffer from one or more comorbid conditions, increasing the risk of competing mortality, which therefore should be accounted for when making treatment decisions. Thus, parallel to the current golden standard: TNM stage, and the promising epigenetic and genetic fingerprint of the tumor, phenotypic profiling should not be neglected in the treatment approach of an individual patient. Lastly, medical specialists involved in cancer management need to join forces to create a collaborative multidisciplinary approach, providing the most efficient, functional and tolerated treatment for each cancer patient.

In order to introduce the individualized cancer treatment approach in to daily medical practice, it is required that the medical society is able to overcome these bumps in the road to precision medicine, with as ultimate goal optimal cancer treatment and control.

PART V. FUTURE PERSPECTIVES

The key for appropriate care in the notoriously heterogeneous older breast cancer population lies in the prediction of who will die *with* and who will die *from* breast cancer.

Therefore, the next phase in oncogeriatric research of breast cancer disease is aimed at personalized, tailored treatment.

General treatment decisions in the medical oncology practice are still largely driven by stratified tumor characteristics, such as hormone receptor and HER-2 status. Great efforts are being made to further define tumor characteristics aiming to individualize therapy for breast cancer patients. In order to facilitate this promising shift in treatment modality, future research should emphasize on investigating determinants and markers for tumor response, preferably in the neoadjuvant setting. Under these circumstances, pre- and post-systemic treatment tumor characteristics can be optimally investigated after surgical resection, and correlated with treatment response. Findings of these studies should shed light on what determines whether a tumor will show treatment response or not. These studies would be of enormous value for the older breast cancer patients with regard to the new, preferably minimally toxic treatment modalities, such as angiogenesis blockers, and metabolic stabilizers or reverser, but also for old drugs with potentially new indications, such as metformin.

A well-known shortcoming in today's medical practice and treatment decisions is the disregard of the older (>65 years of age) patients in the clinical trials on which current breast cancer treatment guidelines are based. Consequently, no proven effective guidelines for the older breast cancer population are in operation. It cannot be expected that clinical trials focusing on the older breast cancer patient will void this knowledge gap in the coming years. Therefore, in order to swiftly gain valuable information on the most optimal treatment of the growing older breast cancer patient, in whom, due to lack of feasibility or personal desire chemotherapy and/or surgery are regularly rejected, population based, observational cohorts consisting of older breast cancer patients should be looted of clinical patient and tumor information for research purposes. Expected is that especially the phenotypical, thus functional patient data, not abnegating the competing risk of mortality of each individual patient, will be important treatment drivers which are currently not always adequately accounted for. Thus, in geriatric oncology, it is recommended that treatment decisions are not (solely) based on calendar age. Currently, a lot of research is done in order to determine whether valuable (bio)markers can be identified which could reliably predict ones biological age. The aim of this development is to concise treatment decisions to: adequate anti-tumor treatment, leading to minimal residual disease or adequate supportive care, in order to maintain quality of life.

Only when we abide by the adages: 'treat first what kills first' and 'treat the patient, not the disease', can we achieve the two main treatment goals in the older breast cancer population: 1. prolong survival and 2. maintain acceptable quality of life. With regard to

the first goal; major developments are expected in the coming years, leading to more adequate tumor targets, resulting in optimized systemic therapies. The second goal can only be achieved if the patient's phenotype or functional status is taken into account. For example, frail hormone receptor positive breast cancer patients, with a high chance of dying from other disease, identified by appropriate aging (bio)markers, should preferably be treated with neoadjuvant hormonal therapy instead of neoadjuvant chemotherapy, in which systemic therapy associated toxicities should not be underestimated and if necessary, adequately dealt with.

Only when all medical specialities bound to the care and cure of older cancer patients join forces, better known as a multidisciplinary oncogeriatric battlefront, these treatment goals, and the implementation into daily clinical practice will be achieved.

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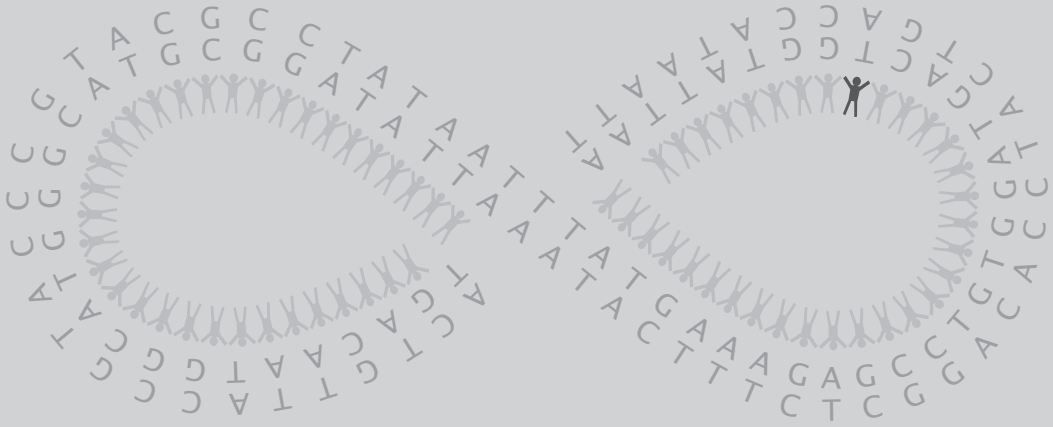

Appendices

Nederlandse Samenvatting
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Dankwoord

NEDERLANDSE SAMENVATTING

Borstkanker is de meest voorkomende maligniteit onder vrouwen in de Westerse wereld en is tevens een van de grootste veroorzakers van kankergerelateerde sterfte [1-3]. De behandeling van borstkanker omvat een multidisciplinaire aanpak, bestaande uit chirurgie, radiotherapie, en systemische therapie zoals chemotherapie, endocriene therapie en immunotherapie.

Ruim een decennium geleden werden de zogeheten '*Hallmarks of Cancer*' gepresenteerd. Hannahan en Weinberg suggereerden dat lichaamscellen zes biologisch verschillende eigenschappen moeten verkrijgen om maligne te ontaarden [4]. Deze veelbesproken eigenschappen zijn: 1. Autonomie voor groeisignalen, 2. Ongevoeligheid voor groei-remmende signalen, 3. Invasieve groei en metastasering, 4. Immortaliteit, 5. Aanhoudende vaatnieuwvorming, en 6. Ongevoeligheid voor geprogrammeerde celdood (apoptose). Recent werden daar twee eigenschappen aan toegevoegd: 7. Reprogrammering van het energiemetabolisme en 8. De mogelijkheid tot ontsnappen aan herkenning door het immuunsysteem [5].

In de huidige klinische setting wordt de keuze voor type operatie en aanvullende behandeling grotendeels bepaald door de tumorstadiëring en klassieke tumor- en patiëntkarakteristieken. Echter, klassieke prognostische factoren zoals leeftijd, tumorgrootte, lymfeklierstatus, histologische graad, hormoonreceptorstatus en *Human Epidermal growth factor Receptor 2* (HER-2) overexpressie resulteren niet in optimale behandeling van borstkankerpatiënten [6]. Tevens moeten de bijwerkingen van adjuvante systemische therapieën niet onderschat worden. Op basis van deze informatie is het zeer aannemelijk dat de huidige tumorstadiëring kan resulteren in zowel onderbehandeling als overbehandeling van borstkankerpatiënten.

Prognostische factoren informeren omtrent de verwachte klinische uitkomst ten tijde van de diagnose, waarbij eventuele aanvullende behandeling buiten beschouwing wordt gelaten. *Predictieve* factoren doen een uitspraak over de verwachte reactie op een therapeutische modaliteit [7;8]. Zowel prognostische als predictieve factoren dienen als leidraad voor klinische beslissingen omtrent behandeling van borstkankerpatiënten. Om over- en onderbehandeling zoveel mogelijk te beperken is het van cruciaal belang dat er nieuwe, meer nauwkeurige prognostische en predictieve factoren onderzocht en geïmplementeerd worden in de huidige klinische setting.

Leeftijd is voor het grootste deel van de Westerse vrouwen de grootste risicofactor voor het verkrijgen van borstkanker. Bijna de helft van de borstkankerpatiënten is 65 jaar of ouder ten tijde van de diagnose [1]. Verwacht wordt dat deze groep in de komende decennia verder in omvang zal toenemen door de vergrijzing. Oudere borstkankerpatiënten vormen een heterogene populatie met een andere tumorbiologie en metabolisme dan de jongere borstkankerpatiënten. Daarbij is er bij de oudere populatie tevens sprake van

een grote diversiteit aan comorbiditeiten en vitaliteit [9-13], hetgeen besluitvorming rond de behandeling van borstkanker in deze patiëntengroep verder bemoeilijkt. Ondanks de hoge incidentie van borstkanker en borstkanker-gerelateerde sterfte in de oudere populatie, is de huidige kennis omtrent de relatie veroudering en oncogenese, en de optimale oncologische behandeling voor de oudere borstkankerpatiënt, nog onvoldoende. De huidige richtlijnen voor de behandeling van borstkanker zijn grotendeels gebaseerd op onderzoek dat verricht is in relatief jonge of fitte oudere patiënten [14;15]. Dit maakt dat de huidige behandelrichtlijnen van ouderen met borstkanker niet *evidence-based* zijn en dat de uitdaging in deze specifieke borstkankerpopulatie ligt in het voorspellen van wie zal sterven *met* borstkanker en wie zal sterven *door* borstkanker. Op basis van deze informatie kan er een weloverwogen besluit genomen worden over wie er op hogere leeftijd nog baat heeft bij agressieve borstkankerbehandeling.

Het doel van dit proefschrift is vierledig. In ***Deel I*** wordt de prognostische waarde van de moleculaire differentiatie van de tumor, de immunogeniciteit, en de aanhoudende proliferatieve activiteit en ongevoeligheid voor apoptose onderzocht. ***Deel II*** bestudeert de prognostische en predictieve waarde van HER-2 en de insulinegroeifactorreceptor-1 (IGF1R) in relatie tot een gerichte behandeling. In ***Deel III*** wordt het effect van veroudering op tumorontwikkeling en de functionele status van de patiënt bestudeerd. Tot slot wordt in ***Deel IV*** het gebruik van predictieve en prognostische biomarkers in de kliniek bestudeerd. Het nut hiervan en de introductie van een op de patiënt toegespitste behandeling wordt ook in dit deel besproken. Het overkoepelend doel van dit proefschrift is het verbeteren van risicostratificatie van de (oudere) borstkankerpatiënt met daaropvolgend de identificatie van de individuele behandelingswinst, waarbij de introductie van gepersonaliseerde therapie in de oudere borstkankerpopulatie zijn intreding krijgt.

DEEL I: PROGNOSTISCHE BIOMARKERS IN MAMMACARCINOOM

Met behulp van biomarkers, die betrokken zijn bij de maligne ontaarding van borstcellen en tumorprogressie, kan een voorspelling gedaan worden over de prognose van een patiënt na primaire tumorbehandeling. Om dit te onderzoeken hebben we biomarkers geselecteerd die behoren tot de, zoals hierboven reeds beschreven, '*Hallmarks of Cancer*'.

In **hoofdstuk 2** hebben we de prognostische waarde van biomarkers die gerelateerd zijn aan apoptose en proliferatie onderzocht. Een verschuiving van de balans tussen deze twee cellulaire processen kan bijdragen aan het ontstaan en onderhoud van tumorgroei [4]. Eerdere studies laten tegenstrijdige resultaten zien wanneer gekeken wordt naar de prognostische waarde van apoptose en/of proliferatie in borstkanker [16;17]. Tumorgroei wordt gekarakteriseerd door de verhouding tussen cellulaire proliferatie en

celdood. Op basis hiervan testen wij in hoofdstuk 2 de hypothese dat de balans tussen deze twee processen een meer nauwkeurige indicatie geeft van tumoragressiviteit dan wanneer er naar de processen afzonderlijk gekeken wordt [4]. In 488 stadium I-III mammacarcinoompatiënten werd de mate van apoptose en proliferatie onderzocht door middel van immunohistochemische kleuringen voor expressie van respectievelijk *p*53, actief caspase-3 en Ki67. Deze studie liet zien dat de patiënten met tumoren waarbij er sprake was van een sterke mate van proliferatie en apoptose de slechtste overleving hadden. Bij combinatie van alle drie de onderzochte markers (Ki67, actief caspase-3 en *p*53) in een zogeheten apoptotisch-proliferatief tumorsubtype, was er sprake van een significante associatie met overleving en tumorrecidief, waarbij patiënten met hoge mate van proliferatie en apoptose in de tumor, gecombineerd met een gemuteerde *p*53 status, de slechtste uitkomst hadden. Interessant was echter dat bij separate analyses naar tumorstadium bleek dat de prognostisch voorspellende waarde van het apoptose-proliferatie tumorsubtype alleen significant bleef in de stadium I mammacarcinoompatiënten. Dit is met name relevant omdat door de toegenomen bewustwording van borstkanker in de maatschappij, en door het bevolkingsonderzoek, er een verschuiving heeft opgetreden naar de detectie van tumoren in een zeer vroeg stadium [18]. Klinische introductie van het hierboven beschreven model zou kunnen leiden tot de identificatie van de vroeg stadium mammacarcinoompatiënten met een ongunstig apoptose-proliferatie tumorprofiel, met een groter klinisch risico waarbij agressieve antitumor behandeling op zijn plaats zou kunnen zijn.

In **hoofdstuk 3, 4 en 5** hebben we de prognostische waarde van belangrijke biomarkers, gerelateerd aan de interacties die er plaatsvinden tussen de borstkankercel en het immuunsysteem onderzocht. In de afgelopen jaren heeft zich bewijs opgestapeld waaruit blijkt dat het immuunsysteem in staat is om tumorontwikkeling en progressie te controleren en te manipuleren [19]. Daarentegen is ook beschreven dat tumoren, door hun intrinsiek genetisch instabiele karakter, eigenschappen kunnen ontwikkelen om te ontsnappen aan dergelijke immuunherkenning en daaropvolgende eliminatie [20]. Eén van de factoren betrokken bij deze continue wisselwerking is *Humane-Leukocyte-Antigen* (HLA) klasse I. HLA klasse I bestaat uit 3 typen (A, B en C), welke tumor-geassocieerde-antigenen kunnen presenteren op het celmembraan van maligne cellen, waardoor Cytotoxische-T-Lymfocyten (CTL) geactiveerd worden en de tumorcellen opgeruimd worden. Om aan deze immuunherkenning te ontsnappen kunnen maligne cellen hun HLA klasse I *downreguleren* [21]. Tumorcellen kunnen, naast verminderde expressie van HLA klasse I moleculen op het celmembraan, HLA-G tot expressie brengen. HLA-G komt zelden voor in gezonde weefsels, maar vertoont wel expressie in tumoren [22]. HLA-E is daarentegen wel aanwezig in verscheidene gezonde weefsels en correleert met expressie van HLA klasse I [23]. Onderzoek heeft aangetoond dat tumorexpressie van HLA-E en HLA-G ervoor zorgt dat *Natural-Killer* (NK) cellen niet geactiveerd kunnen worden, waardoor

tumorcellen ook langs deze weg kunnen ontsnappen aan het immuunsysteem [22-24]. Tot slot kunnen regulatoire-T-cellen (Treg) in de tumoromgeving een immunosuppressief effect uitvoeren op de CTL [19].

Gezien eerdere studies de complexiteit en het belang hebben aangetoond van de interacties tussen het immuunsysteem en de borstkankercel, werd in **hoofdstuk 3** een samengestelde biomarker gecreëerd, genaamd 'tumorimmuuunsubtype', op basis van bovenstaande essentiële markers [25-27]. Voor 293 borstkankerpatiënten in het trainingscohort en 219 borstkankerpatiënten uit het validatie-cohort werd er een statistisch significante associatie gezien met zowel relatieve overleving als borstkanker recidief. Patiënten met tumoren die werden beschouwd als laag immunogeen hadden een slechtere klinische uitkomst in vergelijking met patiënten waar de tumoren als gemiddeld of hoog immunogeen werden beschouwd. Hoog immunogene tumoren werden geïdentificeerd als tumoren met 'HLA klasse I expressie op het celmembraan en CTL positiviteit, zonder Treg infiltratie in de tumor' of 'verlies van HLA klasse I, gecombineerd met verlies van HLA-E en HLA-G waardoor de NK cellen geactiveerd worden, zonder immunosuppressieve Treg infiltratie'. Gemiddelde immunogene tumoren werden gekarakteriseerd door 'HLA klasse I expressie, zonder CTL aanwezigheid of de aanwezigheid van immunosuppressieve Tregs'. Tenslotte werden laag immuun-vatbare tumoren omschreven als 'tumoren waarbij er verlies van HLA klasse I is opgetreden, gecombineerd met het verlies van NK cellen, al dan niet op basis van verhoogde HLA-E en HLA-G expressie of de aanwezigheid van Tregs'. Concluderend kan er gesteld worden dat in bovenstaande studie de complexiteit en veelzijdige wisselwerking tussen het immuunsysteem enerzijds en de tumorcellen anderzijds aangetoond is. De resultaten van dit onderzoek benadrukken nogmaals het grote belang van de interactie tussen het immuunsysteem en de kankercel met betrekking tot prognosticatie en eventueel hierop gebaseerde of hiermee interfererende adjuvante behandeling.

In **hoofdstuk 4** hebben we de prognostische waarde van dezelfde biomarkers als in hoofdstuk 3 onderzocht, waarbij er onderscheid werd gemaakt tussen invasief ductaal carcinoom (IDC) en invasief lobulair carcinoom (ILC). Er is voldoende bewijs dat ILC, in vergelijking met IDC, andere eigenschappen bezit. Zo zijn ILC tumoren vaak groter, vaker hormoonreceptor-positief en hebben ze vaak een minder agressief beloop [28;29]. Echter, tot op heden wordt er in de klinische setting weinig onderscheid gemaakt tussen IDC en ILC. Het doel van deze studie was daarom ook het vergelijken van de relevantie van de tumorimmuunsubtypes in de twee meest voorkomende histologische borsttumor soorten: IDC en ILC. Tevens werden de moleculaire intrinsieke borstkankersubtypes (Luminal A en B, Basal-like en ERBB2) en de mate van apoptose-proliferatie (zoals beschreven in hoofdstuk 2) onderzocht. Er werd aangetoond dat de prognostische waarde van zowel de tumorimmuunsubtypes, de moleculaire intrinsieke borstkankersubtypes, als ook de apoptose-proliferatie tumorsubtypes alleen gelden voor de IDC, en niet van

toepassing zijn op ILC. Verder bleek uit ons onderzoek dat de reeds beschreven tumor-immuunprofielen alleen van toepassing zijn op de Luminal A tumortypes. Deze uitkomst strookt met het feit dat Luminal A tumoren ook het grootste deel uitmaken van de IDC. Op basis van deze uitkomsten kan echter niet gesteld worden dat moleculaire subtypes en histologische borstkankersubtypes identiek zijn. Hoewel frequent gelijk behandeld, is er voldoende bewijs om aan te nemen dat IDC en ILC twee verschillende entiteiten zijn. Om de borstkankerpatiënt in de toekomst van de meeste optimale therapie te voorzien is het van cruciaal belang dat er onderscheid wordt gemaakt tussen deze twee histologisch verschillende borstkankertypes. Toekomstig onderzoek zal zich moeten richten op de therapeutische sensitiviteitsverschillen tussen IDC en ILC, waarbij naast klassieke tumorkarakteristieken ook rekening gehouden moet worden met de immuun- en moleculair gerelateerde eigenschappen van een tumor.

Doordat eerder onderzoek mogelijk een immuunmodulatoir effect van endocriene behandeling liet zien [30] werden in **hoofdstuk 5** de tumorimmuunkarakteristieken bestudeerd in relatie tot klinische uitkomst in een groot, hormoonreceptor positief borstkankercohort, welke met twee verschillende endocriene behandelarmen behandeld zijn (TEAM studie). Patiënten van de TEAM studie werden behandeld met eenmaal daags exemestane 25mg gedurende vijf jaar *of* sequentiële therapie, bestaande uit eenmaal daags tamoxifen 20mg gedurende twee en een half jaar gevolgd door eenmaal daags exemestane 25mg gedurende twee en een half jaar [31]. Uit eerder onderzoek is gebleken dat tamoxifen een immuun-shift teweegbrengt, waarbij de cellulaire immuniteit (T-helper 1) omgezet wordt in humorale immuniteit (T-helper 2) [30] wat tevens een verklaring zou kunnen zijn voor het geobserveerde verschil in behandeleffect tussen aromataseremmers en tamoxifen [30;32]. Onze resultaten lieten alleen bij patiënten in de sequentiële behandelarm een significante associatie zien tussen een hoge mate van FoxP3+ en overleving. Dit resultaat werd tevens ondersteund door een significante interactie tussen FoxP3+ infiltratie en endocriene therapie. Door de kenmerken van de patiënten van de TEAM studie (post-menopausaal: gaat gepaard met een functionele toename van ontstekingsparameters; en hormoonreceptor-positiviteit: waardoor hogere oestrogeenconcentraties in en om de tumor aanwezig zijn, welke leidt tot meer verlies van ontstekingsremmende Adenosine (ADO)), wordt er een voorkeur uitgesproken voor zogeheten 'Natural Tregs' (in tegenstelling tot de 'geïnduceerde Tregs', welke de antitumorimmuunmodulatie beïnvloeden), welke zorgdragen voor onderdrukking van het ontstekingsproces rondom de tumor en hiermee de carcinogenese vertragen [33-35]. Het ontbreken van deze associatie in de exemestane behandelde patiënten kan verklaard worden door het verlies van het hoge gehalte van oestrogeen in en om de tumor. Een vergelijkbaar resultaat werd gezien voor de tumorimmuunsubtypes (laag, gemiddeld en hoog immuun-gevoelige tumoren) in relatie met de twee endocriene

behandelarmen. Gedacht wordt dat ook hier Tregs een belangrijke rol spelen in het verlies van prognostische waarde in de exemestane behandelde patiëntengroep. Reeds werd aangetoond dat met exemestane behandeling er een significante toename van de CD8+/Treg ratio optreedt bij patiënten die goed reageren op aromatase remmende behandeling, en daarnaast werd er ook een afname van FoxP3+ cellen gezien na exemestane behandeling [36;37]. Voorgesteld wordt dat verlies van zeer prognostische Treg cellen leidt tot het gelijktrekken van de klinische uitkomsten van de drie (laag, gemiddeld, hoog) tumorimmuunsubtypes onder exemestane behandeling. Tot de tijd dat dit bewezen wordt, kan er gespeculeerd worden over het grote belang van Treg cellen bij de inhibitie van tumorontwikkeling in de postmenopauzale, hormoonreceptor-positieve borstkankerpatiënt.

Hoofdstuk 6 van dit proefschrift beschrijft de prognostische waarde van de moleculair intrinsieke borstkankersubtypes in de oudere borstkankerpatiënt. De afgelopen jaren heeft de identificatie van de moleculair intrinsieke borstkankersubtypes aangetoond dat borstkanker een heterogene ziekte is met een variatie in de respons op adjuvante systemische behandeling. Daarom is het, zeker in deze oudere borstkankerpopulatie, waar een groot deel van de tumoren hormoonreceptor positief is, met vaak een lage proliferatiegraad, van grote waarde om het prognostische effect van de moleculaire borstkankersubtypes te bepalen.

Er zijn vier veelvoorkomende moleculaire borstkankersubtypes beschreven; Luminal A en B: grotendeels hormoonreceptor positief en met een indolent karakter [38-40]; De basal-like tumoren: triple negatieve tumoren, gecombineerd met expressie van genen betrokken bij de basale epitheliale laag, zoals Cytokeratine 5 en 6; en tot slot de ERBB2 tumorsubtypes, welke veel overeenkomsten tonen met de basal-like subtypes, maar waarbij er hoge mate van HER-2 expressie op de tumor aanwezig is. Deze laatste twee moleculaire tumorsubtypes hebben een agressiever karakter met ongunstige prognose [38]. In deze studie werden, vanwege het gebrek aan in situ hybridisatie en het feit dat de oudere borstkankerpatiënten geen significant verschil in klinische uitkomst toonden in vergelijking met patiënten met HER-2 scores 0 en 1+ (hoofdstuk 8), alle HER-2 2+ tumoren als HER-2 negatief beschouwd.

Eerder onderzoek toonde aan dat moleculaire borstkankersubtypes, in vergelijking met de jongere populatie een ander distributiepatroon had in de oudere borstkankerpopulatie [4], en dat de prognostisch voorspellende waarde verloren ging. Echter, gezien de kleine aantallen patiënten van 65 jaar of ouder in voorgaande studie, welke resulteerde in een klein discriminatief vermogen, waren wij van mening dat het herhalen van deze studie in een grote oudere borstkankerpopulatie van toegevoegde waarde zou zijn.

Resultaten van onze studie toonden aan dat de verdeling van moleculaire subtypes wel van significante prognostische waarde was in de oudere (≥65jaar) borstkanker-

populatie. Zoals verwacht, lieten onze data een hogere mate van ziekte terugkeer zien in de ERBB2 en de basal-like moleculaire tumorsubtypes. Tevens werd er een slechtere relatieve overleving gezien voor alle moleculaire borstkankersubtypes wanneer deze vergeleken werd met de Luminal A subtypes. In vergelijking met de jongere borstkankerpopulatie toonde onze oudere borstkankerpopulatie een hogere prevalentie van de indolente Luminal A tumoren en een lage prevalentie van de agressievere moleculaire tumorsubtypes [41]. Deze observatie is overeenkomstig met de vaak genoemde mildere tumorkarakteristieken op oudere leeftijd.

Dit is de eerste studie, uitgevoerd in een omvangrijk ouder borstkankercohort, welke de significante prognostische waarde van de moleculaire borsttumorsubtypes aantoont, waarbij er rekening wordt gehouden met het grote risico van concurrerende doodsoorzaken. Derhalve ondersteunt deze studie het gebruik van de moleculaire borstkankersubtypes in de oudere borstkankerpatiënt voor prognosticatie en therapiekeuze. Echter, juist in deze oudere borstkankergroep moet het belang van de functionele status en de persoonlijke behandelwens niet bedolven raken onder het moleculaire geweld van de moderne diagnostiek.

DEEL II: PREDICTIEVE BIOMARKERS IN MAMMACARCINOOM EN GERICHTE BEHANDELING

Signalering via de *Insulin-like Growth Factor type 1 Receptor* (IGF1R) speelt een belangrijke rol in de ontwikkeling van vele soorten tumoren, inclusief borstkanker [42;43]. Aangetoond is dat IGF1R expressie gecorreleerd is aan de mate van oestrogeenreceptor expressie [44], en dat 17β-Estradiol, hoewel in de mindere mate dan IGF1, zorg kan dragen voor activatie van de IGF1R, welke op zijn beurt leidt tot activatie van de *Mitogen-Activated Protein Kinase* (MAPK) [45;46]. Deze data leidde tot de hypothese dat patiënten, behandeld met aromataseremmers, door de complete blokkade van oestrogeenproductie deze additionele tumor-groei-stimulerende-*pathway* verliezen. Daarbij zou metformine, reeds bekend om het verlagen van de plasma insuline- en insuline groeifactor-concentratie door het verhogen van de insulinesensitiviteit [47], van additionele therapeutische waarde zijn bij borstkankerbehandeling door de verminderde IGF1 binding op de IGF1R [48;49]. In **hoofdstuk 7** van dit proefschrift, voerden we een substudy analyse uit bij 2.446 Nederlandse patiënten van het TEAM-cohort. Deze studie onderzocht het klinische effect van exemestane en metformine behandeling op de mate van IGF1R expressie van de borsttumor in een hormoonreceptor positief borstkankercohort. Resultaten van deze studie toonden, in vergelijking met sequentieel behandelde patiënten, een significante verbetering van de ziektevrije overleving aan voor patiënten die behandeld waren met exemestane monotherapie waarbij de tumoren een hoge mate van IGF1R tot

expressie brachten. Er werd geen associatie gezien voor patiënten van wie de tumoren een lage expressie hadden van IGF1R. Het additionele gebruik van metformine, naast exemestane behandeling, resulteerde in een verdere verbetering van de ziektevrije overleving en *overall* overleving in patiënten waarvan de tumoren een hoge mate van IGF1R tot expressie brachten. Deze interessante bevindingen staan in schril contrast met de hoofdbevindingen van de TEAM studie, waarbij er geen verschil gezien werd in *overall survival, breast cancer specific survival* of *disease free survival* tussen de twee behandelarmen [31]. Een mogelijke verklaringen voor onze bevinding is het toenemend bewijs voor de mogelijkheid van oestrogeen om, naast de binding en activatie van de klassieke oestrogeenreceptoren, tevens de capaciteit te bezitten om IGF1R te fosforyleren en activeren [46]. Onze resultaten, namelijk de sterke interactie tussen de mate van IGF1R expressie op het tumoroppervlak en de effectiviteit van exemestane, doen vermoeden dat het effect grotendeels afhankelijk is van de afgenomen oestrogeenproductie, waardoor de hierboven besproken oestrogeen geïnduceerde activatie van de IGF1R verstoord wordt. Deze hypothese wordt ook ondersteund door de observatie dat patiënten met hoge mate van IGF1R expressie op hun tumoroppervlak, maar behandeld werden met tamoxifen, geen klinisch voordeel hadden. Dit laatste berust waarschijnlijk op het feit dat zij nog circulerend oestrogeen hebben, welke de IGF1R kan activeren en zo de groei van borstkanker kan stimuleren. Een tweede hypothese-onderbouwende observatie is het feit dat er geen klinisch voordeel gezien werd voor patiënten behandeld met exemestane waarbij de tumoren een lage mate van IGF1R expressie toonden. Dit laatste zou kunnen berusten op het feit dat de oestrogeen geïnduceerde groeistimulatie van IGF1R te klein is in deze tumoren. Daarnaast stellen we voor dat het additieve effect van metformine behandeling geïnduceerd wordt door de directe verlaging van de IGF concentratie. Patiënten met hoge IGF1R expressie op het tumoroppervlak, die behandeld zijn met zowel exemestane als metformine, hebben een dubbele blokkade van de ER-IGF1R *crosstalk*, welke resulteert in een betere klinische uitkomst. Voor de alledaagse kliniek betekent dit dat door het stratificeren van de patiënten op basis van de IGF1R tumorexpressie, we patiëntsubgroepen kunnen identificeren die veel voordeel kunnen hebben van gecombineerde therapie. Deze bevinding is een ondersteuning van de hedendaagse trend waarbij individualisering van de behandeling, met als doel de klinische uitkomst te verbeteren, centraal staat in de heterogene borstkankerpopulatie. Tenslotte is dit een uitkomst, gezien het grote risico op chemotherapie toxiciteit, die van groot belang zou kunnen zijn voor de oudere borstkankerpatiënt [11], Daarnaast zijn ongeveer 80% van de tumoren in de oudere borstkankerpopulatie hormoonreceptorpositief, welke louter met endocriene therapie behandeld worden [50]. Gezien borstkankersterfte toeneemt met de leeftijd, wat toegeschreven kan worden aan zowel onder- als overbehandeling [51], zijn nieuwe behandelstrategieën, bij voorkeur met een laag toxiciteitsprofiel zoals metformine, zeer gerechtvaardigd.

In **hoofdstuk 8** werd het potentiële herstel van de klinische interesse voor anti-HER-2 therapie in de oudere borstkankerpatiënten onderzocht. Het is reeds bekend dat HER-2 overexpressie geassocieerd is met een agressiever tumor fenotype [52], met als gevolg een slechtere klinische uitkomst [53;54]. Behandeling van HER-2 overexpressie op de tumor verbeterd de klinische uitkomst in zowel lymfeklier negatieve als lymfeklier positieve borstkanker [55;56]. Aberrante activatie van de Phosphatidylinositol 3-kinase (PI3K)/ AKT pathway door PIK3CA mutaties, welke vaak voorkomt in combinatie met HER-2 overexpressie, resulteert ook in tumorgroei [57], en verkleint de mate van response op HER-2-gerichte therapieën [58;59]. Een bekende tekortkoming van het huidige klinische onderzoek naar de waarde van de biologische tumormarkers is dat het grootste deel van de studies uitgevoerd wordt in een relatief jonge borstkankerpopulatie, wat extrapolatie naar de oudere borstkankerpatiënt bemoeilijkt. De hoge incidentie van cardiale bijwerkingen van anti-HER-2 behandeling leidt tot de vaak voorkomende angst voor het voorschrijven van anti-HER-2 behandeling in de oudere borstkankerpatiënt. Echter, tot op heden is er geen wetenschappelijk bewijs voor het achterwege laten van de anti-HER-2 behandeling in deze specifieke borstkankersubgroep. Gezien het gebrek aan wetenschappelijke onderbouwing en het gebrek aan klinische richtlijnen, zijn wij van mening dat onderzocht moet worden of de huidige *non-evidence based* behandelingsstrategieën met betrekking tot HER-2, bevestigt of weerlegt moeten worden.

Onze studie, bestaande uit 1.698 borstkankerpatiënten van 65 jaar of ouder (FOCUS cohort), toonde aan dat 5-jaar na diagnose, patiënten met een HER-2 score van 3+ een significant hoger risico hadden op ziekteterugkeer en een slechtere 10-jaars relatieve overleving hadden in vergelijking met HER-2 negatieve patiënten, zelfs als er rekening werd gehouden met een grotere kans op sterfte gezien de oudere leeftijd. Interessant genoeg, bleek dat patiënten met HER-2 2+ tumoren geen hoger risico hadden op ziekte terugkeer dan borstkanker patiënten *zonder* HER-2 overexpressie. PIK3CA mutaties waren niet van prognostische waarde in deze specifieke borstkankerpopulatie.

Bovenstaand resultaat impliceert dat oudere patiënten met HER-2 3+ tumoren mogelijk wel baat zouden hebben bij anti-HER-2 behandeling. Bovendien heeft recent onderzoek bewezen dat de vaak ernstig gevreesde, hoofdzakelijk cardiale bijwerkingen van anti-HER-2 behandeling in de praktijk minder ernstig en minder vaak voorkomen dan voorheen gedacht werd [60;61]. In de huidige medische setting wordt anti-HER-2 behandeling vaak achterwege gelaten bij de oudere borstkankerpatiënt vanwege de gevreesde bijwerkingen en de vaak reeds beperkte levensverwachting. Echter, gezien de uitkomst van deze studie zou er gesuggereerd kunnen worden dat anti-HER-2 behandeling wel degelijk van meerwaarde zou kunnen zijn in de fitte, oudere borstkankerpatiënten. Daarnaast zou (dubbele) HER-2 blokkade in oudere patiënten, met een slechtere klinische conditie, of met een sterke voorkeur om chemotherapie achterwege te laten, een waardevolle optie kunnen zijn. Eén van de kenmerken van de oudere

kankerpatiënten is de heterogeniteit binnen dezelfde chronologische leeftijd. Onder de huidige omstandigheden zal, indien er geen verschil gemaakt wordt tussen fitte en zwakke(re) oudere patiënten, de zorg voor deze fitte populatie tekortschieten, met uiteindelijk oneerlijke overlevingskansen. Toekomstig onderzoek moet gaan uitwijzen of het haalbaar is om een effectieve anti-HER-2 behandeling met minimale toxiciteit voor de oudere borstkankerpatiënten te bewerkstelligen.

DEEL III: VEROUDERING IN DE BORSTKANKERPATIËNT

Van alle factoren die bijdragen aan de oncogenese brengt veroudering het grootste risico met zich mee [62]. De *multi-hit*, ook wel de hypothese van Knudson genoemd, beweert dat kanker vaker voorkomt op oudere leeftijd omdat er tijd nodig is om genetische mutaties op te lopen en cellen over de mutagene drempel heen te duwen [63]. Echter, wat bovenstaande hypothese niet kan verklaren is waarom het risico op kanker fors verlaagd blijkt bij patiënten die zich aan een caloriebeperkt dieet houden en veel aan lichamelijke beweging doen [64]. Tijdens de veroudering wordt er een afname van de nucleair gelokaliseerde nicotinamide adenine dinucleotide (NAD^+) gezien, wat resulteert in een afname van Sirtuin 1 (SIRT1) activiteit in de celkern waardoor Von Hippel-Lindau (VHL) afneemt en hypoxia-inducible factor-1α (HIF-1α) zich stabiliseert [65]. Deze door leeftijd geïnduceerde stabilisatie van HIF-1α resulteert in een pseudo-hypoxische cel-staat, welke verstoring van de oxidatieve fosforylering (OXPHOS) als gevolg heeft, en hiermee een zogeheten Warburg-effect veroorzaakt. De hieropvolgende toename van reactieve zuurstofradicalen (ROS) resulteren in een mutageen milieu met als gevolg carcinogenese. Bovenstaande bevindingen zouden een verklaring kunnen zijn voor het exponentieel toegenomen risico op kanker op oudere leeftijd [65;66]. Deze leeftijd gedreven metabole achteruitgang als een belangrijke oorzaak van tumorgenese wordt ook wel "geroncogenese" genoemd.

Hoewel hypoxie toxisch is voor een cel, kunnen kankercellen zich aanpassen door middel van genetische modificatie en hiermee de overleving en proliferatietendens beïnvloeden onder deze stressvolle omstandigheden. Een reeds bekend cellulair adaptatie mechanisme in geval van (pseudo) hypoxie in de weefsels is opregulatie van HIF-1. Bij (pseudo)hypoxie, bindt HIF-1 zich aan zogeheten hypoxie-respons elementen (HREs), welke op hun beurt de expressie van verscheidene hypoxische respons genen beïnvloeden [67]. Bekende HIF-1 *target* genen zijn betrokken bij celproliferatie, angiogenese, inflammatie, metabolisme, apoptose, immortalisatie en migratie [67;68]. In **hoofdstuk 9** laten we, in vergelijking met normaal borstweefsel, een toename zien van de HIF-1α mRNA expressie en zijn *target* genen in het borstkankerweefsel. Deze observatie gold echter alleen voor patiënten van 65 jaar of ouder, ongeacht de overeenkomsten op het

gebied van pathologisch tumorstadium, tumorgraad en tumormorfologie tussen de leeftijdsgroepen (<65jr of ≥65jr). Opvallend was dat voor de jongere patiëntengroep wel dezelfde trend te zien was voor de mate van expressie van HIF-1α en de beschreven *targets* als voor de oudere borstkankerpopulatie. Dit doet vermoeden dat HIF-1α en bijbehorende *targets* waarschijnlijk wel een rol spelen bij de tumorgenese van de jongere borstkankerpatiënt maar dat deze van minder belang zijn dan in de patiëntengroep van 65 jaar of ouder. Een mogelijke verklaring hiervoor werd beschreven door Gomes *et al.* In deze publicatie wordt gepleit voor een zogenaamde '*priming*' van de gezonde cellen op oudere leeftijd met als resultaat hoge mate van expressie van HIF-1α door de zogeheten leeftijd-geïnduceerde HIF-1α stabilisatie door de pseudo-hypoxische staat [65]. Tumorontwikkeling, bekend vanwege de hoge mate van HIF-1α expressie [69], in een milieu met reeds verhoogde HIF-1α waarden, resulteert in een exponentiële toename van HIF-1α in de tumor. Voorgesteld wordt dat deze significant verhoogde mate van HIF-1α expressie in de borsttumoren van de oudere patiënten een belangrijke rol speelt in het agressievere, en minder therapiegevoelige karakter van de mammatumor van de oudere patiënt [51]. Therapeutische blokkade van HIF, door middel van antisense HIF-1α [70], of opregulatie van het VHL gen [71], zou kunnen leiden tot een afname van de tumorgroei middels verstoring van de neovascularisatie en metabole reprogrammering, met als doel de klinische uitkomst verbeteren. Indien succesvol, zou dit een veelbelovende nieuwe farmacologische behandeltechniek zijn welke, gezien de resultaten van onze studie, van groot belang zou zijn voor de oudere borstkankerpatiënt.

Bovengenoemde metabole verandering waarbij oxidatieve fosforylering wordt vervangen door aerobe glycolyse is grotendeels afhankelijk van het glycolytisch enzyme pyruvate kinase (PK) [72]. Gezonde lichaamscellen brengen overwegend de pyruvate kinase M1 isoform (PKM1) tot expressie, terwijl tumorcellen hoofdzakelijk de M2 isoform (PKM2) tot expressie brengen.

PKM2 katalyseert de laatste stap van de glycolyse en reprogrammeert de glycolytische flux wat van groot belang is voor prolifererende cellen [72]. Het laatste decennium trekt PKM2 steeds meer de aandacht als veelbelovende therapeutische *target* voor kankerbehandeling, en zou het tevens ook een prominente rol kunnen aannemen in anti-verouderingsbehandeling.

In **hoofdstuk 10** onderzochten we het verschil in de mate van expressie van HIF-1α en de geassocieerde *target* genen, waaronder PKM1 en 2, in patiënten tussen de 65 en 80 jaar en patiënten van ouder dan 80 jaar, in zowel gezond borstweefsel als tumorweefsel. Daarnaast onderzochten we of de mate van expressie, oftewel metabole reprogrammering, geassocieerd is met klinische kenmerken van veroudering en uitkomst. Onze resultaten toonden aan dat HIF1-α significant hoger tot expressie komt in het normale borstweefsel van de oudere patiënt, en dat de mate van HIF1-α expressie in het normale borstweefsel geassocieerd is met een hogere tumorgraad in de patiënt.

Verder was PKM2 significant geassocieerd met surrogaatmarkers voor functionaliteit zoals polyfarmacie en moeite met lopen, waarbij hoge mate van PKM2 expressie in het normale borstweefsel vaker geassocieerd is met slechte functionele uitkomsten, met een potentieel negatief effect op overleving.

Bovenstaande bevindingen ondersteunen de hypothese dat ontregeling van de HIF1-α metabole pathway, leidend tot een toename van ROS, een belangrijke rol speelt bij de hoge kankerincidentie van de oudere populatie.

Daarentegen toonde onze studie aan dat hoge mate van PKM2 eiwitexpressie in de borsttumoren geassocieerd was met een significant betere ziektevrije overleving en werd er een statische trend gezien voor minder ziekteterugkeer dan in patiënten met weinig PKM2 eiwitexpressie in de tumor. Deze uitkomst komt overeen met de resultaten van een eerdere studie, waarbij aangetoond werd dat activatie van PKM2 het oncogene metabolisme *in vitro* veranderde welke leidde tot vertraagde xenograft tumorgroei [73]. Een mogelijke verklaring hiervoor is het tekort aan *precursors* voor de synthese van bouwblokken, begunstigd door dimerisch PKM2, noodzakelijk in hoog proliferatieve cellulaire omstandigheden. Activatie van PKM2 in tetramere vorm leidt tot inhibitie van de cellulaire proliferatie [73;74], welke resulteert in minder kankerontwikkeling en -verspreiding. Op basis van bovenstaande bevindingen pleiten sommigen voor PKM2-activatie als een veelbelovende adjuvante behandelingsmodaliteit. Echter, de aanwezigheid van PKM2, en de rol die het vervult in het verouderingsproces moet niet onderschat worden en zou de antikanker therapeutische effectiviteit kunnen verstoren.

Er zal meer onderzoek moeten geschieden om de precieze rol, functie en bijdrage van HIF1-α en PKM2 op het verouderingsproces en de mogelijke invloed op tumorigenese te achterhalen.

Als metabole verandering inderdaad een belangrijke '*driver*' blijkt van veroudering en geroncogenese, zullen moleculen die metabole veroudering voorkomen, afremmen of terugdraaien een belangrijke rol gaan innemen in anti-veroudering en anti-kankerbehandeling. Veelbelovende ontwikkelingen hebben reeds plaatsgevonden met betrekking tot HIF1-α *inhibitors*, SIRT *activators*, en zowel PKM2 inhiberende, waarbij hnRNPA1, hnRNPA2 en PTB getarget worden, als PKM2 activerende behandeling [74-77]. Op basis van de huidige kennis is het aannemelijk dat behandeling welke leidt tot vertragen of terugdraaien van veroudering en leeftijd gerelateerde aandoeningen zoals kanker, een snelle ontwikkeling zal doormaken, met grote klinische consequenties in de komende jaren.

DEEL IV: GEPERSONALISEERDE KANKERBEHANDELING

Hedendaagse classificatie middels de TNM-stadiëring, waarbij rekening gehouden wordt met de tumor (T), de betrokken lymfeklieren (N), en de metastasering op afstand

(M), blijkt geen ideaal handvat te zijn voor clinici om een behandelingsstrategie te bepalen. Om deze reden is het van groot belang om additionele biomarkers te onderzoeken die de huidige tumorstadiëring kunnen verbeteren. Met de introductie van de aanvullende biomarkers naast de huidige tumorstadiëring zou de prognose van een patiënt beter kunnen worden ingeschat en kan de multidisciplinair bepaalde behandeling per individu worden geoptimaliseerd. Het uiteindelijke doel is om voor deze gepersonaliseerde benadering de moleculaire, (epi)genetische en klinische tumoreigenschappen met patiëntkarakteristieken te integreren, om zo betere preventieve, diagnostische en therapeutische methoden te ontwikkelen.

Eén van de ontwikkelingen die het afgelopen decennium een grote vlucht heeft genomen is de zogeheten '*genome-wide-approach*'. Hierbij worden RNA-expressie profielen met prognostische en predictieve waarde per tumortype ontwikkeld. De twee bekendste profielen, die reeds hun intrede op de diagnostische markt hebben gemaakt zijn Onco*type* DX (Genomic Health Inc., Redwood City, CA, USA) en de MammaPrint (Agendia BV, Amsterdam, Nederland).

De laatste jaren heeft bovenstaande ontwikkeling een belangrijke voet aan de grond gekregen voor de behandeling van mammacarcinoom en colorectaalcarcinoom.

Alhoewel de toegevoegde waarde van genotypering voor het bepalen van systemische therapie duidelijk lijkt, is de impact op chirurgisch vlak onduidelijk. In **hoofdstuk 11** wordt de rol van genotypering in de chirurgische besluitvorming bediscussieerd. Behoudens enkele afzonderlijke genetische mutaties die een directe invloed hebben op chirurgisch ingrijpen, zoals *BRCA* mutaties in borstkanker waarvoor een profylactische bilaterale mastectomie wordt geadviseerd, is er tot op heden geen directe relatie tussen genotypering en chirurgische besluitvorming. Indirect is er voor genotypering wel een chirurgische besluitvormingsrol weggelegd. Zo kan het genotype van een preoperatief biopt leiden tot een gerichte neo-adjuvante therapie, leidend tot optimale tumorregressie, met als gevolg dat de timing en uitgebreidheid van de operatie kan worden beïnvloed. Indien door gerichte neo-adjuvante therapie complete remissie optreedt, zou zelfs een '*wait-and-see*' benadering tot de mogelijkheden behoren. Om uiteindelijk de veelbelovende gepersonaliseerde behandeling met behulp van biomarkers en genotypering van kankerpatiënten te bereiken, moeten er nog belangrijke stappen worden genomen. Ten eerste is het voor de ontwikkeling en validatie van biomarkers en genotyperingsprofielen belangrijk om gestandaardiseerde methoden en vergelijkbare patiëntcohorten te gebruiken, waarmee de klinische integratie verbeterd kan worden. Ten tweede is het, gezien de vergrijzing van onze populatie, niet onbelangrijk om het fenotype van een patiënt voor ogen te houden, waarbij zowel de comorbiditeiten als de effecten van deze comorbiditeiten op de tumorontwikkeling en de behandeling moeten worden meegewogen in het bepalen van de patiëntgerichte behandelingsstrategie. Ten slotte is het van cruciaal belang dat alle medische specialisten die betrokken zijn bij

de kankerbehandeling hun krachten bundelen in een multidisciplinair oncogeriatrisch-front, waardoor iedere individuele patiënt de meest efficiënte en draaglijke behandeling krijgt en de kankerbehandeling naar een hoger niveau getild kan worden.

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Charla Chábeli Engels was born on February 24th 1986 in Willemstad, Curaçao.

After graduating from the Peter Stuyvesant College in 2004, she moved to the Netherlands to commence with her medical studies at the Leiden University Medical Center. After completing her preclinical training in medicine, she started with her clinical rotations in December 2008. After receiving her medical degree in November 2010, she joined Professor Jeff Bulte's lab at the institute for cell engineering (ICE) at the Johns Hopkins University, School of Medicine in Baltimore, MD, USA. During her six-month research fellowship, she studied enhanced cerebral targeting of magnetically labelled glial precursor cells using the VLA-4/VCAM-1 adhesion pathway. In December 2011 she started with her PhD training at the department of Surgical Oncology at the Leiden University Medical Center in the Netherlands, under the supervision of Professor C.J.H. van de Velde, Dr. G.J. Liefers and Dr. P.J.K. Kuppen. During her graduate training Charla was able to join the laboratory of Professor David Sinclair at the Harvard Medical School in Boston, MA, USA. During this research fellowship she studied the role of HIF-1α and its metabolic targets in geroncogenesis in breast cancer patients. The results of her studies are presented in this thesis and have been published in several international journals. After finalizing her graduate program, she started working at the department of Surgery of the Alrijne Hospital, Leiderdorp, under the supervision of Dr. A.M. Zeillemaker. In April 2016, Charla will start her residency training in Radiology at the MCH Westeinde hospital under the supervision of Drs. E.G. Coerkamp.

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