Prognostication in young and elderly breast cancer patients

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Chapter 1

Introduction and outline of thesis

Breast cancer

Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death from cancer in women in the western world[1]. Women in these countries have a 12-13% risk of developing breast cancer in their life and incidence rates are increasing, due to changes in reproductive factors (use of postmenopausal hormone therapy), increase in breast cancer screening and population graying[1, 2]. On the other hand, mortality rates are decreasing due to early detection through mammography and advances in breast cancer treatment[3, 4].

Treatment of breast cancer

Treatment of early stage breast cancer consists of loco-regional control and prevention of development of distant metastases. Loco-regional control is managed through removal of the tumor in the breast and spread to the lymph nodes with surgery with or without radiotherapy. The cause of breast cancer-related deaths are distant metastases, which are thought to develop from tumor cells that have detached from the primary tumor and circulate in the blood or already have formed undetectable micro metastases at time of surgery[5, 6]. Adjuvant systemic therapy, i.e. chemotherapy, endocrine therapy and targeted trastuzumab therapy, are aimed at eradicating these circulating tumor cells and micro metastases in order to prevent development of distant metastases. Data from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) has shown that administration of adjuvant systemic therapy results in a statistically significant beneficial disease-free and overall survival of breast cancer patients[7-10]. On the other hand, adjuvant systemic therapy can cause a wide range of acute and long-term side effects[11]. It is therefore of crucial importance to identify patient that will develop distant metastases and who may benefit from adjuvant systemic treatment and at the same time identify patients who will not develop distant metastases in order to spare those from unnecessary side effects of these therapies. Prognostic and predictive factors are needed that aid in the estimation of patients' prognosis and response to adjuvant systemic therapy.

Prognostication in breast cancer

Prognostic and predictive factors are aimed at estimating which patients necessitate adjuvant systemic treatment by estimating the patients' risk of developing distant metastases and response to treatment[12]. Nowadays, clinical and pathological factors, such as age, menopausal status, tumor size, lymph node status, tumor differentiation grade, hormone receptor status and human epidermal growth factor receptor 2 (HER2) overexpression, are used in daily practice to select patients that might benefit from adjuvant systemic treatment[13]. However, these prognostic and predictive factors,

separately or in combination with one another (e.g. St. Gallen recommendations, Nottingham Prognostic Index, Adjuvant! Tool) still do not provide an optimal patient stratification and consequently recommendations for adjuvant systemic treatment are not accurate[14-16]. As a result, a proportion of patients that does need systemic treatment, but are classified as "good prognosis", inadequately does not receive systemic treatment and is therefore undertreated. On the other hand, a substantial proportion of patients that will be cured by surgery and radiotherapy alone do receive systemic treatment and are therefore over treated and unnecessarily exposed to these treatment's toxicities. There is therefore a great need for new and more accurate prognostic and predictive factors.

There are several new pathological and molecular variables in development that are consistently associated with outcome or response to loco regional and systemic treatment. In 2007, the American Society of Clinical Oncology Committee recommended the following new prognostic markers in clinical practice for breast cancer patients: urokinases plasminogen activator (uPA); plasminogen activator inhibitor-1 (PAI-1); and multiparameter gene expression assays, mammaprint and oncotypeDX[17-19]. The prognostic value and clinical application of these factors are currently being evaluated in clinical trials[18, 19]. However, these new prognostic factors have several limitations. First, they are not suitable for all tumors, since fresh frozen material is often needed, which is not always available. In addition, the major critique of microarray-based prognostic tools is the fact that these gene prints were constructed using top-down analyses and were not defined based on a biological rationale[20]. The better understanding of underlying breast cancer biology aids in distinguishing biologically differing breast tumors. Biomarkers predictive for patient prognosis and treatment efficacy, which are based on these differences in biology, provide more solid tools for prognostication and treatment response prediction.

PART I: PROGNOSTIC BIOMARKERS IN THE INTERACTIONS BETWEEN THE HOST'S IMMUNE SYSTEM AND BREAST CANCER

The first part of this thesis focuses on the interactions taking place between breast tumors and the immune system. There is strong evidence that the host's adaptive immune system is able to control tumor progression[21]. On the other hand, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from such immune recognition[22]. Various interactions underlie this balance between tumor immune control and escape. We investigated the expression and prognostic effect of various crucial immunological markers and their interactions in a well-described large

cohort of breast cancer patients primarily treated with surgery at the Leiden University Medical Center, with long-term follow-up data.

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 by CTL, cancer cells may lose expression of classical HLA class I[23]. Another tumor escape mechanism from immune surveillance is attraction and induction of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment[24]. In **Chapter 2** these tumor escape mechanisms, classical HLA class I down regulation and attraction of Treg, are related to patients' outcome especially concerning response to chemotherapy treatment.

Loss of expression of classical HLA class I on the tumor cell surface makes malignant cells prone to natural killer (NK) cell recognition[25]. Non-classical HLA class I molecules (HLA-E, HLA-G) also 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[25-27]. The prognostic role of tumor expression of HLA-E and HLA-G in relation to classical HLA class I expression is described in **Chapter 3**.

The activating receptor NK cell lectin-like receptor gene 2D (NKG2D) is a stimulatory immune receptor that is expressed on NK cells, NKT cells and T cells [28]. Ligands which bind NKG2D receptors comprise major histocompatibility complex class I chain-related proteins A and B (MIC-AB) and unique long 16 (UL16) binding proteins 1-6 (ULBP1-6)[29, 30]. Expression of these ligands may be induced upon infection and other inducers of cellular stress, such as malignant transformation, and is unusual in normal cells[31]. By binding to the NKG2D receptors on NK and T cells, the NKG2D ligands may initiate an immune response against cells expressing these ligands. Overexpression and shedding of NKG2D ligands have been reported[31]. It is unclear whether up regulation of NKG2D ligands on tumor cells results in activation of an immune response or leads to overstimulation and down regulation of NKG2D on immune cells [28] and the effects of up regulation of the ligands on patient prognosis has been found to variate between tumor types. The prognostic effect of NKG2D ligands expression in breast cancer is described in **Chapter 4**.

A variety of immune reactions have been found to date in breast cancer. Studies have demonstrated that breast cancer is highly immunogenic, but on the other hand also capable of evading immune recognition. This suggests that various interactions exist between breast tumors and the immune system and that in order to get a good perspective on the effects of the immune system on tumor progression and patient outcome in these cancer patients, such interactions should be accounted for. This emphasizes the importance of research on combinations of markers of immune surveillance together with markers of tumor immune escape. In **Chapter 5**, tumor immune subtypes were constructed, considering various interactions that can take place between tumor and immune system and reflecting the various stages of tumor immune escape from high

immune susceptibility to high immune evasion. In this study, the prognostic effect of the tumor immune subtypes in breast cancer was evaluated.

PART II: PROGNOSTIC BIOMARKERS IN ELDERLY BREAST CANCER PATIENTS

Breast cancer in the elderly

Because of a graying population, breast cancer is increasingly becoming a disease affecting older women[32]. This older breast cancer population differs clinically in many aspects from younger breast cancer patients. Due to patient co-morbidity and the potential for therapy to amplify pre-existing medical conditions, the balance between treatment toxicity and benefits is uncertain[33]. In addition, life expectancy is significantly shorter in elderly breast cancer patient resulting in elderly breast cancer patients dying more often "with the disease" instead of "from the disease"[34-36]. These competing risks of death highly influence treatment significance. Furthermore, patient preferences are different in older breast cancer patients compared to their younger counterparts. In addition to clinical aspects, there are indications that elderly breast cancer differs in underlying biology. Characteristics such as hormone receptor status, human epidermal growth factor receptor 2 (HER2) status and amount of tumor cell proliferation have been found to differ considerably in tumors from elderly compared to young patients[37-39]. However, though these significant differences between elderly and young breast cancer patients exist, evidence-based treatment guidelines specific for elderly patients are lacking. Translational cancer research, which lies on the basis of evidence-based treatment, is in the elderly still rare but therefore urgently needed.

Breast cancer stem cells

Cancer stem cells, defined as a small subset of tumor cells with stem cell-like features, including epithelial-to-mesenchymal transition, have the capacity of self-renewal and differentiation; giving rise to a heterogeneous tumor cell population[40]. Various putative markers of breast cancer stem cells have been proposed, including aldehyde dehydrogenase-1 (ALDH1) activity, CD44+/CD24-, CD133, and ITGA6.[40-43] In particular, ALDH1 expression has shown promise as a clinically relevant marker for unfavorable clinical prognosis.[42, 44, 45]

It is unknown whether expression of ALDH1 is associated with age and has influence on clinical outcome in elderly breast cancer patients. **Chapter 6** describes the age

distribution of ALDH1 expression and its prognostic role in young and elderly breast cancer patients in our above-described cohort.

Molecular subtypes

Gene expression studies have identified several distinct breast cancer subtypes based on gene expression patterns, that showed marked differences in patient prognosis[46-48]. This "intrinsic" classification proposes four different classes of breast tumors: Luminal A and B, which are mostly hormone receptor-positive and show high expression of genes characteristic of the luminal epithelial cell layer, including expression of ER, GATA3 and genes regulated by these[47, 48], Basal-like tumors, which typically are triple-negative tumors (ER, PR, and HER2 negative) and exhibit high expression of genes characteristic of the basal epithelial cell layer such as cytokeratin (CK) 5, 6 and 17 [46] and the ERBB2 tumor subtype, which clusters near the basal-like tumor, are mostly hormone receptor-negative and show high overexpression of HER2 and high HER2 gene amplification[47, 48]. Concerning outcome, hormone receptor-positive tumors are associated with the best patient outcome where, compared to Luminal B tumors, Luminal A tumors seem to be the most indolent tumors[47]. Hormone receptor-negative intrinsic subtypes, ERBB2 and Basal-like tumors have an aggressive natural history, resulting in an unfavorable patient outcome[47]. The distribution and prognostic effect of intrinsic breast cancer subtypes specific in the elderly breast cancer population compared to younger breast cancer patients is still unknown. Using immunohistochemical (IHC) surrogates, which we validated against gene expression determined intrinsic subtypes, **Chapter 7** describes the identification of breast tumor intrinsic subtypes in our breast cancer cohort and the distribution and prognostic effect of these intrinsic subtypes in elderly compared to their younger counterparts.

Tumor immune subtypes

Among others, age-specific immune surveillance may contribute to the strong association between breast cancer and increasing age. The mechanisms involved in immune surveillance have been shown to alter with ageing[50]; a decline in immune system functioning, which is commonly defined as immunosenescence[51]. It has been suggested that thymic involution, intrinsic changes due to cell damage leading to altered signaling, and chronic antigen stimulation during life are the main underlying causes for immuosenescence[50]. Among others, immunosenescence comprises the decrease in production of new T cells and oligoclonal expansion of CD8+ memory T cells, which may limit the ability to respond to newly encountered viruses [52, 53] and may result in a decreased exportation of naïve T cells to peripheral tissue[54, 55]. Consequently further restriction of the ability to renew the immune repertoire occurs. In addition, a decreased

toxicity and a decreased IL-2 production have been observed for NK cells [50] and in animal studies it has been shown that a high number of immune suppressive Tregs were found in old mice[56, 57]. Preclinical data therefore suggest that immunosenescence may impair immune surveillance and consequently tumor immune surveillance may be affected in elderly[50]. **Chapter 8** describes a study where the distribution of key markers for cellular immune response, classical HLA class I, HLA-E, HLA-G, CD8, NK cells, and Treg were compared between elderly and young breast cancer patients and the age-specific prognostic effect of previously described tumor immune subtype was assessed.

Finally, **Chapter 9** includes a summary of this thesis as well as conclusions and discussion on future perspectives. **Chapter 10** provides a summary in Dutch.

Reference List

1. Jemal,A. et al. Cancer statistics, 2008. CA Cancer J. Clin. **58**, 71-96 (2008).
2. Althuis,M.D., Dozier,J.M., Anderson,W.F., Devesa,S.S., & Brinton,L.A. Global trends in breast cancer incidence and mortality 1973-1997. Int. J. Epidemiol. **34**, 405-412 (2005).
3. Berry,D.A. & Ravdin,P.M. Breast cancer trends: a marriage between clinical trial evidence and epidemiology. J. Natl. Cancer Inst. **99**, 1139-1141 (2007).
4. Jatoi,I. & Miller,A.B. Why is breast-cancer mortality declining? Lancet Oncol. **4**, 251-254 (2003).
5. de,B.M. et al. Micrometastases or isolated tumor cells and the outcome of breast cancer. N. Engl. J. Med. **361**, 653-663 (2009).
6. Pagani,O. et al. International guidelines for management of metastatic breast cancer: can metastatic breast cancer be cured? J. Natl. Cancer Inst. **102**, 456-463 (2010).
7. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet **351**, 1451-1467 (1998).
8. Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet **352**, 930-942 (1998).
9. Clarke,M. et al. Adjuvant chemotherapy in oestrogen-receptor-poor breast cancer: patient-level meta-analysis of randomised trials. Lancet **371**, 29-40 (2008).
10. Early breast cancer Trialists' Collaborative Group Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet **365**, 1687-1717 (2005).
11. Eifel,P. et al. National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1-3, 2000. J. Natl. Cancer Inst. **93**, 979-989 (2001).
12. Weigelt,B., Peterse,J.L., & van,'., V Breast cancer metastasis: markers and models. Nat. Rev. Cancer **5**, 591-602 (2005).
13. Harris,J. Staging of Breast Cancer. ed. LippmanME, M. M. H. S. Disease of the Breast. 457-459. 2009. Lippincot-Raven, New York, 1996.
14. Goldhirsch,A. et al. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann. Oncol. **20**, 1319-1329 (2009).
15. Ravdin,P.M. et al. Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer. J. Clin. Oncol. **19**, 980-991 (2001).
16. Todd,J.H. et al. Confirmation of a prognostic index in primary breast cancer. Br. J. Cancer **56**, 489-492 (1987).
17. Harris,L. et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J. Clin. Oncol. **25**, 5287-5312 (2007).
18. Michiels,S., Koscielny,S., & Hill,C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. Lancet **365**, 488-492 (2005).
19. van de Vijver M.J. et al. A gene-expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. **347**, 1999-2009 (2002).
20. Sotiriou,C. & Pusztai,L. Gene-expression signatures in breast cancer. N. Engl. J. Med. **360**, 790-800 (2009).
21. Zitvogel,L., Tesniere,A., & Kroemer,G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat. Rev. Immunol. **6**, 715-727 (2006).
22. Dunn,G.P., Old,L.J., & Schreiber,R.D. The three Es of cancer immunoediting. Annu. Rev. Immunol. **22**, 329-360 (2004).
23. Algarra,I., Garcia-Lora,A., Cabrera,T., Ruiz-Cabello,F., & Garrido,F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol. Immunother. **53**, 904-910 (2004).
24. Cerwenka,A., Baron,J.L., & Lanier,L.L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc. Natl. Acad. Sci. U. S. A **98**, 11521-11526 (2001).
25. Wischhusen,J., Waschbisch,A., & Wiendl,H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin. Cancer Biol. **17**, 459-468 (2007).
26. Khong,H.T. & Restifo,N.P. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat. Immunol. **3**, 999-1005 (2002).
27. Marin,R. et al. Analysis of HLA-E expression in human tumors. Immunogenetics **54**, 767-775 (2003).
28. Champsaur,M. & Lanier,L.L. Effect of NKG2D ligand expression on host immune responses. Immunol. Rev. **235**, 267-285 (2010).
29. Bauer,S. et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science **285**, 727-729 (1999).

30. Cosman,D. et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. **14**, 123-133 (2001).
31. Stern-Ginossar,N. & Mandelboim,O. An integrated view of the regulation of NKG2D ligands. Immunology **128**, 1-6 (2009).
32. Petrakis,I.E. & Paraskakis,S. Breast cancer in the elderly. Arch. Gerontol. Geriatr. **50**, 179-184 (2010).
33. Albrand,G. & Terret,C. Early breast cancer in the elderly: assessment and management considerations. Drugs Aging **25**, 35-45 (2008).
34. Wildiers,H. et al. Management of breast cancer in elderly individuals: recommendations of the International Society of Geriatric Oncology. Lancet Oncol. **8**, 1101-1115 (2007).
35. Bastiaannet,E. et al. Breast cancer in elderly compared to younger patients in The Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. Breast Cancer Res. Treat. **124**, 801-807 (2010).
36. Bastiaannet,E. et al. Lack of survival gain for elderly women with breast cancer. Oncologist. **16**, 415-423 (2011).
37. Daidone,M.G., Coradini,D., Martelli,G., & Veneroni,S. Primary breast cancer in elderly women: biological profile and relation with clinical outcome. Crit Rev. Oncol. Hematol. **45**, 313-325 (2003).
38. Diab,S.G., Elledge,R.M., & Clark,G.M. Tumor characteristics and clinical outcome of elderly women with breast cancer. J. Natl. Cancer Inst. **92**, 550-556 (2000).
39. Remvikos,Y., Magdelenat,H., & Dutrillaux,B. Genetic evolution of breast cancers. III: Age-dependent variations in the correlations between biological indicators of prognosis. Breast Cancer Res. Treat. **34**, 25-33 (1995).
40. Al-Hajj,M., Wicha,M.S., Benito-Hernandez,A., Morrison,S.J., & Clarke,M.F. Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U. S. A **100**, 3983-3988 (2003).
41. Cariati,M. et al. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. Int. J. Cancer **122**, 298-304 (2008).
42. Ginestier,C. et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell **1**, 555-567 (2007).
43. Wright,M.H. et al. Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. Breast Cancer Res. **10**, R10 (2008).
44. Resetkova,E. et al. Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. Breast Cancer Res. Treat. **123**, 97-108 (2010).
45. Tanei,T. et al. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. Clin. Cancer Res. **15**, 4234-4241 (2009).
46. Perou,C.M. et al. Molecular portraits of human breast tumours. Nature **406**, 747-752 (2000).
47. Sorlie,T. et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A **98**, 10869-10874 (2001).
48. Sorlie,T. et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. U. S. A **100**, 8418-8423 (2003).
49. Carey,L.A. et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA **295**, 2492-2502 (2006).
50. Fulop,T. et al. Potential role of immunosenescence in cancer development. Ann. N. Y. Acad. Sci. **1197**, 158-165 (2010).
51. Agarwal,S. & Busse,P.J. Innate and adaptive immunosenescence. Ann. Allergy Asthma Immunol. **104**, 183-190 (2010).
52. Dorshkind,K., Montecino-Rodriguez,E., & Signer,R.A. The ageing immune system: is it ever too old to become young again? Nat. Rev. Immunol. **9**, 57-62 (2009).
53. Dorshkind,K. & Swain,S. Age-associated declines in immune system development and function: causes, consequences, and reversal. Curr. Opin. Immunol. **21**, 404-407 (2009).
54. Dorshkind,K., Montecino-Rodriguez,E., & Signer,R.A. The ageing immune system: is it ever too old to become young again? Nat. Rev. Immunol. **9**, 57-62 (2009).
55. Dorshkind,K. & Swain,S. Age-associated declines in immune system development and function: causes, consequences, and reversal. Curr. Opin. Immunol. **21**, 404-407 (2009).
56. Nishioka,T., Shimizu,J., Iida,R., Yamazaki,S., & Sakaguchi,S. CD4+CD25+Foxp3+ T cells and CD4+CD25-Foxp3+ T cells in aged mice. J. Immunol. **176**, 6586-6593 (2006).
57. Sharma,S., Dominguez,A.L., & Lustgarten,J. High accumulation of T regulatory cells prevents the activation of immune responses in aged animals. J. Immunol. **177**, 8348-8355 (2006).

Part I

PROGNOSTIC BIOMARKERS IN THE INTERACTIONS BETWEEN THE HOST'S IMMUNE SYSTEM AND BREAST CANCER

Chapter 2

The predictive value of hla class I tumor cell expression and presence of intratumoral tregs for chemotherapy in patients with early breast cancer

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ABSTRACT

Purpose We hypothesized that T cell immune interaction affects tumor development and thus clinical outcome. Therefore, we examined the clinical impact of human leukocyte antigen (HLA) class I tumor cell expression and regulatory T cell (Treg) infiltration in breast cancer.

Experimental Design Our study population (n=677) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1994. Formalin-fixed paraffin-embedded tumor tissue was immunohistochemically stained using HCA2, HC10 and Foxp3 monoclonal antibodies.

Results HLA class I expression was evaluated by combining results from HCA2 and HC10 antibodies and classified into three groups: loss, downregulation and expression. Remarkably, only in patients who received chemotherapy, both presence of Treg ($p=0.013$) and higher HLA class I expression levels ($p=0.002$) resulted in less relapses, independently of other parameters. Treg and HLA class I were not of influence on clinical outcome in patients who did not receive chemotherapy.

Conclusions We showed that HLA class I and Treg both affect prognosis, exclusively in chemotherapy-treated patients and are therefore one of the few predictive factors for chemotherapy response in early breast cancer patients. Chemotherapy may selectively eliminate Treg, thus enabling Cytotoxic T-lymphocytes to kill tumor cells that have retained HLA class I expression. As a consequence, HLA class I and Treg can predict response to chemotherapy with high discriminative power. These markers could be applied in response prediction to chemotherapy in breast cancer patients.

INTRODUCTION

Breast cancer is the most common cancer in women: it affects one in nine women. Systemic treatment improves disease free survival (DFS) and overall survival (OS) in patients with early breast cancer.[1] Decisions regarding this systemic therapy are depending on prognostic and predictive factors, which divide patients into different risk-groups.[2] With the current classifications, however, prediction of outcome is still not optimal and additional prognostic and predictive factors are needed to improve tailored treatment.

It is widely accepted that the adaptive immune system plays an important role in controlling tumor growth and spread.[3] Cytotoxic T-lymphocytes (CTL) are capable to affect tumor development. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from CTL recognition. Among these properties are downregulation or complete loss of Human Leukocyte Antigen (HLA) class I expression. In addition, immunosuppressive regulatory T cells (Treg) may be induced.[4]

HLA class I molecules play a pivotal role in CTL-mediated immune responses and have been found to be a prognostic factor in various types of cancer.[5-7] Previous studies have demonstrated that HLA class I expression is frequently down-regulated in breast cancer.[8, 9] However, the reports on prognostic influence of HLA class I expression in breast cancer have contradictory results.[10-12] Some found no significant correlation between percentage of tumor cells expressing HLA class I and survival of breast cancer patients.[10, 12] In contrast, another study found that total loss of HLA class I was an independent indicator of good prognosis.[11]

Treg act as immunosuppressors and maintain immunological self-tolerance. Numbers of tumor-infiltrating Treg are known to be increased in several malignancies and a correlation was found with worse disease stage and prognosis in cancer. [13, 14]In breast cancer, the presence of Treg in the tumor environment has been found in several studies.[15-17] Moreover, these studies found a higher prevalence of Treg in tumor microenvironment and in peripheral blood of patients suffering breast cancer compared to healthy donors.[15-17] One study found higher numbers of Treg to be correlated with worse disease stage and shorter survival.[15] Interestingly, chemotherapy has been found to be involved in presence and prognostic influence of Treg.[16] The numbers of Treg in tumor tissue decreased after chemotherapy administration and there was an association between disappearance of Treg and pathologic complete response to preoperative chemotherapy.

The purpose of our study was to analyze the prognostic relevance of HLA class I expression and Treg infiltration in a large cohort of early breast cancer patients. In addition, we explored the predictive value of these markers for chemotherapy response.

MATERIALS AND METHODS

Patients and tumors

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1994 (n=677). 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 differentiation grade and morphology, TNM stage, local and systemic therapy, locoregional/distant tumor recurrence, secondary tumor, alive/death, estrogen receptor (ER), progesterone receptor (PgR),Ki67, and human epidermal growth factor receptor 2 (HER2). All tumors were graded according to current pathological standards, by one pathologist (VS).

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 which recognize HLA class I heavy chains (kindly provided by Prof. Dr. J. Neefjes) were used. The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24) as well as some HLA-B, HLA-C, HLA-E, HLA-F, HLA-G chains.[18, 19] HC10 reacts mostly with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, -A28, -A29, -A30, -A31, -A32, -A33.[20, 21] Mouse antibodies against human Foxp3 (ab20034 clone 236A/E7: AbCam, UK) were used for Treg identification. The reactivity spectrum of Foxp3 comprises Treg and may include small numbers of CD8+ cells [22], but so far it is the best single marker of Treg.[23]

Immunohistochemistry

For HLA class I staining, slides of 4 μm were cut from a priory constructed tissue micro array (TMA). For staining of Treg sections of 4 μm were cut from the original formalin-fixed paraffin-embedded (FFPE) tumor blocks. Tissue sections were deparaffinised and rehydrated. Endogenous peroxidase was blocked for 20 minutes in hydrogen-peroxide methanol. For antigen retrieval, 0.01 M citrate buffer (pH6.0) was used for 10 minutes at maximum power in a microwave oven. Sections were incubated overnight with HCA2 or HC10 at room temperature using predetermined optimal concentrations. After incubation with secondary antibody envision anti-mouse (Dako Cytomation K4001), sections were visualised using DAB-solution (25ml DAB in 225 ml 0.05M Tris-HCl). Tissue section were counterstained with haematoxylin, dehydrated and mounted in malinol. All slides were stained simultaneously to avoid inter-assay variation. For each patient normal epithelium, stromal cells or lymphoid cells served as internal positive

	Total		HLA Class I						Treg			
			Loss		Downregulation		Expression		Absence		Presence	
	N	%	N	%	N	%	N	%	N	%	N	%
Age								P: 0.449				P: 0.902
<40	48	8.4	8	6.6	15	7.5	19	9.4	24	7.5	21	8.8
40-50	145	25.3	37	30.3	58	29.0	44	21.8	84	26.2	61	25.5
50-60	132	23.0	31	25.4	42	21.0	45	22.3	77	24.1	53	22.2
>60	249	43.4	46	37.7	85	42.5	94	46.5	135	42.2	104	43.5
Grade								P: <0.001				P: 0.047
I	80	14.2	28	23.7	26	13.1	16	8.0	52	16.5	27	11.4
II	282	49.9	55	46.6	114	57.6	87	43.3	163	51.7	113	47.7
III	203	35.9	35	29.7	58	29.3	98	48.8	100	31.7	97	40.9
Histological type								P: 0.135				P: 0.290
Ductal	513	89.4	102	86.4	178	89.9	190	94.5	286	90.8	215	90.3
Lobular	46	8.0	14	11.9	16	8.1	10	5.0	27	8.6	18	7.6
Other	7	1.2	2	1.7	4	2.0	1	0.5	2	0.6	5	2.1
Tumor stage								P: 0.760				P: 0.850
pT1	211	38.0	46	39.3	73	37.4	71	36.0	120	38.7	87	37.5
pT2	272	49.0	55	47.0	92	47.2	103	52.3	151	48.7	112	48.3
pT3/4	72	13.0	16	13.7	30	15.4	23	11.7	39	12.6	33	14.2
Nodal stage								P: 0.871				P: 0.831
pN0	307	55.1	64	53.3	107	55.4	111	56.3	170	54.5	128	55.4
pN+	250	43.6	56	46.7	86	44.6	86	43.7	142	45.5	103	44.6
Estrogen receptor								P: 0.004				P: 0.465
Negative	203	37.6	37	30.8	64	33.0	93	46.5	109	36.3	88	39.5
Positive	337	62.4	83	69.2	130	67.0	107	53.5	191	63.7	135	60.5
Progesterone receptor								P: <0.001				P: 0.140
Negative	223	41.6	44	37.6	64	32.8	105	52.5	116	38.8	100	45.2
Positive	313	58.4	73	62.4	131	67.2	95	47.5	183	61.2	121	54.8
Ki67 expression								P: 0.161				P: 0.001
Negative	458	85.4	103	88.0	169	87.1	161	81.3	270	90.0	176	80.0
Positive	78	14.6	14	12.0	25	12.9	37	18.7	30	10.0	44	20.0
HER2 overexpression								P: 0.147				P: 0.403
No overexpression	378	89.6	92	94.8	128	87.7	148	88.1	213	88.8	157	91.3
Overexpression	44	10.4	5	5.2	18	12.3	20	11.9	27	11.2	15	8.7
Local therapy								P: 0.051				P: 0.985
MST-radiotherapy	223	38.9	46	37.7	75	37.5	85	42.1	125	39.2	90	38.0
MST+radiotherapy	108	18.8	25	20.5	27	13.5	46	22.8	60	18.8	44	18.6
BCS-radiotherapy	5	0.9	2	1.6	2	1.0	0	0	3	0.9	2	0.8
BCS+radiotherapy	238	41.5	49	40.2	96	48.0	71	35.1	131	41.1	101	42.6
Systemic therapy								P: 0.426				P: 0.294
Chemotherapy	112	19.5	20	16.4	39	19.5	48	23.8	64	20.0	48	20.1
Endocrine therapy	75	13.1	12	9.8	25	12.5	28	13.9	49	15.3	24	10.0
Both	18	3.1	3	2.5	9	4.5	6	3.0	11	3.4	7	2.9
No systemic therapy	369	64.3	87	71.3	127	63.5	120	59.4	196	61.2	160	66.9

Table 1 Correlations between HLA class I expression and presence of Treg and well-established prognostic factors using chi-square test.

Abbreviations N number of patients; % percentage; HLA class I human leukocyte antigen class I; Treg regulatory T cell; HER2 human epidermal growth factor receptor 2; MST Mastectomy; BCS breast conservative surgery.

control for HLA class I antibody reactivity. Slides from human tonsil tissue served as positive control for Treg staining. For each staining, slides that did undergo the whole immunohistochemical staining procedure, but without primary antibodies served as negative controls.

Evaluation of immunostaining

Microscopic analysis of HCA2 and HC10 was assessed by two independent observers (EdK and QT) in a blinded manner. Percentage of tumor cells that showed membranous staining was assessed. HCA2 and HC10 staining were scored in 5 categories according to the defined standard method of the International HLA and Immunogenetics Workshop (IHIWS, score 1: 0-5 percent of tumor cells positively stained; score 2: 5-25; score 3: 25-50; score 4: 50-75; score 5: 75-100).[24] Quantification of Treg within the tumor was microscopically assessed in 10 high power fields (hpf) by two observers (EdK: 100%; AS: 30%) in a blinded manner. Treg was scored into two categories: absence and presence of Treg infiltration.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 15.0 for Windows, Spps Inc, Chicago, IL, USA). Cohen's kappa coefficient revealed a satisfactory agreement in classification (kappa=0.73). The χ^2 test was used to evaluate associations between various clinicopathological parameters and HLA class I expression and infiltration of Treg. Relapse-free period (RFP) was the time from date of surgery until a locoregional recurrence and/or distance recurrence, whichever came first. Clinical follow-up policy was equal for all patients in the study. Overall survival (OS) was defined from date of surgery until death. The Kaplan–Meier method was used for calculation of survival probabilities and the log-rank test for comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk.[25] Cox regression was used for univariate and multivariate analysis for RFP and OS. Significant or close to significant variables ($p<0.1$) in univariate analysis were included in multivariate analysis. To analyze the predictive effect of HLA class I and Treg, analyses were performed in which was stratified for adjuvant chemotherapy administration.

RESULTS

Patient and tumor characteristics

Tumor material was available and incorporated in the TMA of 86% (574/677) of the patients. Clinicopathological and treatment characteristics are shown in table 1. Median age of patients was 57 years (range= 23-96 years). Median follow-up of patients alive

was 19 years (range=0-23 years). Chemotherapy treatment consisted of a combination of cytostatic drugs, always containing cyclophosphamide.

Expression of HLA class I and infiltration of Treg

Microscopical quantification was successful in 94% (538/574) of tumors for HC10 and in 96% (548/574) for HCA2 (figures 1 A, B). A total of 523/574 tumors (91%) could be quantified for both and were therefore available for total HLA class I expression evaluation. Three groups were defined for HLA class I expression: (1) HLA class I loss (both HCA2 and HC10 scored 0-5%), 23% of tumors, (2) HLA class I downregulation (either HCA2 or HC10 scored 0-5%), 38% of tumors and (3) HLA class I expression (both HCA2 and HC10 scored 5-100%), 39% of tumors (table1).

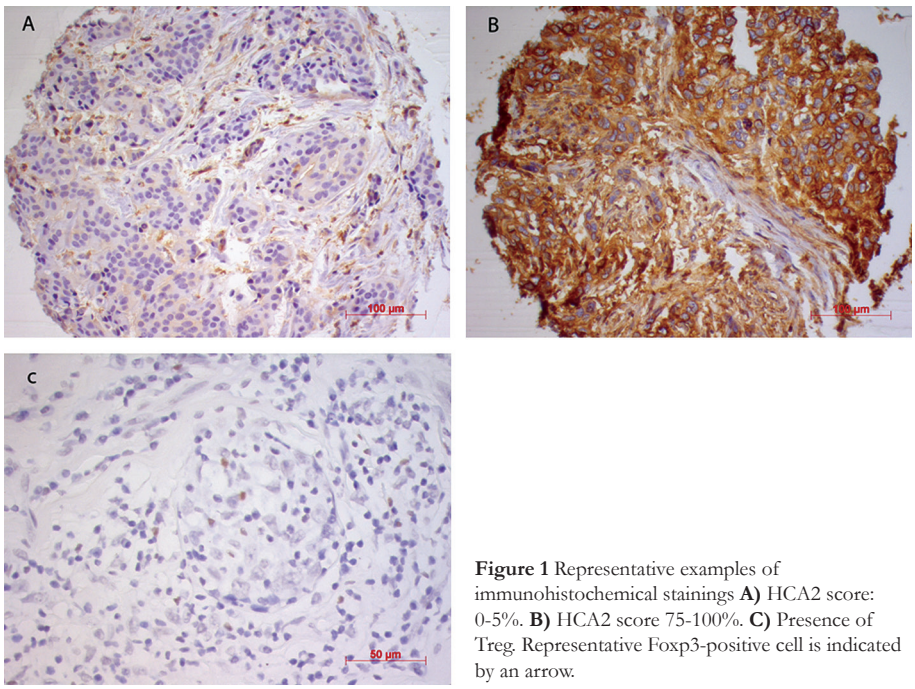

Figure 1 Representative examples of immunohistochemical stainings **A)** HCA2 score: 0-5%. **B)** HCA2 score 75-100%. **C)** Presence of Treg. Representative Foxp3-positive cell is indicated by an arrow.

A total of 556/574 (97%) tumors could be evaluated for Treg infiltration (figures 1 C,). Tumors with absence of Treg (0Treg/10hpf) and presence of Treg (≥1 Treg/10hpf) were seen in 57% and 43% of patients respectively (table 1).

Prognostic value of HLA class I and Treg

In order to analyze the prognostic effect of HLA class I and Treg, all patients who did not receive any systemic therapy were analyzed. There were no statistically significant differences in outcome for RFP (logrank: p=0.27) or OS (logrank: p=0.55) between different HLA class I expression levels (figures 2a, b). Treg showed no differences, neither in outcome for RFP (logrank: p=0.93) nor OS (logrank: p=0.14) between intra-tumoral

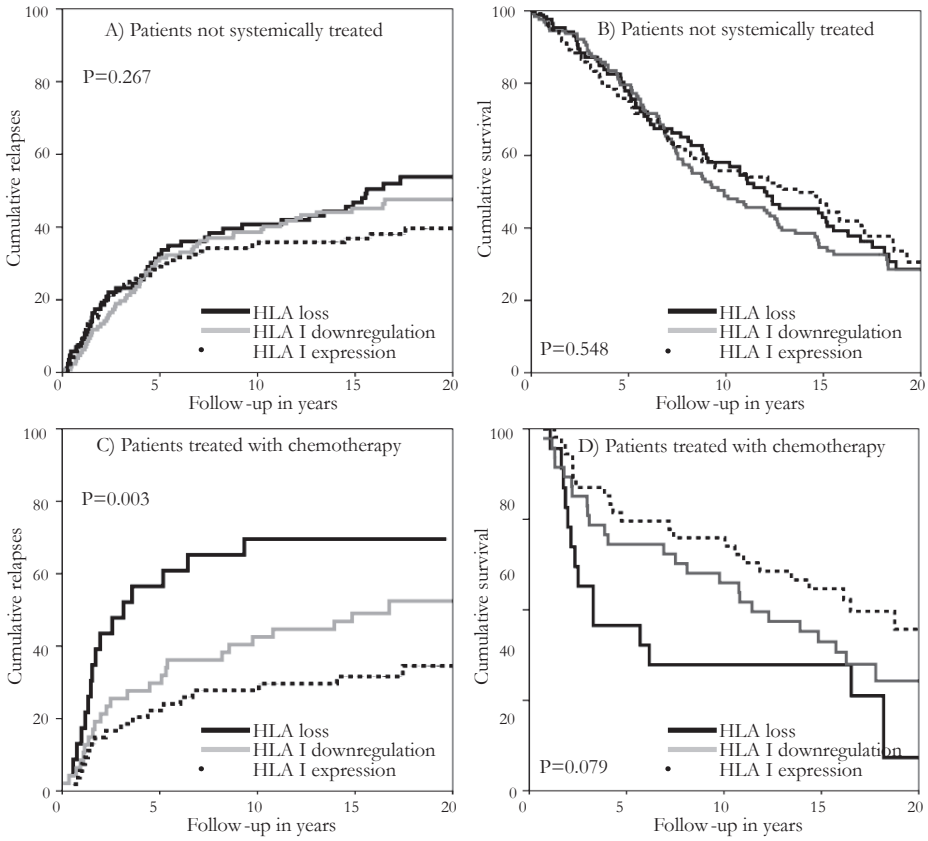

Figure 2 HLA class I tumor expression and clinical outcome. Relapses over time (A) and overall survival (B) of non-systemically treated patients. Relapses over time (C) and overall survival (D) of chemotherapy-treated patients.

absence and presence (figures 3a, b). In contrast with the data in patients who did not receive systemic treatment, analysis of HLA class I expression in chemotherapy-treated patients showed statistically significant differences for RFP between groups (logrank: p=0.003) (figure 2c). Of patients with expression of HLA class I, 68% were relapse free after 15 years, compared to 51% and 30% for downregulation and loss of HLA class I expression respectively. Infiltration of Treg showed, similarly to HLA class I, moderate differences in outcome between groups among chemotherapy-treated patients (logrank: p=0.06) (figure 3c). Patients with intra-tumoral infiltration of Treg had less relapses compared to patients with no infiltration of Treg. Cox proportional multivariate analysis was performed with data from chemotherapy-treated patients including the parameters that showed a trend of influence on outcome ($p<0.1$) in Cox proportional univariate analysis; lymph node status, HLA class I and Treg (table 2). This analysis revealed that lymph node status, HLA class I (p= 0.002; downregulation, HR 2.11, 95%CI 1.13-3.95;

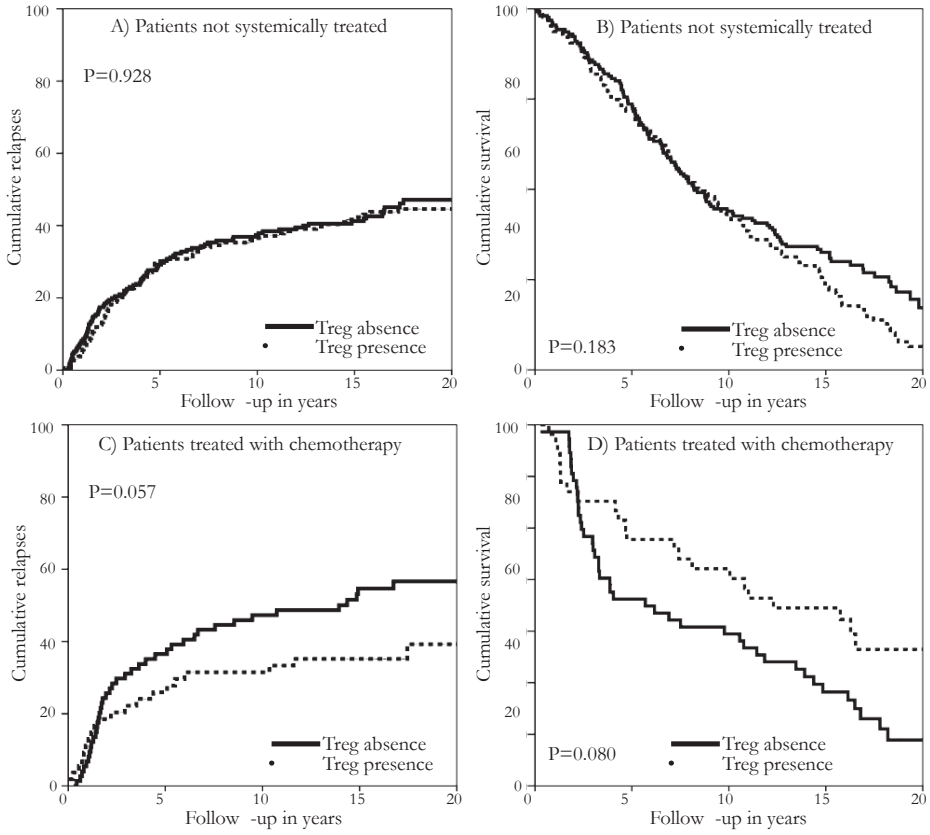

Figure 3 Treg tumor infiltration and clinical outcome. Relapses over time (A) and overall survival (B) of non-systemically treated patients. Relapses over time (C) and overall survival (D) of chemotherapy-treated patients.

loss, HR 3.34, 95%CI 1.67-6.67) and Treg (p=0.01, HR 2.04, 95%CI 1.16-3.57) were all independent prognostic factors for RFP among chemotherapy treated patients.

Predictive value of HLA class I and Treg

In order to prove that HLA class I and Treg were statistically significant cooperating with chemotherapy, an interaction term was introduced in Cox regression analysis. This analysis showed that both HLA class I (p< 0.001; Downregulation HR: 2.15, 95%CI: 1.17-3.96; Loss HR: 3.15, 95%CI: 1.92-5.15) and Treg (p<0.001; HR 2.47, 95%CI 1.54-3.95) significantly interacted with chemotherapy administration. These data indicated that both HLA class I expression and Treg tumor infiltration possess prognostic value specifically in breast cancer patients that are treated with chemotherapy.

		UNIVARIATE			MULTIVARIATE	
	N	HR	95% CI	p-value	HR	95% CI	p-value
Age							
<40	25	1.00		0.370			
40-50	57	1.01	0.537-1.888				
50-60	30	0.67	0.303-1.472				
>60	18	0.51	0.183-1.413				
Grade							
I	15	1.00		0.887			
II	57	1.12	0.490-2.558				
III	57	0.99	0.428-2.271				
Histological type							
Ductal	117	1.00		0.453			
Other	12	0.453	0.594-3.209				
Tumor stage							
pT1	35	1.00		0.416			
pT2	70	0.88	0.491-1.560				
pT3/4	20	1.39	0.657-2.950				
Nodal stage							
pN-	38	1.00		0.002	1.00		0.001
pN+	92	2.92	1.480-5.741		3.08	1.539-6.179	
Estrogen receptor							
Negative	58	1.00		0.207			
Positive	65	1.41	0.828-2.390				
Progesterone receptor							
Negative	57	1.00		0.377			
Positive	69	1.27	0.750-2.138				
Ki67 expression							
Negative	101	1.00		0.866			
Positive	23	0.68	0.438-1.712				
HER2 overexpression							
Negative	82	1.00		0.497			
Positive	17	1.29	0.622-2.663				
Local treatment							
MST-Radiotherapy	33	1.00		0.109			
MST +radiotherapy	35	1.78	0.889-3.548				
BCS +radiotherapy	62	1.01	0.524-1.955				
Endocrine therapy							
Negative	112	1.00		0.501			
Positive	18	0.76	0.347-1.678				
HLA							
Expression	54	1.00		0.005	1.00		0.002
Downregulation	47	1.71	0.929-3.159		2.11	1.127-3.947	
Loss	23	3.11	1.577-6.116		3.34	1.671-6.670	
Treg							
>0	54	1.00		0.060	1.00		0.013
0	75	1.67	0.979-2.857		2.04	1.164-3.568	

Table 2 Cox univariate and multivariate analysis for recurrence free period (RFP) of patients who did receive chemotherapy.

Abbreviations N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; HLA class I human leukocyte antigen class I; Treg regulatory T cell; HER2 human epidermal growth factor receptor 2; MST mastectomy; BCS breast conserving surgery.

DISCUSSION

Our study showed that HLA class I and Treg are independent prognostic markers for chemotherapy-treated patients with substantial discriminative power. Parameters that are able to determine which breast cancer patients may benefit from adjuvant chemotherapy are few.[26] Known factors which tend to indicate better chemotherapy response are negative ER, high tumor grade and high proliferative activity, but their predictive value is marginal.[27] In our study, independently of those factors, both high levels of HLA class I expression and presence of Treg resulted in statistically significant less relapses over time. Most importantly, these results can be explained by underlying biology and correspond with results of previous studies.

In concordance with previous studies, downregulation and loss of HLA class I was frequently seen in our study.[9-11] Prior studies indicate that breast cancer is immunogenic and induces tumor associated antigen (TAA)-specific CTL.[28] These findings may imply that breast cancer cells with downregulation or loss of HLA class I expression escaped from immune destruction and therefore selectively grew out.[29] This seems quite a common phenomenon in breast cancer considering the fact that we and others, found HLA class I downregulation or loss in more than half of the tumors. In addition, Treg were found in a significant number of tumors. Tumors may either attract these immune-suppressing cells in order to evade attack from effector T cells, or Treg may consider tumor cells as normal cells and thus prevent immune attack. Our data indicate that the immune system is closely involved in the development of breast cancer. At the time of a clinically manifest tumor, the balance between immune attack and tumor growth obviously is at the site of the tumor. [29]

We showed that expression levels of HLA class I had a specific prognostic effect, but only in chemotherapy-treated patients. Previous studies on HLA class I expression in breast cancer did not stratify for systemic therapy. A total of 3 studies have evaluated HLA class I expression and its effect on prognosis in breast cancer.[10-12] Two studies found that HLA class I expression levels had no influence on the prognosis of patients, which is in concordance with our findings in patients that were not systemically treated. [10, 12]Our study also showed that infiltration of Treg was a predictive marker for chemotherapy response in breast cancer patients. These finding are supported by Ladoire *et al.* who found that the number of Treg declined due to chemotherapy, showing that chemotherapy affects Treg and thus may counteract by restrained CTL. More importantly, complete absence of Treg after chemotherapy administration, resulted in a better response with higher rates of pathological complete response (pCR), further supporting our findings of a predictive role of Treg. [16] Other studies have shown that infiltration of Treg in breast tumors resulted in a worse prognosis in terms of relapses and survival.[15, 30] Our study could not statistically prove such a relation in patients who

did not receive systemic treatment. In order to unravel the complex tumor-immune system interactions during tumor development, further studies are needed.
The specific prognostic effects found for HLA class I and Treg among chemotherapy-treated patients can be explained by the following biological explanation. In our population, choice of chemotherapy comprised cyclophosphamide which positively influences host immune responses against cancer.[31-33] It is hypothesized that several mechanism are the basis for this phenomenon: enhanced homeostatic expansion of antigen-specific T cells by creation of a niche in the immune system, stimulation of dendritic cells (DC), induction of T cell growth factors such as type I interferons (IFN) and selective elimination of Treg. [31-33]Ceasing of Treg through cyclophosphamide effects, results amongst other things in enhanced expansion and function of responding of CTL.[32, 33] Ladoire *et al.* found that after preoperative chemotherapy absolute numbers of tumor-infiltrating Treg significantly declined, and numbers of effector T cells and CTL remained stable. In addition, a pathologic complete response was associated with a combination of absence of infiltration of Treg and presence of CTL after chemotherapy.[16] In our study this phenomenon was associated only in tumors that retained HLA expression, suggesting that upon counteraction of Treg, CTL are able to affect tumor metastases development.

In summary, HLA class I and Treg have an independent prognostic effect for chemotherapy-treated patients, which can be explained by underlying biology. Both factors resulted in a very high differentiation in sensitivity to chemotherapy. Predictive factors for chemotherapy response in breast cancer are highly necessitated. Therefore, we conclude that HLA class I and Treg are candidate markers for further investigation in randomized studies and may be applied for chemotherapy response prediction.

REFERENCES

1. Early breast cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365(9472):1687-1717.
2. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007;18(7):1133-1144.
3. Van Pel A, Boon T. Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. Proc Natl Acad Sci U S A 1982;79(15):4718-4722.
4. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6(10):715-727.
5. Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren GJ. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. Cancer Immunol Immunother 2008;57(2):197-206.
6. Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. Lab Invest 2002;82(12):1725-1733.
7. Speetjens FM, de Bruin EC, Morreau H et al. Clinical impact of HLA class I expression in rectal cancer. Cancer Immunol Immunother 2008;57(5):601-609.
8. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53(10):904-910.
9. Cabrera T, Angustias FM, Sierra A et al. High frequency of altered HLA class I phenotypes in invasive breast carcinomas. Hum Immunol 1996;50(2):127-134.
10. Gudmundsdottir I, Gunnlaugur JJ, Sigurdsson H, Olafsdottir K, Tryggvadottir L, Ogmundsdottir HM. Altered expression of HLA class I antigens in breast cancer: association with prognosis. Int J Cancer 2000;89(6):500-505.
11. Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. Int J Cancer 2005;117(2):248-255.
12. Redondo M, Garcia J, Villar E et al. Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. Hum Pathol 2003;34(12):1283-1289.
13. Salama P, Phillips M, Grieu F et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 2009;27(2):186-192.
14. Wolf D, Wolf AM, Rumpold H et al. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. Clin Cancer Res 2005;11(23):8326-8331.
15. Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006;24(34):5373-5380.
16. Ladoire S, Arnould L, Apetoh L et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res 2008;14(8):2413-2420.
17. Perez SA, Karamouzis MV, Skarlos DV et al. CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. Clin Cancer Res 2007;13(9):2714-2721.
18. Seitz C, Uchanska-Ziegler B, Zank A, Ziegler A. The monoclonal antibody HCA2 recognises a broadly shared epitope on selected classical as well as several non-classical HLA class I molecules. Mol Immunol 1998;35(13):819-827.
19. Sernee MF, Ploegh HL, Schust DJ. Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. Mol Immunol 1998;35(3):177-188.
20. Hutter H, Hammer A, Blaschitz A et al. Expression of HLA class I molecules in human first trimester and term placenta trophoblast. Cell Tissue Res 1996;286(3):439-447.
21. Perosa F, Luccarelli G, Prete M, Favoino E, Ferrone S, Dammacco F. Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. J Immunol 2003;171(4):1918-1926.
22. Hill JA, Feuerer M, Tash K et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. Immunity 2007;27(5):786-800.
23. Generali D, Bates G, Berruti A et al. Immunomodulation of FOXP3+ regulatory T cells by the aromatase inhibitor letrozole in breast cancer patients. Clin Cancer Res 2009;15(3):1046-1051.
24. Chew SF, Kanaan C, Tait BD. HLA expression and cancer--14th IHIWS immunohistochemistry quality control exercise exchange results. Tissue Antigens 2007;69 Suppl 1:248-251.

25. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 2007;26(11):2389-2430.
26. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. J Clin Oncol 2003;21(17):3357-3365.
27. Ross JS, Linette GP, Stec J et al. Breast cancer biomarkers and molecular medicine. Expert Rev Mol Diagn 2003;3(5):573-585.
28. Sotiropoulou PA, Perez SA, Iliopoulou EG et al. Cytotoxic T-cell precursor frequencies to HER-2 (369-377) in patients with HER-2/neu-positive epithelial tumours. Br J Cancer 2003;89(6):1055-1061.
29. Ferrone S, Whiteside TL. Tumor microenvironment and immune escape. Surg Oncol Clin N Am 2007;16(4):755-74, viii.
30. Gobert M, Treilleux I, driss-Vermare N et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res 2009;69(5):2000-2009.
31. Dudley ME, Wunderlich JR, Robbins PF et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298(5594):850-854.
32. Ghiringhelli F, Larmonier N, Schmitt E et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34(2):336-344.
33. Proietti E, Greco G, Garrone B et al. Importance of cyclophosphamide-induced bystander effect on T cells for a successful tumor eradication in response to adoptive immunotherapy in mice. J Clin Invest 1998;101(2):429-441.

Chapter 3

HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients

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ABSTRACT

Non-classical human leukocyte antigens (HLA), HLA-E and HLA-G, are known to affect clinical outcome in various tumor types. We examined the clinical impact of HLA-E and HLA-G expression in early breast cancer patients, and related the results to tumor expression of classical HLA class I.

Our study population (n=677) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1995. Tissue micro array (TMA) sections of arrayed tumor and normal control material were immunohistochemically stained for HLA-E and HLA-G. For evaluation of HLA-E and HLA-G and the combined variable, HLA-EG, a binary score was used. Expression of classical HLA class I molecules was previously determined.

HLA-E, HLA-G and HLA-EG on breast tumors were classified as expression in 50%, 60% and 23% of patients respectively. Remarkably, only in patients with loss of classical HLA class I tumor expression, expression of HLA-E ($p=0.027$), HLA-G ($p=0.035$) or HLA-EG ($p=0.001$) resulted in a worse relapse free period. An interaction was found between classical and non-classical HLA class I expression ($p=0.002$), suggestive for a biological connection.

We have demonstrated that, next to expression of classical HLA class I, expression of HLA-E and HLA-G is an important factor in the prediction of outcome of breast cancer patients. These results provide further evidence that breast cancer is immunogenic, but also capable of evading tumor eradication by the host's immune system, by up- or down regulation of HLA class Ia and class Ib loci.

INTRODUCTION

There has been strong evidence that tumor progression is controlled by the host's immune system 1. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from immune recognition 2. These poorly immunogenic clones frequently have lost expression of classical human leukocyte antigen (HLA) class I (HLA-A, HLA-B, HLA-C) which enables them to escape cytotoxic T lymphocyte (CTL) attack. However, in that case they may be vulnerable to natural killer (NK) cell elimination. Expression of non-classical HLA class I molecules (HLA-E, HLA-G), which play a pivotal role in immune surveillance by NK-cells, may therefore also determine outcome of tumor immune interaction 3. Under normal circumstances, expression of the HLA-E molecule is found in most tissues that express HLA-A, -B, -C or -G molecules and is thought to provide an important "self-signal" to the immune system by accommodating and presenting peptide fragments from leader sequences of these molecules [3, 4]. HLA-G expression, on the other hand, has very restricted tissue expression and has been mostly found in extravillous trophoblastic cells, where it mediates semi-allograft immunotolerance during pregnancy 5. Expression of HLA-E and HLA-G on the cell surface can respectively bind with the inhibitory receptors CD94/NKG2A and KIR2DL4/p49 of NK cells, and thereby cause inhibition of their proliferation and cytotoxic effector functions [6, 7]. HLA-E also binds activating CD94/NKG2C receptors, present on T and NK cells, however with a 6-fold lower affinity 8.

Tumors may acquire or upregulate expression of HLA-E and HLA-G as protective property against immune recognition and elimination of tumors 3. HLA-E is regularly expressed in various healthy tissues and correlates with expression of classical HLA class I molecules. This physiological correlation with classical HLA class I molecules has been found to be disturbed in tumors, suggesting that malignant cells which escape T cell immune recognition by downregulation of classical HLA class I expression, may further escape immune recognition by upregulation of HLA-E 9. In addition, expression of HLA-G protects against "missing self" recognition of NK. Expression of this molecule, which is rarely found in healthy tissues, is frequently observed in pathological conditions such as in tumors [10, 11]. Previous studies showed that both HLA-E and HLA-G had increased expression in different types of tumor 12-15. Studies on the prognostic value of HLA-E expression in colorectal and cervix cancer showed that expression of this molecule correlated to tumor progression and had a trend towards a worse clinical outcome. The prognostic value of HLA-G expression has been investigated in colorectal, gastric, esophageal squamous cell carcinoma and non-small cell lung **cancer and** revealed it to be an independent prognostic factor for poor clinical outcome 16-19. **In addition,** expression of HLA-G has also been found in breast cancer, however no statistically significant associations were found with outcome of patients 20-22.

The prognostic effect of HLA-E and HLA-G expression in breast cancer is unknown. The purpose of this study was to analyze the prognostic relevance of expression of HLA-E and HLA-G in a large cohort of early breast cancer patients. Previously, we determined classical HLA class I expression in the same patient cohort. Therefore, we were able to stratify patients based on classical HLA class I expression of tumors and to analyze the impact of HLA-E and HLA-G expression on clinical outcome of early breast cancer patients.

PATIENTS AND METHODS

Patients and tumors

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1994 (n=677). 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, locoregional/distant tumor recurrence, secondary tumor, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) 23. All tumors were graded according to current pathological standards, by a pathologist (VS). In addition, for about half the cohort of patients (n=266) a TMA of paired histologically normal breast tissue was available. Normal breast tissue originated from the cancer-affected breast, but localized more distal from the tumor tissue.

Immunohistochemistry

MEM-E/02 (AbCam, UK) and 4H84 (Nuclilab, NL) antibodies were used to recognize HLA-E and HLA-G respectively. MEM-E/02 reacts specifically with the denatured heavy chain of human HLA-E24. The 4H84 antibody recognizes denatured HLA-G molecules and has been described to react with classical HLA class I molecules 25-27. Tissue section of 4 μm were cut from a previously constructed tissue micro array (TMA) of formalin-fixed paraffin-embedded tumors 23. Tissue sections were deparaffinized and rehydrated. For antigen retrieval, 0.01 M Trizma EDTA (TE) buffer (pH6.0) was used for 10 minutes at maximum power in a microwave oven. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen-peroxide methanol. Sections were incubated overnight with primary monoclonal antibodies using predetermined optimal concentrations. After 30 minutes incubation with secondary antibody Envision anti-mouse (Dako Cytomation K4001), sections were visualised using DAB-solution. Tissue section were counterstained with haematoxylin, and then dehydrated and finally mounted in malinol. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation. For each staining, placenta tissue slides served as positive

control. Negative controls were placenta tissue slides that did undergo the whole immunohistochemical staining without primary antibodies. Sections of paired normal tissue TMA were stained with MEM-E/02 and 4H84 in order to assess frequency of staining in normal breast tissue samples.
Tumor staining for classical HLA class I using the mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLAB/C respectively) was previously described 28.

Evaluation of immunostaining

Microscopic analysis of HLA-E and HLA-G was assessed by two independent observers (AS and EdK) in a blinded manner. Both markers were scored in a binary manner, considering any specific staining of tumor cells as positive expression and no staining as no expression. A combined variable of HLA-E and HLA-G scores was created: HLA-EG. HLA-EG expression was considered positive when both HLA-E and HLA-G were expressed and negative when either HLA-E or HLA-G was not expressed.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA). Cohen's kappa coefficient was used to asses inter-observer agreement in quantification. This revealed a substantial agreement in classification for HLA-E (kappa=0.72) and a very good agreement in classification for HLA-G (kappa=0.90). The χ^2 test was used to evaluate associations between various clinicopathological parameters and HLA-E and HLA-G expression. Relapse free period (RFP) was the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). Overall survival (OS) was defined as date of surgery until death. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk 29. Cox regression was used for univariate and multivariate analysis for RFP and OS. Significant variables ($p<0.1$) in univariate analysis were included in multivariate analysis. To analyze the independent prognostic effect of HLA-E and HLA-G on clinical outcome, tumors were stratified based on a previously determined expression characteristics of classical HLA class I molecules.
We finally analyzed whether the specificity of the anti-HLA-G antibody would interfere with the results of our survival analyses by separately analyzing the set of patients in which those who stained positive for this antibody on normal breast tissue were excluded.

RESULTS

Patient and tumor characteristics

Tumor material was available and incorporated in the TMA of 86% (574/677) of the patients. Paired normal breast tissue was available on TMA in 46% (266/574) of the patients. Median age of patients was 57 years (range= 23-96 years). Median follow-up of patients alive was 19 years (range=14-23 years). Clinicopathological and treatment characteristics are shown in table I.

Expression of HLA-E and HLA-G

Microscopical quantification was successful in 86% (493/574) of tumors for HLA-E and in 87% (501/574) for HLA-G. Respectively 14% and 13% of tumors were damaged or lost on the TMA slides, a problem associated with preparation, staining and mounting of TMA slides. Two groups, expression versus no expression, were defined for HLA-E and HLA-G (figure 1 A-D). Expression was found in 50% (247/493) and in 60% (299/501) of tumors for HLA-E and HLA-G respectively (table I). Expression of HLA-EG was found in 23 % (100/428) of tumors. HLA-G stained positive in 1% (3/266) of normal tissue samples (figure 1E, F), while HLA-E showed positive staining in all normal tissue samples (figure 1 G).

HLA-E, HLA-G and HLA-EG and prognostic associations with outcome

In the whole cohort of patients HLA-E, HLA-G and HLA-EG showed no statistically significant difference in outcome between expression versus no expression for RFP (log rank p-values respectively: 0.52, 0.95, 0.72) or OS (log rank p-values respectively: 0.86, 0.74, 0.27) (figures 2 A, D, G and 3 A, D, G).

Next, we stratified patients based on classical HLA class I tumor expression, classified as expression versus loss. Among the subgroup of classical HLA class I expression results were similar as in the whole cohort of patients: neither for HLA-E, HLA-G, nor HLA-EG a statistically significant difference was found for different expression levels in outcome for RFP (log rank p-values respectively: 0.73, 0.69, 0.51) or OS (log rank p-values respectively: 0.64, 0.74, 0.22) (figures 2 B, E, H and 3 B, E, H). Interestingly, among the subgroup of patients with loss of tumor expression of classical HLA class I, HLA-E and HLA-G expression showed significant differences for RFP (log rank p-values respectively: 0.03, 0.04) and OS (log rank p-values respectively: 0.03, 0.12) between both expression groups (figures 2 C, F and 3 C, F). Of the patients with no tumor expression of HLA-E or HLA-G, respectively 60% and 56% of patients were relapse free after 10 years, whereas of the patients with tumor expression of HLA-E or HLA-G, respectively 35% and 39% of patients were relapse free after 10 years. The combination variable HLA-EG showed, similarly to HLA-E and HLA-G separately, differences in outcome between expression and no expression among the subgroup of classical HLA class I loss, but at a much higher level of significance than each separately

	Total		HLA-E					HLA-G				
			No expression		Expression		p-value	No expression		Expression		p-value
	N	%	N	%	N	%		N	%	N	%	
Age							0.378					0.221
<40	48	8,4	17	6,9	28	11,4		22	7,4	16	7,9	
40-50	145	25,3	64	25,9	59	24		74	24,7	59	29,2	
50-60	132	23	57	23,1	56	22,8		61	20,4	50	24,8	
>=60	249	43,4	109	44,1	103	41,9		142	47,5	77	38,1	
Grade							<0.001					0.242
I	80	14,2	44	18,1	29	12		40	13,4	23	11,8	
II	282	49,9	132	54,3	105	43,6		158	53	92	47,2	
III	203	35,9	67	27,6	107	44,4		100	33,6	80	41	
Histological type							0.094					0.465
Ductal	513	90,6	214	87,7	225	93,4		266	89	180	92,3	
Lobular	53	9,4	30	12,3	16	6,6		33	10,1	15	7,7	
Tumor stage							0.094					0.616
pT1	211	38	96	40,2	87	36,6		112	38,8	67	34,4	
pT2	272	49	108	45,2	128	53,8		142	49,1	103	52,8	
pT3/4	72	13	35	14,6	23	9,7		35	12,1	25	12,8	
Nodal stage							0.332					0.151
pN0	307	55,1	138	57,7	129	53,5		159	54,3	112	57,7	
pN1-3	250	44,9	101	42,3	112	46,5		134	45,7	82	42,3	
Estrogen receptor							0.004					0.095
Negative	203	37,6	72	31,4	106	44,7		100	35,3	82	42,9	
Positive	337	62,4	157	68,6	131	55,3		183	64,7	109	57,1	
Progesterone receptor							0.021					0.499
Negative	223	41,6	81	35,1	106	45,9		115	41,1	84	44,2	
Positive	313	58,4	150	64,9	125	54,1		165	58,9	106	55,8	
Her2 overexpression							0.008					0.014
No overexpression	435	80,9	200	87,7	186	78,5		236	84,6	145	75,5	
Overexpression	103	19,1	28	12,3	51	21,5		43	15,4	47	24,5	
Classical HLA I							0.003					<0.001
Negative	112	21,3	68	30,1	40	17,9		78	28,4	28	14,6	
Positive	401	69,9	158	69,9	183	82,1		197	71,6	164	85,4	
Local Therapy							0.407					0.661
MAST-RT	223	38,9	109	44,1	92	37,4		116	38,8	78	38,6	
MAST+RT	108	18,8	41	16,6	50	20,3		52	17,4	43	21,3	
BCS-RT	5	0,9	2	0,8	1	0,4		2	0,7	2	1	
BCS+RT	238	41,5	95	38,5	103	41,9		129	43,1	79	39,1	
Systemic therapy							0.076					0.004
Chemotherapy	112	19,5	37	15	57	23,2		43	14,4	52	25,7	
Endocrine therapy	75	13,1	42	17	32	13		52	17,4	20	9,9	
Both	18	3,1	7	2,8	10	4,1		12	4	6	3	
None	369	64,3	161	65,2	147	59,8		192	64,2	124	61,4	
Total	574	100	247	100	246	100		299	100	202	100	

TABLE 1. Correlations between HLA-E and HLA-G expression and well-established prognostic factors using chi-squared test. *Abbreviations* N number of patients; % percentage; HLA-E human leukocyte antigen E; HLA-G human leukocyte antigen G; HER2 human epidermal growth factors receptor 2; MAST Mastectomy; BCS breast conservative surgery.

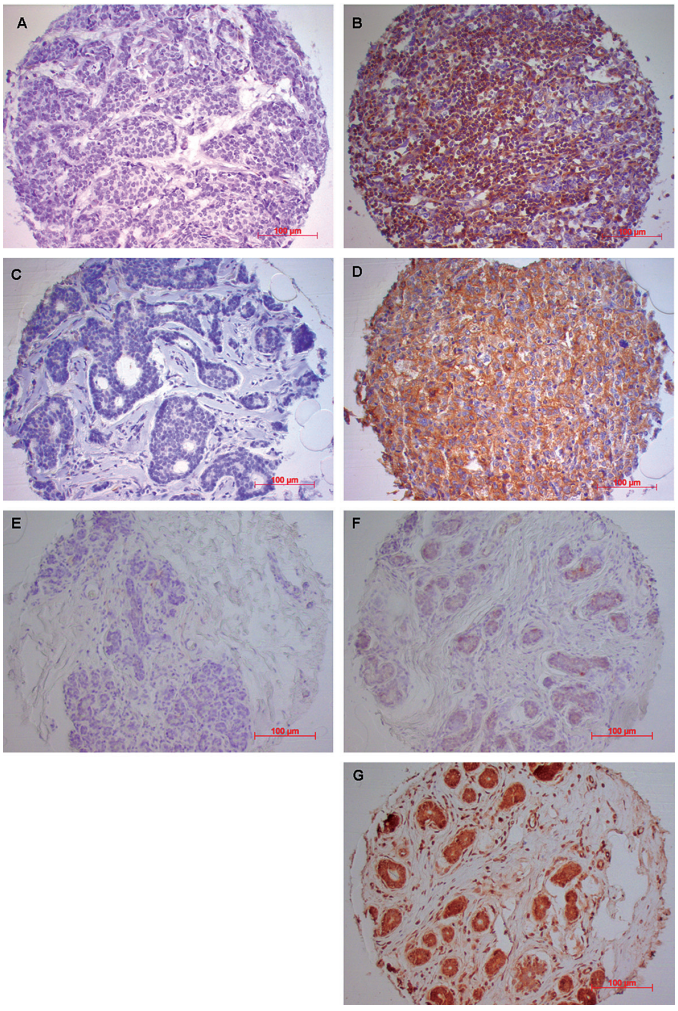

Figure 1 Representative examples of immunohistochemical stainings with MEM-E/02 and 4H84 antibodies on mammary tissues, performed according to standard protocols (details in Materials and Methods) **A)** HLA-E negative tumor **B)** HLA-E positive tumor **C)** HLA-G negative tumor **D)** HLA-G positive tumor **E)** HLA-G negative normal tissue **F)** HLA-G positive normal tissue **G)** HLA-E positive normal tissue.

(log rank p-values: RFP:0.001; OS: 0.007) (figures 2 I and 3 I). Among the patients with no expression of HLA-EG, 55% were relapse free after 10 years, compared to 17% for expression of HLA-EG. Cox proportional multivariate analysis was performed for relapses over time including the following factors: tumor stage, lymph node status, ER-status, HER2 expression, local therapy, endocrine therapy and HLA-EG. This analysis revealed that lymph node status and HLA-EG (p= 0.011, Hazard Ratio (HR): 2.87, 95% Confidence interval (CI): 1.28-6.43) were independent factors for RFP among the subgroup of classical HLA class I loss patients (table II). These data showed that HLA-EG possesses a specific prognostic effect, but only among classical HLA class I loss patients. In order to prove that classical HLA class I and HLA-EG were significantly cooperating variables, an interaction term was introduced in Cox regression analysis. This analysis showed a statistically significant interaction (p= 0.002) between the two

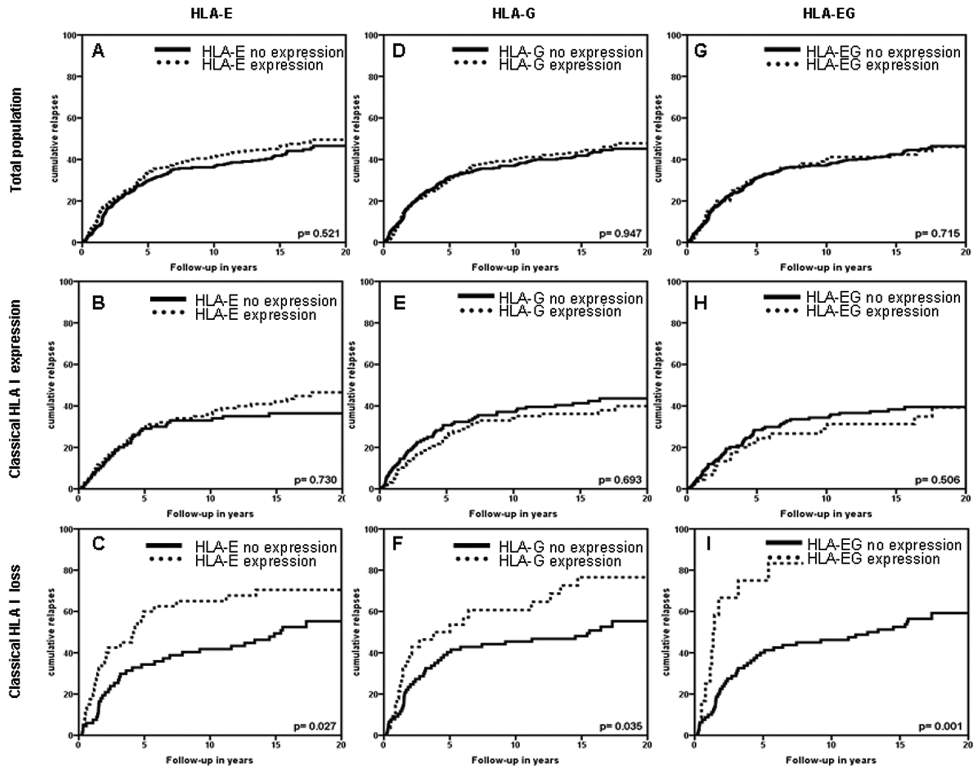

Figure 2 Relapses over time related with HLA-E **(A,B,C)**, HLA-G **(D,E,F)** and HLA-EG **(G,H,I)** tumor expression, among the total population **(A,D,G)**, patients with classical HLA class I tumor expression **(B,E,H)**, and patients with loss of classical HLA class I tumor expression **(C,F,I)**. Remarkably, only in patients with loss of classical HLA class I expression, HLA-E, HLA-G and HLA-EG affect relapses over time. Log-rank p-values are shown in each graph.

markers, suggesting that there is a biological connection between classical HLA class I and HLA-EG.

Correction for specificity of antibodies

The 4H84 antibody has been described to occasionally cross-react with classical HLA class I molecules 27. Therefore, we performed additional immunohistochemical analyses to examine whether this cross-reaction would interfere with our survival results.

Expression on paired normal breast tissue of half the cohort was found in 1% (3/266) for HLA-G. These 3 patients who showed weakly positive staining for HLA-G on normal breast tissue, also stained positive for classical HLA class I on normal and tumor tissue, indicating that the 4H48 antibody possibly occasionally cross-reacted with these classical HLA class I molecules. It should be noted however that the staining on normal tissue was only modest when compared to tumor staining with the 4H84 antibody (compare fig 1F with 1D). In order to examine whether the occasional cross-reaction of the 4H84 antibody would interfere with our results, we performed a sub-analysis

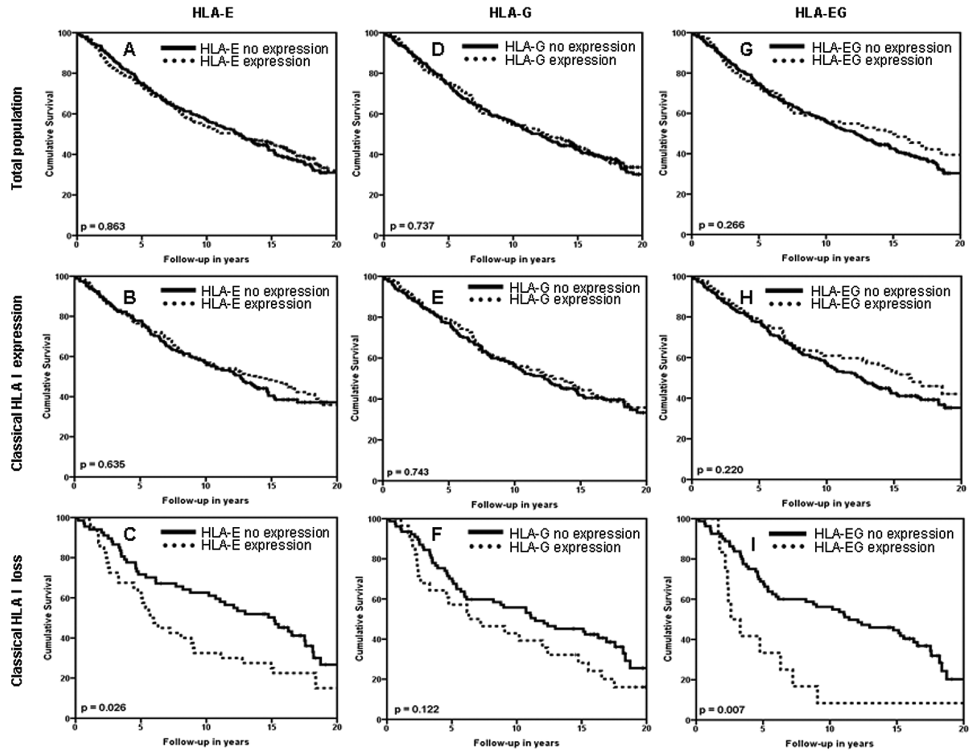

Figure 3 Kaplan Meier analysis of overall survival related with HLA-E **(A,B,C)**, HLA-G **(D,E,F)** and HLA-EG **(G,H,I)** tumor expression, among the total population **(A,D,G)**, patients with classical HLA class I tumor expression **(B,E,H)**, and patients with loss of classical HLA class I tumor expression **(C,F,I)**. Remarkably, only in patients with loss of classical HLA class I expression, HLA-E, HLA-G and HLA-EG affect overall survival. Log-rank p-values are shown in each graph.

by selecting only the tumors of the 266 patients of whom paired normal tissue was available. In this analysis, we excluded the 3 cases which showed positive staining for HLA-G on normal breast tissue (the presumed cases which showed cross-reaction for the 4H84 antibody) and examined whether survival analyses would reveal similar results as to when these cases would not be excluded. When excluding these 3 cases, no survival analyses reached statistical significance (log rank p-values ≥ 0.426) in neither the total population of patients nor the patient population with expression of classical HLA class I. This was concordant with the results found without exclusion of these cases (log-rank p-values ≥ 0.693). Importantly, no expression was seen of HLA-G in normal breast tissue of patients whose tumor showed no classical HLA class I expression, but resulted positive for HLA-G expression. Together, these results suggests that the occasional cross-reaction of 4H84 with classical HLA class I molecules did not interfere with our results.

		Univariate			Multivariate	
	N	HR	95% CI	p-value	HR	95% CI	p-value
Age							
<40	8	1.00		0.580			
40-50	37	1.23	0.426-3.544				
50-60	31	1.54	0.526-4.508				
>60	46	1.02	0.349-2.949				
Grade							
I	28	1.00		0.068			
II	55	1.29	0.661-2.507				
III	35	2.09	1.048-4.172				
Histological type							
Ductal	102	1.00		0.884			
Other	16	0.95	0.470-1.917				
Tumor stage							
pT1	46	1.00		0.006	1.00		0.679
pT2	55	1.71	0.994-2.953		1.25	0.590-2.644	
pT3/4	16	2.99	1.526-5.870		0.88	0.260-2.964	
Nodal stage							
pN-	64	1.00		<0.001	1.00		<0.001
pN+	56	4.10	2.482-6.783		3.60	1.812-7.165	
ER-status							
Negative	37	1.00		0.057	1.00		0.237
Positive	83	0.62	0.376-1.014		0.70	0.385-1.266	
PgR-status							
Negative	44	1.00		0.202			
Positive	73	0.73	0.445-1.186				
HER2							
No overexpression	102	1.00		0.075	1.00		0.069
Overexpression	10	1.73	0.947-3.176		2.21	0.939-5.217	
Ki67							
Ki67-	91	1.00		0.841			
Ki67+	26	0.94	0.523-1.695				
Local therapy							
MAST-RT	46	1.00		<0.001	1.00		0.320
MAST+RT	25	2.97	1.631-5.422		2.15	0.796-5.813	
BCS	51	0.96	0.542-1.703		1.23	0.572-2.663	
Endocrine therapy							
ET+	15	1.00		0.048	1.00		0.471
ET-	107	0.52	0.273-0.994		0.74	0.318-1.698	
Chemotherapy							
CT+	23	1.00		0.130			
CT-	99	0.65	0.371-1.136				
HLA-EG							
No expression	81	1.00		0.002	1.00		0.011
Expression	12	3.08	1.512-6.251		2.87	1.278-6.430	

TABLE 2. Cox univariate and multivariate analysis for relapses free period (RFP).
Abbreviations N number of patients; HR hazard ratio; 95%CI 95% confidence interval; HER2 human epidermal growth factor receptor 2; MAST Mastectomy; BCS breast conservative surgery.

DISCUSSION

Tumor-immune interaction may be of great importance for clinical outcome 2. In this study we showed that in tumors devoid of classical HLA class I expression, HLA-E and HLA-G expression were of statistically significant influence on outcome of breast cancer patients independently of known clinicopathological parameters, with an almost 3 times higher risk of relapse over time for patients with expression of HLA-EG compared to patients with no expression of HLA-EG. This is the first study providing evidence for a prognostic value of non-classical HLA class I molecule expression in a large cohort of breast cancer patients. In addition, to our knowledge we are the first to report that such an effect on outcome of patients interplays with expression of classical HLA class I molecules. Importantly, these results can be explained by underlying biology and support and add to previous studies on tumor-immune interaction in breast cancer [3, 12-19].

Previous studies have found elevated expression levels of the non-classical HLA class I molecules, HLA-E and HLA-G, in tumor tissues [3, 12-19]. Normally, HLA-G is not expressed on non-malignant cells. Corresponding to this fact, we found in our study that 4H84 HLA-G antibody did stain in a considerable number of tumor tissues, but in a negligible number of normal mammary tissues. Under normal circumstances HLA-E surface expression is dependent on the availability of HLA class I signal sequence-derived peptides. Therefore, HLA-E surface expression is usually found to be co-expressed with classical HLA class I, which comes to expression in almost all healthy tissues [3, 4]. Corresponding to this fact, we did not find any normal mammary tissue that did not express HLA-E molecules. In some tumor tissue however, HLA-E expression seems to be independent of the availability of classical HLA class I sequence-derived peptides and can be expressed in cells that lack classical HLA class I expression [9, 30]. Indeed, we found cytoplasmic expression of HLA-E in classical HLA class I negative tumors in our study. The disturbed balances of expression of classical HLA class I, HLA-E and HLA-G, as found in our study, suggests a cooperation between these molecules in evading immune recognition. According to the immunoediting hypothesis, tumors may become shaped through interaction with the immune system, leading to the selective outgrowth of highly tumorigenic clones that escape from immune recognition and elimination 31. Downregulation of classical HLA class I expression in tumors, with simultaneous loss of cell surface expression of HLA-E due to lack of peptide fragments which it can bind, is believed to reflect CTL immune escape 3. However, these tumor cells become highly vulnerable to NK cells, which recognize these "missing self" cells 14. Through a variety of factors, such as epigenetic control, hypoxia, stress and cytokines, expression of HLA-G and HLA-E may be upregulated and counteract this susceptibility to NK cells [3, 10, 32, 33]. Supportive for a specific NK cell inhibition of the non-classical HLA class I molecules, for both HLA-E and HLA-G an inverse

correlation was found with NK cell infiltrate in a colorectal cancer and gastric cancer study respectively [34, 35]. In addition, in various studies using colon cancer and melanoma cell lines it was demonstrated that overexpression of HLA-E and HLA-G respectively directly inhibited NK-mediated cell lysis 35-39. The statistical interaction between HLA-E and HLA-G with classical HLA class I molecules, as found in our study, adds to this evidence, suggesting that specifically in tumors devoid of classical HLA class I expression, upregulation of HLA-E and HLA-G expression counteracts the resulting NK cell susceptibility, leading to immune escape of tumor cells. Our study supports and adds to previous findings, suggesting that HLA-E and HLA-G contribute to tumor immune escape, specifically NK cells, a phenomenon that is likely to have impact on clinical outcome of patients.

Prognostic associations of HLA-E and HLA-G have been studied in various types of tumors [16-19, 30, 35]. In cervical cancer HLA-E expression increased with the progression of the lesion. One study analyzed the prognostic effect of HLA-E expression in colorectal cancer. A statistically significant association with outcome was noticed where high expression of HLA-E resulted in a worse disease free survival of patients 35. HLA-G expression showed a positive correlation with higher histological grade and clinical stage in colorectal cancer, gastric cancer, epithelial squamous cell carcinomas (ESCC) and cutaneaous T cell lymphoma. In addition, expression of HLA-G was an independent prognostic factor for a worse outcome of patients in colorectal cancer, ESCC and non small cell lung cancers [16, 18, 19]. We described that tumor expression of HLA-E and HLA-G has an independent prognostic influence in breast cancer patients, resulting in a worse patient outcome. Previously, similar results for disease free survival were found for breast cancer, albeit that these results did not reach statistical significance 20. This study was similar to ours in terms of patients selection criteria and immunohistochemical staining methods, but was probably limited by the small number of breast cancer patients studied (n=43). The results of our study demonstrate for the first time a statistically significant association of HLA-E and HLA-G expression with clinical outcome in a large cohort of breast cancer patients, which is particularly revealed in patients with tumors lacking expression of classical HLA class I molecules. Moreover, patients with tumors with simultaneous expression of HLA-E and HLA-G had an increased risk of relapses compared to patients with tumors expressing either HLA-E or HLA-G, a phenomenon that has been previously described as well 13. In addition we were able to demonstrate a statistical interaction in outcome analyses, indicating that the effect on outcome of HLA-E and HLA-G expression and the effect on outcome of HLA class I expression do not only operate simultaneously, but that the combined effect on outcome of these molecules is more than additive. These data correspond to the hypothesis that tumor expression of the non-classical HLA class I molecules E and G may indeed serve to protect tumor cells from NK-cell attack, but

this is mostly relevant in a situation that NK cells are activated, i.e. in case classical HLA class I molecule expression is downregulated 10.

Together, these results provide new insights in breast cancer tumorigenesis and provide further evidence that the immune system is able to recognize and eliminate breast cancer cells. However, it is also evident that breast cancer cells are capable of escaping immune attack. A better understanding of the various phases of tumor immune interactions in breast cancer, i.e. elimination, equilibrium and finally escape, may lead to a better prediction of clinical outcome of patients. Furthermore, this knowledge may be used for the development of tailored immunotherapeutic treatment modalities.

REFERENCES

1. Van Pel A, Boon T. Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. Proc Natl Acad Sci U S A 1982;79(15):4718-4722.
2. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3(11):999-1005.
3. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53(10):904-910.
4. Wei XH, Orr HT. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. Hum Immunol 1990;29(2):131-142.
5. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990;248(4952):220-223.
6. Braud VM, Allan DS, O'Callaghan CA et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 1998;391(6669):795-799.
7. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. J Exp Med 1999;189(7):1093-1100.
8. Kaiser BK, Pizarro JC, Kerns J, Strong RK. Structural basis for NKG2A/CD94 recognition of HLA-E. Proc Natl Acad Sci U S A 2008;105(18):6696-6701.
9. Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. Hum Immunol 2005;66(1):1-12.
10. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007;17(6):459-468.
11. Menier C, Rouas-Freiss N, Favier B, LeMaoult J, Moreau P, Carosella ED. Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule. Tissue Antigens 2010;75(3):201-206.
12. Bianchini M, Levy E, Zucchini C et al. Comparative study of gene expression by cDNA microarray in human colorectal cancer tissues and normal mucosa. Int J Oncol 2006;29(1):83-94.
13. Malmberg KJ, Levitsky V, Norell H et al. IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. J Clin Invest 2002;110(10):1515-1523.
14. Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. Immunogenetics 2003;54(11):767-775.
15. Rouas-Freiss N, Moreau P, Ferrone S, Carosella ED. HLA-G proteins in cancer: do they provide tumor cells with an escape mechanism? Cancer Res 2005;65(22):10139-10144.
16. Ye SR, Yang H, Li K, Dong DD, Lin XM, Yie SM. Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer. Mod Pathol 2007;20(3):375-383.
17. Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of human leukocyte antigen G (HLA-G) correlates with poor prognosis in gastric carcinoma. Ann Surg Oncol 2007;14(10):2721-2729.
18. Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of HLA-G is associated with prognosis in esophageal squamous cell carcinoma. Am J Clin Pathol 2007;128(6):1002-1009.
19. Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of human leucocyte antigen G (HLA-G) is associated with prognosis in non-small cell lung cancer. Lung Cancer 2007;58(2):267-274.
20. Kleinberg L, Florenes VA, Skrede M et al. Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch 2006;449(1):31-39.
21. Lefebvre S, Antoine M, Uzan S et al. Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. J Pathol 2002;196(3):266-274.
22. Singer G, Rebmann V, Chen YC et al. HLA-G is a potential tumor marker in malignant ascites. Clin Cancer Res 2003;9(12):4460-4464.
23. van Nes JG, de Kruijf EM, Faratian D et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res Treat 2010.
24. Lo ME, Sibilio L, Melucci E et al. HLA-E: strong association with beta2-microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides. J Immunol 2008;181(8):5442-5450.

25. Menier C, Saez B, Horejsi V et al. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. Hum Immunol 2003;64(3):315-326.
26. Paul P, Rouas-Freiss N, Moreau P et al. HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. Hum Immunol 2000;61(11):1177-1195.
27. Polakova K, Kuba D, Russ G. The 4H84 monoclonal antibody detecting beta2m free nonclassical HLA-G molecules also binds to free heavy chains of classical HLA class I antigens present on activated lymphocytes. Hum Immunol 2004;65(2):157-162.
28. de Kruijf EM, van Nes JG, Sajet A et al. The Predictive Value of HLA Class I Tumor Cell Expression and Presence of Intratumoral Tregs for Chemotherapy in Patients with Early Breast Cancer. Clin Cancer Res 2010;16(4):1272-1280.
29. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 2007;26(11):2389-2430.
30. Goncalves MA, Le DM, Simoes RT et al. Classical and non-classical HLA molecules and p16(INK4a) expression in precursors lesions and invasive cervical cancer. Eur J Obstet Gynecol Reprod Biol 2008;141(1):70-74.
31. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-360.
32. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med 1998;187(5):813-818.
33. Sheu J, Shih I. HLA-G and immune evasion in cancer cells. J Formos Med Assoc 2010;109(4):248-257.
34. Ishigami S, Natsugoe S, Miyazono F et al. HLA-G expression in gastric cancer. Anticancer Res 2006;26(3B):2467-2472.
35. Levy EM, Bianchini M, Von Euw EM et al. Human leukocyte antigen-E protein is overexpressed in primary human colorectal cancer. Int J Oncol 2008;32(3):633-641.
36. Adrian CF, Moreau P, Riteau B et al. HLA-G expression in human melanoma cells: protection from NK cytolysis. J Reprod Immunol 1999;43(2):183-193.
37. Munz C, Holmes N, King A et al. Human histocompatibility leukocyte antigen (HLA)-G molecules inhibit NKAT3 expressing natural killer cells. J Exp Med 1997;185(3):385-391.
38. Riteau B, Menier C, Khalil-Daher I et al. HLA-G1 co-expression boosts the HLA class I-mediated NK lysis inhibition. Int Immunol 2001;13(2):193-201.
39. Yu Y, Wang Y, Feng M. Human leukocyte antigen-G1 inhibits natural killer cytotoxicity through blocking the activating signal transduction pathway and formation of activating immunologic synapse. Hum Immunol 2008;69(1):16-23.

Chapter 4

NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: and observational study

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ABSTRACT

Background Cell surface NKG2D ligands (NKG2DL) bind to the activating NKG2D receptor present on NK cells and subsets of T cells, thus playing a role in initiating an immune response. We examined tumor expression and prognostic effect of NKG2DL in breast cancer patients.

Methods Our study population (n=677) consisted of all breast cancer patients primarily treated with surgery in our center between 1985 and 1994. Formalin-fixed paraffin-embedded tumor tissue was immunohistochemically stained with antibodies directed against MIC-A/MIC-B (MIC-AB), ULBP-1, ULBP-2, ULBP-3, ULBP-4, and ULBP-5.

Results NKG2DL were frequently expressed by tumors (MIC-AB, 50% of the cases; ULBP-1, 90%; ULBP-2, 99%; ULBP-3, 100%; ULBP-4, 26%; ULBP-5, 90%) and often showed co-expression: MIC-AB and ULBP-4 ($p=0.043$), ULBP-1 and ULBP-5 ($p=0.006$), ULBP-4 and ULBP-5 ($p<0.001$). MIC-AB ($p=0.001$) and ULBP-2 ($p=0.006$) expression resulted in a statistically significant longer relapse free period (RFP). Combined expression of these ligands showed to be an independent prognostic parameter for RFP ($p<0.001$, HR 0.41). Combined expression of all ligands showed no associations with clinical outcome.

Conclusions We demonstrated for the first time that NKG2DL are frequently expressed and often co-expressed in breast cancer. Expression of MIC-AB and ULBP-2 resulted in a statistically significant beneficial outcome concerning RFP with high discriminative power. Combination of all NKG2DL showed no additive or interactive effect of ligands on each other, suggesting that similar and co-operative functioning of all NKG2DL can not be assumed. Our observations suggest that among driving forces in breast cancer outcome are immune activation on one site and tumor immune escape on the other site.

BACKGROUND

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 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, 3]. However, current prognostic and predictive factors still do not provide optimal risk-stratification. Therefore, additional prognostic and predictive information could result in an improved tailored treatment for patients with breast cancer.

There is strong evidence that the immune system plays a role in tumor growth and progression [4, 5]. An effective immune response may lead to recognition of tumor cells, resulting in their eradication. However, due to their genetic unstable nature, tumor cells may arise which display properties that enables them to escape from immune recognition [4, 5]. Indeed, downregulation or loss of proteins that are crucial for immune responses, like classical human leukocyte antigens (HLA) class I, or upregulation of proteins that confer resistance to immune recognition, like non-classical HLA class I, are frequently found in various types of tumors [6-10].

The activating receptor natural killer cell lec-tin-like receptor gene 2D (NKG2D) is a stimulatory immune receptor that is expressed on natural killer (NK) cells, NKT cells, $\gamma\delta^+$ T cells and CD8+ T cells [11]. Ligands which bind NKG2D receptors comprise major histocompatibility complex class I chain-related proteins A and B (MIC-AB) and unique long 16 (UL16) binding proteins 1-6 (ULBP1-6) [12, 13]. Expression of these ligands may be induced upon infection and other inducers of cellular stress and is unusual in normal cells [14]. By binding to the NKG2D receptors on NK and T cells, the NKG2D ligands may initiate an immune response against cells expressing these ligands. Overexpression and shedding of NKG2D ligands have been reported [14]. It is, however, unclear whether these features also results in activation of an immune response or lead to overstimulation and downregulation of NKG2D on immune cells [11].
Malignant transformation of cells may be among stimuli inducing expression of NKG2D ligands as such expression has been found in various tumor types [8-10, 15-18]. This may be a mechanism for preventing tumor growth by advancing an anti-tumor immune response. Convincing evidence has been found in *in vivo* studies, which have shown that in mouse models transfection with NKG2D ligands resulted in a NKG2D-mediated tumor rejection [19, 20]. Other studies showed that downregulation or complete knockout of NKG2D in mice resulted in an impaired immune response against tumor cells, higher expression levels of NKG2D ligands, and an increased incidence of certain tumors [21, 22].

A few studies have investigated tumor expression of NKG2D ligands and associations with clinical outcome in human breast, colorectal, and ovarian cancer [8-10, 15, 16]. Expression of MIC-A was frequently found in all tumors studied and resulted in a statistically significant favorable patient's prognosis in colorectal cancer, while it was not statistically significantly associated with outcome in breast cancer and ovarian cancer [8-10, 16]. ULBP1-5 expression was also found to be expressed in many tumor samples of colorectal and ovarian cancer [9, 10, 15]. In colorectal cancer expression of ULBP5 was an independent prognostic factor for a favorable clinical outcome [9]. In contrast to these results, expression of ULBP2 and ULBP4 were found to be independent prognostic factors for a worse outcome of ovarian cancer patients [10, 15]. Taken together, several studies suggest that evasion of NKG2D-mediated immune regulation plays an important role in tumor progression, but some studies contradict this suggestion. Contradictory results may be explained by assuming functional differences in immune regulation of the different ligands. Moreover, expression of NKG2D ligands may behave different among different tumor types [9]. It is known that overexpression or shedding of these ligands leads to overstimulation and downregulation of NKG2D on immune cells [10, 15], thereby evading an immune response.

In breast cancer, the prognostic effect of NKG2D ligands and their mutual relationship is largely unknown. Therefore, the purpose of this study was to analyze the clinical prognostic value of MIC-AB and ULBP1-5 in a large patient cohort of early stage breast cancer.

PATIENTS AND METHODS

Patients and tumors

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery between 1985 and 1994 at the Leiden University Medical Center (n=677). 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 morphology and differentiation grade, TNM stage, type of local and systemic therapy, recurrence and survival status, estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor 2 (HER2) expression (Table1). All these parameters were determined according to current pathology standards. A tissue micro array (TMA) of available formalin-fixed paraffin-embedded (FFPE) tumors of the patient cohort has been previously constructed and described (n=574) [23]. Approval 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).

Immunohistochemistry

Antibodies specific against MIC-AB (ab54413; Abcam), ULBP-1 (HPA007547; Atlas antibodies), ULBP-2 (af1298; R&D systems), ULBP-3 (CUMO3-100; BAMOMAB), ULBP-4 (RAET1E) and ULBP-5 (RAET1G, both kindly provided by Dr. Robert A Eagle, Cambridge, UK) [9, 24] were used for immunohistochemical staining of tumor tissue. The specificity of anti-ULBP-2 antibody has been previously determined, which showed occassional cross-reactity with highly related molecules RAET1L and to a lesser extent with RAET1G, but a good recognition of ULBP-2 [25]. We are not aware of antibodies which can specifically discriminate between ULBP2, RAET1L and RAET1G extracellular domains.

TMA sections of 4μm were cut, deparaffinized and rehydrated. Endogenous peroxidase was blocked in 0.3% hydrogen-peroxide methanol for 20 minutes. Heat-induced antigen retrieval for 10 minutes at maximum power in a microwave oven was performed. Sections were incubated overnight with primary antibodies using predetermined optimal dilutions and incubations times. Sections for ULBP-2 staining were incubated with Rabbit Anti-Goat Immunoglobulins (DAKO) followed by StreptABComplex (DAKO) for 30 minutes. Sections for all other stainings were incubated with secondary antibody Envision (Dako cytomation K4001 or K4003) for 30 minutes. Stainings were visualized using DAB-solution (Dako cytomation K3468), counterstained with haematoxylin, dehydrated, and finally mounted in malinol. For each type of antibody, all tissue sections were stained simultaneously to avoid inter-assay variation.

Evaluation of immunostaining

Microscopic analysis of MIC-AB, ULBP-1, ULBP-2, ULBP-3, ULBP-4 and ULBP-5 expression was performed by two independent observers in a blinded manner. Since staining of tumors was relatively homogenous, for each tumor the overall intensity of staining (negative (0), weak (1), intermediate (2) or strong (3)) was determined.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA). Cohen's kappa coefficient was used to assess inter-observer agreement in quantification. This revealed a moderate agreement for ULBP-5 (kappa=0.410), a substantial agreement in classification for MIC-AB (kappa=0.790) and ULBP-4 (kappa=0.650), and an almost perfect agreement for ULBP-1 (kappa=0.913), ULBP-2 (kappa=0.940), and ULBP-3 (kappa=0.869). The χ^2 test was used to evaluate associations between expression of the different NKG2D ligands. Relapse free period (RFP) was the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk [26].

	Total		MICAB				ULBP1				ULBP2			
			Low		High		Low		High		Low		High	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Age														
<40	48	8.4	4	4.0	31	8.7	22	10.0	10	6.1	34	8.7	9	7.3
40-50	145	25.3	28	28.0	86	24.0	62	28.2	32	19.4	85	21.7	42	34.1
50-60	132	23.0	26	26.0	78	21.8	45	20.5	44	26.7	100	25.6	20	16.3
>=60	249	43.4	42	42.0	163	45.5	91	41.4	79	47.9	172	44.0	52	42.3
Grade														
I	80	14.2	22	22.0	38	10.9	39	18.1	9	5.5	41	10.7	27	22.1
II	282	49.9	42	42.0	181	51.7	109	50.5	80	49.1	188	49.0	65	53.3
III	203	35.9	36	36.0	131	37.4	68	31.5	74	45.4	155	40.4	30	24.6
Histological type														
Ductal	513	90.6	91	91.0	322	91.7	194	89.8	151	92.6	354	92.2	106	86.9
Lobular	53	9.4	9	9.0	29	8.3	22	10.2	12	7.4	30	7.8	16	13.1
T-status														
T1	211	38.0	40	41.7	124	35.7	96	44.9	37	23.3	128	33.7	59	50.4
T2	272	49.0	44	45.8	176	50.7	87	40.7	96	60.4	198	52.1	46	39.3
T3/4	72	13.0	12	12.5	47	13.5	31	14.5	26	16.4	54	14.2	12	10.3
N-status														
N0	307	55.1	60	61.2	181	52.8	118	54.9	69	44.5	196	51.7	74	62.2
N1-3	250	44.9	38	38.8	162	47.2	97	45.1	86	55.5	183	48.3	45	37.8
ER-status														
Negative	203	37.6	33	33.7	137	39.3	95	43.8	55	34.8	147	38.2	45	37.5
Positive	337	62.4	65	66.3	212	60.7	122	56.2	103	65.2	238	61.8	75	62.5
PgR-status														
Negative	223	41.6	33	33.3	147	42.6	88	40.9	68	43.0	169	43.8	40	33.9
Positive	313	58.4	66	66.7	198	57.4	127	59.1	90	57.0	217	56.2	78	66.1
Her2-status														
No overexpression-	378	89.6	78	92.9	264	88.0	174	89.7	125	89.3	291	90.9	79	84.9
Overexpression	44	10.4	6	7.1	36	12.0	20	10.3	15	10.7	29	9.1	14	15.1
Local Therapy														
MAST-RT	223	38.9	41	41.0	146	40.8	80	36.4	79	47.9	149	38.1	53	43.1
MAST+RT	108	18.8	17	17.0	66	18.4	46	20.9	33	20.0	83	21.2	15	12.2
BCS-RT	5	0.9	0	0.0	5	1.4	2	0.9	2	1.2	5	1.3	0	0.0
BCS+RT	238	41.5	42	42.0	141	39.4	92	41.8	51	30.9	154	39.4	55	44.7
Systemic therapy														
CT alone	112	19.5	17	17.0	73	20.4	44	20.0	25	15.2	80	20.5	24	19.5
HT alone	75	13.1	8	8.0	54	15.1	31	14.1	29	17.6	54	13.8	16	13.0
CT&HT	18	3.1	1	1.0	13	3.6	3	1.4	9	5.5	14	3.6	3	2.4
None	369	64.3	74	74.0	218	60.9	142	64.5	102	61.8	243	62.1	80	65.0
Total	574	100	100	100	358	100	220	100	165	100	391	100	123	100

Table 1 Correlations between MIC-A-B, ULBP-1, ULBP-2 expression and well-established prognostic factors. Missing values are not shown.

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

Cox regression was used for univariate and multivariate analysis for RFP. Significant variables ($p<0.1$) in univariate analysis were included in multivariate analysis.

RESULTS

Patient and tumor characteristics

Median age of patients was 57 years (range: 23-96 years). Median follow-up of patients alive was 19 years (range: 14-23 years). Clinicopathological and treatment characteristics are shown in table 1.

Expression of NKG2D ligands

Most of the NKG2D ligands examined in this study were frequently expressed among the breast tumor cohort: MIC-AB in 50% of the cases; ULBP-1 in 90%; ULBP-2 in 99%; ULBP-3 in 100%; ULBP-4 in 26%; and ULBP-5 in 90%. A broad distribution of immunohistochemical staining-intensities was seen for ULBP-2, ULBP-3 and ULBP-5, while MIC-AB, ULBP-1 and ULBP-4 showed a skewed distribution of staining-intensities where most tumors stained weakly positive (representative examples of staining: Figure 1). Therefore, the median intensity was taken as a cut-off value for all ligands to categorize low and high expression resulting in respectively 50%, 43%, 24%, 27%, 26%, 10% of tumors with high expression of MIC-AB (Figure 1B), ULBP-1 (Figure 1D), ULBP-2 (Figure 1F), ULBP-3 (Figure 1H), ULBP-4 (Figure 1J) and ULBP-5 (Figure 1L) and respectively 50%, 57%, 76%, 73%, 90% of the tumors with low expression of MIC-AB (Figure 1A), ULBP-1 (Figure 1C), ULBP-2 (Figure 1E), ULBP-3 (Figure 1G), ULBP-4 (Figure 1I) and ULBP-5 (Figure 1K).
NKG2D ligands were found to be frequently co-expressed: MIC-AB positively correlated with ULBP-4 ($p=0.043$); ULBP-1 showed a positive correlation with ULBP-5 ($p=0.006$); ULBP-4 had a positive correlation with ULBP-5 ($p<0.001$).

Association of NKG2D ligands with clinicopathological parameters

High expression of NKG2D ligands was generally associated with favorable clinicopathological parameters (table 1 and 2): statistically significant associations were found between high expression of MIC-AB and lower tumor grade ($p=0.012$); high expression of ULBP-1 and higher tumor grade ($p<0.001$), smaller tumor size ($p<0.001$) and more lymph node positive tumors ($p=0.049$); high expression of ULBP-2 and younger age ($p=0.022$), lower tumor grade ($p<0.001$), smaller tumor size ($p=0.005$) and more lymph node negative tumors ($p=0.046$); high expression of ULBP-3 and higher tumor grade ($p=0.001$); high expression of ULBP-4 and smaller tumor size ($p=0.001$); high expression of ULBP-5 and more PgR negative tumor status ($p=0.016$).

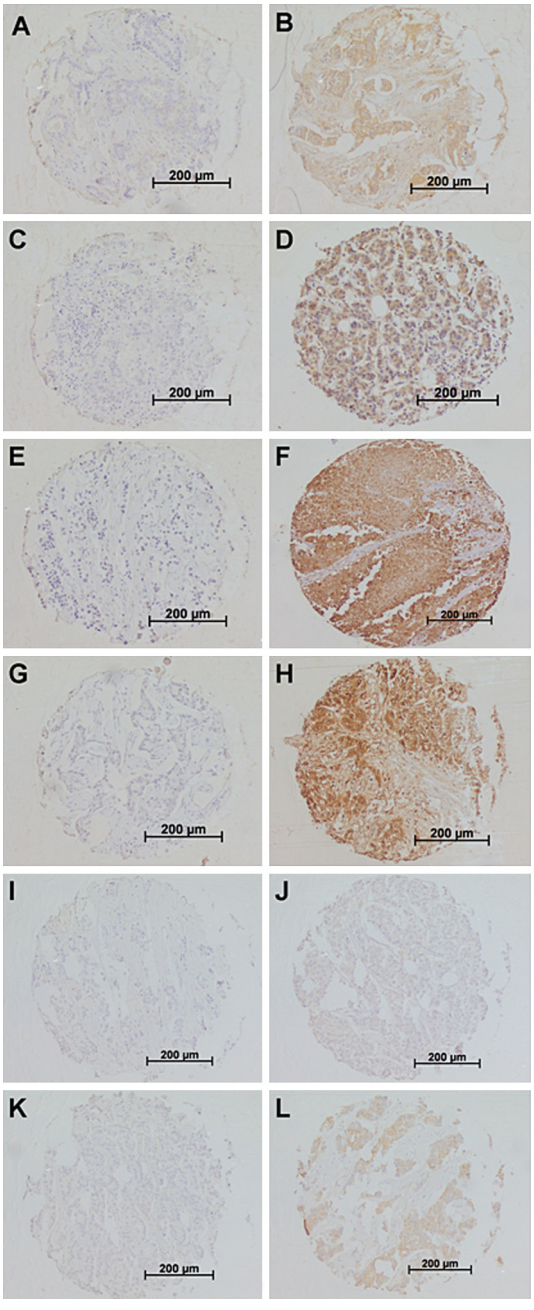

Figure 1 Representative examples of immunohistochemical stainings of primary breast cancer tissues for respectively no expression and high expression of MIC-AB (**A**: intensity 0 (negative); **B**: intensity 2 (intermediate)), ULBP-1 (**C:** intensity 0 (negative); **D**: intensity 2 (intermediate)), ULBP-2 (**E**: intensity 0 (negative); **F**: intensity 3 (strong)), ULBP-3 (**G**: intensity 0 (negative); **H**: intensity 3 (strong)), ULBP-4 (**I:** intensity 0 (negative); **J**: intensity 1 (weak)), and ULBP-5 (**K**: intensity 0 (negative); **L**: intensity 3 (strong)) in breast cancer. Immunohistochemistry was performed according to standard protocols as described in Materials and Methods.

Associations with outcome of NKG2D ligands

When analyzed separately, MIC-AB and ULBP-2 showed statistically significant results on outcome analyses (log rank p-values respectively: 0.001, 0.006), where high expression of MIC-AB and ULBP-2 showed to have fewer relapses over time compared to low expression (Figure2 A, C). For MIC-AB low expression, 51% of patients were relapse

	Total		ULBP3				ULBP4				ULBP5			
			Low		High		Low		High		Low		High	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Age														
<40	48	8.4	25	8.2	11	9.8	29	7.8	15	11.8	38	8.5	3	6.4
40-50	145	25.3	84	27.5	24	21.4	91	24.6	33	26.0	119	26.7	9	19.1
50-60	132	23.0	68	22.3	22	19.6	86	23.2	30	23.6	103	23.1	12	25.5
>=60	249	43.4	128	42.0	55	49.1	164	44.3	49	38.6	185	41.6	23	48.9
Grade														
I	80	14.2	45	15.0	10	9.1	51	13.9	13	10.6	62	14.2	2	4.3
II	282	49.9	160	53.2	43	39.1	181	49.5	67	54.5	219	50.0	21	45.7
III	203	35.9	96	31.9	57	51.8	134	36.6	43	35.0	157	35.8	23	50.0
Histological type														
Ductal	513	90.6	281	93.4	102	92.7	330	89.9	113	91.9	396	90.2	43	93.5
Lobular	53	9.4	20	6.6	8	7.3	37	10.1	10	8.1	43	9.8	3	6.5
T-status														
T1	211	38.0	113	38.4	40	36.0	118	33.0	57	46.0	159	36.8	19	40.4
T2	272	49.0	142	48.3	54	48.6	180	50.3	61	49.2	209	48.4	26	55.3
T3/4	72	13.0	39	13.3	17	15.3	60	16.8	6	4.8	64	14.8	2	4.3
N-status														
N0	307	55.1	158	53.9	61	55.5	193	53.2	66	54.5	237	54.7	24	53.3
N1-3	250	44.9	135	46.1	49	44.5	170	46.8	55	45.5	196	45.3	21	46.7
ER-status														
Negative	203	37.6	113	38.8	37	33.6	135	37.8	43	35.0	152	35.3	21	45.7
Positive	337	62.4	178	61.2	73	66.4	222	62.2	80	65.0	278	64.7	25	54.3
PgR-status														
Negative	223	41.6	130	45.0	42	38.2	141	39.6	51	41.5	158	37.2	26	55.3
Positive	313	58.4	159	55.0	68	61.8	215	60.4	72	58.5	267	62.8	21	44.7
Her2-status														
Overexpression -	378	80.9	207	89.6	82	89.1	256	88.6	93	93.9	311	90.7	35	85.4
Overexpression +	44	19.1	24	10.4	10	10.9	33	11.4	6	6.1	32	9.3	6	14.6
Local Therapy														
MAST-RT	223	38.9	116	38.0	43	38.4	141	38.1	46	36.2	165	37.1	24	51.1
MAST+RT	108	18.8	56	18.4	24	21.4	86	23.2	14	11.0	94	21.1	3	6.4
BCS-RT	5	0.9	3	1.0	1	0.9	1	0.3	3	2.4	4	0.9	0	0.0
BCS+RT	238	41.5	130	42.6	44	39.3	142	38.4	64	50.4	182	40.9	20	42.6
Systemic therapy														
CT alone	112	19.5	65	21.3	23	20.5	81	21.9	20	20.3	93	20.9	8	17.0
HT alone	75	13.1	32	10.5	10	8.9	52	14.1	16	12.6	60	13.5	5	10.6
CT&HT	18	3.1	10	3.3	2	1.8	13	3.5	1	0.8	11	2.5	1	2.1
None	369	64.3	198	64.9	77	68.8	224	60.5	90	70.9	281	63.1	33	70.2
Total	574	100	305	100	112	100	370	100	127	100	445	100	47	100

Table 2 Correlations between ULBP-3, ULBP-4 and ULBP-5 expression and well-established prognostic factors. Missing values are not shown.

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

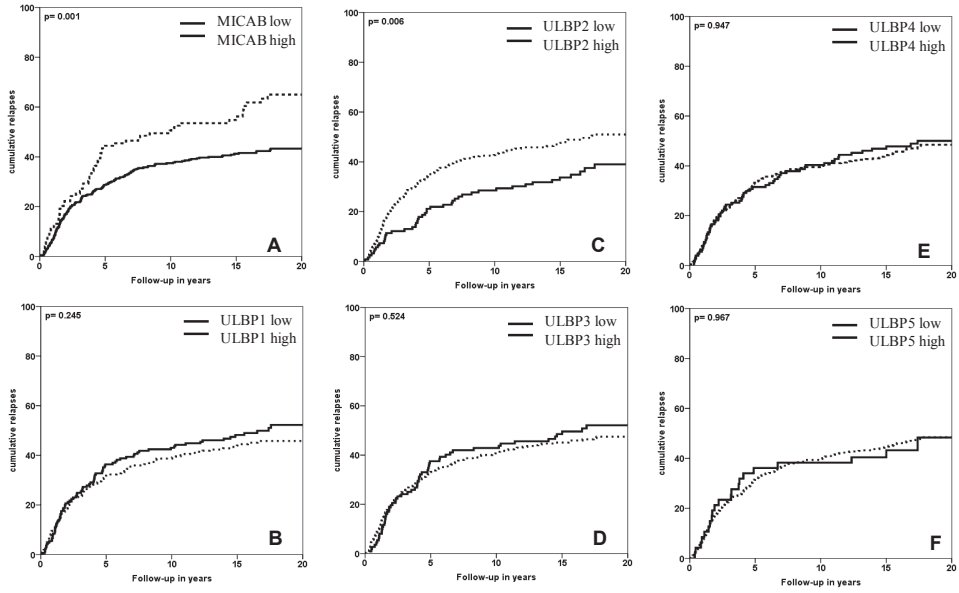

Figure 2 Relapses over time related with expression of MIC-AB **(A)**, ULBP-1 **(B)**, ULBP-2 **(C)**, ULBP-3 **(D)**, ULBP-4 **(E)**, and ULBP-5 **(F)**. X-axis represents patient follow-up in years; Y-axis represents cumulative relapses in %. Log-rank p-values are shown in each graph. Only expression of MIC-AB and ULBP-2 resulted in statistically significantly favorable relapse-free period (RFP).

free after 20 years, while of patients with high expression of MIC-AB 27% showed a relapse within 20 years. For ULBP-2, 20 year RFP rates for low expression versus high expression were respectively 56% and 43%. No statistically significant associations with outcome were seen for ULBP-1, ULBP-3, ULBP-4 and ULBP-5 (Figure 2 B, D-F).

Cox univariate regression analysis was performed for expression of each type of ligand. MIC-AB (Hazard ratio (HR) 0.60, 95% confidence interval (95%CI) 0.448-0.810, p=0.001) and ULBP-2 (HR 0.63, 95%CI 0.454-0.869, p=0.005) showed statistically significant results for a favorable RFP, while all other types of ligands did not reach statistical significance (data not shown).

To seek how combined expression of MIC-AB and ULBP2 ligands would predict patient outcome a new variable was made representing expression of both ligands: (1) Both MIC-AB and ULBP-2 low expression; (2) either MIC-AB or ULBP-2 high expression; (3) both MIC-AB and ULBP-2 high expression. Combined expression of MIC-AB and ULBP-2 resulted in a prognostic factor (log rank p-value: <0.001; Figure 3), where low expression of both ligands versus high expression of either ligand versus high expression of both ligands resulted in respectively 23%, 48% and 60% of patients to be relapse free after 20 years. Cox proportional multivariate analysis showed the combined ligand variable to be statistically significant for RFP independently of known clinicopathological parameters (MIC-AB and ULBP-2 both low versus either high: HR

0.54, 95%CI 0.380-0.757; MIC-AB and ULBP-2 both low versus both high: HR 0.41, 95%CI 0.246-0.682; p-value<0.001) (Table 3).

Relapse Free Period		UNIVARIATE			MULTIVARIATE		
	N	HR	95% CI	p-value	HR	95% CI	p-value
Age							
<40	48	1.00		0.422			
40-50	145	0.97	0.612-1.539				
50-60	132	1.17	0.734-1.853				
>60	249	0.90	0.574-1.408				
Grade							
I	80	1.00		0.001	1.00		0.473
II	282	1.43	0.945-2.172		1.18	0.711-1.948	
III	203	2.02	1.326-3.078		1.34	0.802-2.231	
Histological type							
Ductal	513	1.00		0.291			
Other	53	1.24	0.832-1.846				
Tumor stage							
pT1	211	1.00		<0.001	1.00		0.298
pT2	272	1.59	1.205-2.093		1.17	0.832-1.637	
pT3/4	72	2.49	1.706-3.635		1.45	0.908-2.316	
Nodalstage							
pN-	307	1.00		<0.001	1.00		<0.001
pN+	250	3.06	2.379-3.945		2.70	1.987-3.669	
ER-status							
Negative	203	1.00		0.725			
Positive	337	1.05	0.808-1.359				
PgR-status							
Negative	223	1.00		0.744			
Positive	313	0.96	0.743-1.236				
HER2							
No overexpression	378	1.00		0.401			
Overexpression	44	1.21	0.776-1.883				
Endocrine therapy							
ET-	481	1.00		0.197			
ET+	93	1.24	0.896-1.705				
Chemotherapy							
CT-	444	1.00		0.839			
CT+	130	0.97	0.730-1.291				
MIC-AB & ULBP-2							
Both Low	68	1.00		<0.001	1.00		<0.001
Either one high	275	0.59	0.426-0.820		0.54	0.380-0.757	
Both high	64	0.38	0.230-0.612		0.41	0.246-0.682	

Table 3 Cox univariate and multivariable analysis for recurrence free period (RFP) for combined expression of MIC-AB and ULBP-2. Missing values are not shown.
Abbreviations N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; ER estrogen receptor; PgR progesterone receptor; HER2 human epidermal growth factor receptor 2; ET endocrine therapy; CT chemotherapy.

In order to analyze the frequencies and prognostic effect of number of co-expressed and amount of co-expression of NKG2D ligands, two new variables were constructed. First, the total number of the different NKG2D ligands that were expressed. For that purpose, the number of NKG2D ligands with high expression was counted. So for each tumor, this resulted in a minimal and maximal possible score of respectively 0 and 6. Second, the total amount of NKG2D ligand expression. For that purpose, the intensity of staining (ranging from 0 to 3) of NKG2D ligands was added, obtaining a total NKG2D ligand intensity score. So for each tumor, this resulted in a minimal and maximal possible score of respectively 0 and 18.
The median number of NKG2D ligands with high expression was 1 (range 0-6). For statistical reasons (too small patient groups) in outcome analyses, the groups with 3, 4,

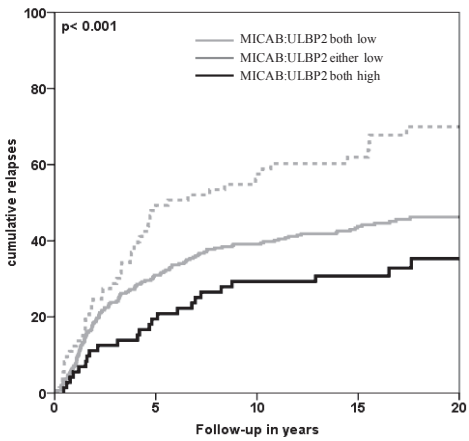

Figure 3 Relapses over time related with combined expression of MIC-AB and ULBP-2. X-axis represents patient follow-up in years; Y-axis represents cumulative relapses in %. Log-rank p-values are shown in the graph. Combined low expression of MIC-AB and ULBP-2 resulted in the worst outcome of patients concerning relapse-free period (RFP); while combined high expression of both ligands resulted in the most favorable outcome of patients.

5 and 6 numbers of different NKG2D ligands highly expressed were combined as one single group: ≥ 3 ligands of high expression.
No associations were seen for the number of NKG2D ligands with high expression for RFP outcome analyses (log rank p-value: 0.967); patients with tumors with a low number of NKG2D ligands with high expression resulted in a similar RFP compared to a high number of NKG2D ligands with high expression (Figure 4A).
The median total amount of NKG2D ligand intensity score was 8 (range 4-16). No tumors showed complete lack (score 0) or high intensity expression of all NKG2D ligands (score 18).
For outcome analyses, NKG2D scores 14-16 were combined and classified as ≥ 14, since these subgroups separately contained only one patient. No association was seen for amount of total NKG2D ligand expression and RFP (log rank p-value: 0.721); high total NKG2D ligand expression resulted in some cases in a worse RFP (e.g. score 11) while in others it resulted in a favorable RFP (e.g. score 13) and vice versa, low total NKG2D ligand expression resulted for some patients in a favorable RFP (e.g. score 6) and for other patients in a worse RFP (e.g. score 4) (Figure 4B).

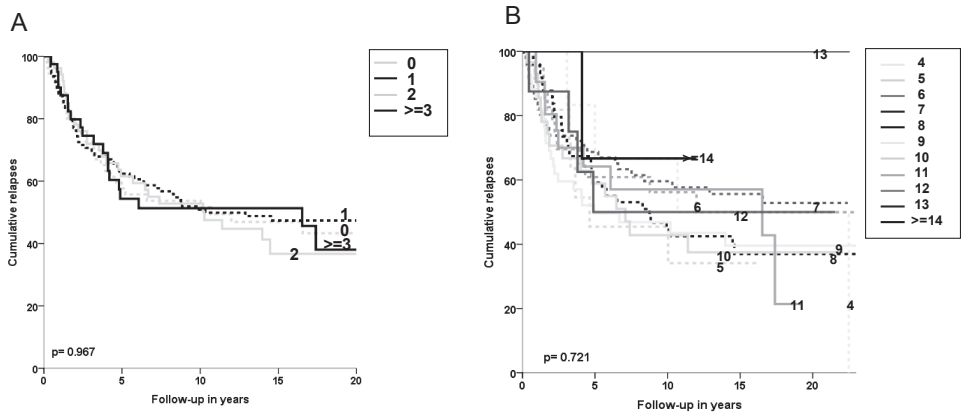

Figure 4 Relapses over time related with combined number of NKG2D ligands with high expression **(A)** and amount of expression of NKG2D ligands **(B)**. **(A)** legends in graph show total number of NKG2D ligands with high expression; **(B)** legends in graph show total intensity score of all NKG2D ligand expression. X-axis represents patient follow-up in years; Y-axis represents cumulative relapses in %. Log-rank p-values are shown in the graph. No associations were found with outcome concerning RFP for either combined number of expressed **(A)** or combined amount of expression **(B)** of ligands.

DISCUSSION

The importance of interaction between tumor development and the immune system for cancer outcome is highlighted by an overwhelming number of studies, performed *in vitro*, *in vivo* and using patient cohorts. Recent studies have shown that NKG2D ligands may play an important role in cancer immunosurveillance and cancer immunoediting [8-10, 15-22]. In this study, we examined the impact of tumor expression of NKG2D ligands on the prognosis of breast cancer patients. The data of our study indicate that NKG2D ligands are frequently high expressed in breast tumors and that this expression influences prognosis of patients. We were able to statistically prove that high expression levels of MIC-AB and ULBP-2 resulted in a RFP benefit. Combining expression of MIC-AB and ULBP-2 resulted in a very accurate stratification of patients for prognosis concerning RFP. The prognostic potential of this combined variable was comparable to that of lymph node status: patients with low tumor expression of both ligands had an almost 2.5 times increased risk of developing relapses compared to patients with high tumor expression of both ligands.

NKG2D ligands are expressed on the cell surface in response to stress or malignant transformation [11]. Our study confirms that breast cancer tumor cells show frequent and high expression of NKG2D ligands, as has been found in other studies for various types of tumors such as ovarian cancer, colorectal cancer and breast cancer [8-10, 15, 16]. Though all studies show consistently frequent expression of NKG2D ligands, very diverse prognostic effects have been described for these types of ligands in different cancer

types [8-10, 15, 16]. This may be explained by functional differences in immune regulation for varying expression levels of different ligands in different environments. Expression of NKG2D ligands may induce an immune response through binding to the NKG2D receptor, present on NK cells and a subset of T cells [11]. Therefore, selective outgrowth of malignant cells that do not express these NKG2D ligands may be a mechanism of tumor immune escape. On the other hand, overexpression of NKG2D ligands could lead to overstimulation and thereby insensibility or anergy of immune cells, which would result in evasion of immune attack by tumors overexpressing NKG2D ligands [11]. Adding to this hypothesis, it has been reported that NKG2D ligands on the cell membrane may be cleaved and produce soluble molecules. This shedding of NKG2D ligands could systemically downregulate NKG2D receptor expression and thereby result in an impaired anti-tumor reactivity of NK and T cells [11, 27]. Taken together, the mechanisms by which NKG2D ligands mediate immune function or dysfunction may be diverse in different tumors and differ according to circumstances. The contradictory results on the prognostic effect of NKG2D ligands found between different studies on different tumors may be reflected by the functional and mechanistic implications of interaction between NKG2D and its ligands. In ovarian cancer expression of NKG2D ligands resulted in a worse patient outcome, probably due to chronic overexpression and shedding of these ligands, leading to overstimulation and downregulation of the NKG2D receptor of NK and T cells and, therefore, an impaired immune response [10, 15]. Supporting the hypothesis that elevated expression of NKG2D ligands results in immune escape in ovarian cancer, one study found elevated levels of MIC-AB and ULBP-2 to be positively correlated to less intra-tumor epithelial CD57+ cells. The results found in breast cancer in the present study are contradictory to the results found in ovarian cancer, but similar to those found in colorectal cancer [9, 16]. The results in our study and colorectal cancer are supported by the theory that expression of NKG2D ligands results in activation of immune cells which is reflected in a patient beneficial outcome for high ligand expression [9, 16]. We found frequent and high expression of ligands in our study and statistically significant associations between expression levels of these ligands, indicating their cooperation with each other. Adding to the hypothesis that low expression of these ligands is a result of selective pressure by the immune system that results in cancer immune evasion or immunoediting, low expression of MIC-AB and ULBP-2 were prognostic factors for an unfavorable RFP of patients. When expression of MIC-AB and ULBP-2 were combined they showed to add to each others prognostic effect which is in line with the results found in previous studies [9, 10] and suggests that NKG2D ligands operate together and in a similar manner.

Since the exact functioning of all NKG2D ligands and their cooperative function is largely unknown, we performed outcome analyses with two different variables that represented combined number of highly co-expressed ligands and amount of co-expression of all ligands. The results of these analyses revealed no patterns of any

cooperative functioning between all ligands, as both variables showed no consistent and significant relationship with clinical outcome of disease. This suggests that the original hypothesis of all NKG2D ligands having a similar functioning and additive effect on each other's functioning in activating or evading an immune response, may be too simplistic. Considering our results and those as found in literature, altogether, each NKG2D ligand analysed separately does not show equal effects on clinical outcome, and different ligands show varying prognostic effects in different tumors. Specific combinations of ligands (e.g. MIC-AB and ULBP-2 in our study, ULBP2 and ULBP4 in ovarian cancer [10]) do show additive effects or statistical interactions on prognostic value. However, as highlighted by our combined analyses, a simple additive effect of all NKG2D ligands, by considering a similar or cooperative functioning of all these ligands, can not be assumed. This indicates the complexity of NKG2D ligands functioning and emphasizes the importance of further research on the precise mechanisms of actions of NKG2D ligands, separately, in combination with each other, and under different circumstances.

CONCLUSIONS

We have shown in this study, for the first time, that breast tumors may express all of the known NKG2D ligands and that expression of MIC-AB and ULBP-2 results in a favorable outcome concerning RFP. A variable combining MIC-AB and ULBP-2 expression has shown to be a prognostic parameter independently of known clinicopathological parameters and with high discriminative power. Our results suggest that NKG2D ligands play a crucial role in tumor immunoediting in breast cancer and provide further evidence that tumor-immune interactions play an important role in breast cancer. In addition, by NKG2D ligand combined analyses we highlight the importance of further studies on unraveling the precise separate functioning of these ligands.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.
2. Early breast cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365(9472):1687-1717.
3. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007;18(7):1133-1144.
4. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21(2):137-148.
5. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-360.
6. Cabrera T, Angustias FM, Sierra A et al. High frequency of altered HLA class I phenotypes in invasive breast carcinomas. Hum Immunol 1996;50(2):127-134.
7. de Kruijf EM, van Nes JG, Sajet A et al. The Predictive Value of HLA Class I Tumor Cell Expression and Presence of Intratumoral Tregs for Chemotherapy in Patients with Early Breast Cancer. Clin Cancer Res 2010;16(4):1272-1280.
8. Madjd Z, Spendlove I, Moss R et al. Upregulation of MICA on high-grade invasive operable breast carcinoma. Cancer Immun 2007;7:17.
9. McGilvray RW, Eagle RA, Watson NF et al. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. Clin Cancer Res 2009;15(22):6993-7002.
10. McGivary RW, Eagle RA, Rolland P, Jafferji I, Trowsdale J, Durrant LG. ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. Int J Cancer 2010.
11. Champsaur M, Lanier LL. Effect of NKG2D ligand expression on host immune responses. Immunol Rev 2010;235(1):267-285.
12. Bauer S, Groh V, Wu J et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285(5428):727-729.
13. Cosman D, Mullberg J, Sutherland CL et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 2001;14(2):123-133.
14. Stern-Ginossar N, Mandelboim O. An integrated view of the regulation of NKG2D ligands. Immunology 2009;128(1):1-6.
15. Li K, Mandai M, Hamanishi J et al. Clinical significance of the NKG2D ligands, MICA/B and ULBP2 in ovarian cancer: high expression of ULBP2 is an indicator of poor prognosis. Cancer Immunol Immunother 2009;58(5):641-652.
16. Watson NF, Spendlove I, Madjd Z et al. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. Int J Cancer 2006;118(6):1445-1452.
17. Pende D, Rivera P, Marcenaro S et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 2002;62(21):6178-6186.
18. Raffaghello L, Prigione I, Airoldi I et al. Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma. Neoplasia 2004;6(5):558-568.
19. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A 2001;98(20):11521-11526.
20. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature 2001;413(6852):165-171.
21. Guerra N, Tan YX, Joncker NT et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 2008;28(4):571-580.
22. Wiemann K, Mittrucker HW, Feger U et al. Systemic NKG2D down-regulation impairs NK and CD8 T cell responses in vivo. J Immunol 2005;175(2):720-729.
23. Johanna G.H.van Nes, Esther M.de Kruijf, Dana Faratian et al. Analysis of untreated early breast cancer challenges the prognostic role of COX2 expression. 2009.
24. Eagle RA, Flack G, Warford A et al. Cellular expression, trafficking, and function of two isoforms of human ULBP5/RAET1G. PLoS One 2009;4(2):e4503.
25. Eagle RA, Traherne JA, Hair JR, Jafferji I, Trowsdale J. ULBP6/RAET1L is an additional human NKG2D ligand. Eur J Immunol 2009;39(11):3207-3216.
26. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 2007;26(11):2389-2430.
27. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002;419(6908):734-738.

Chapter 5

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 primarily 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

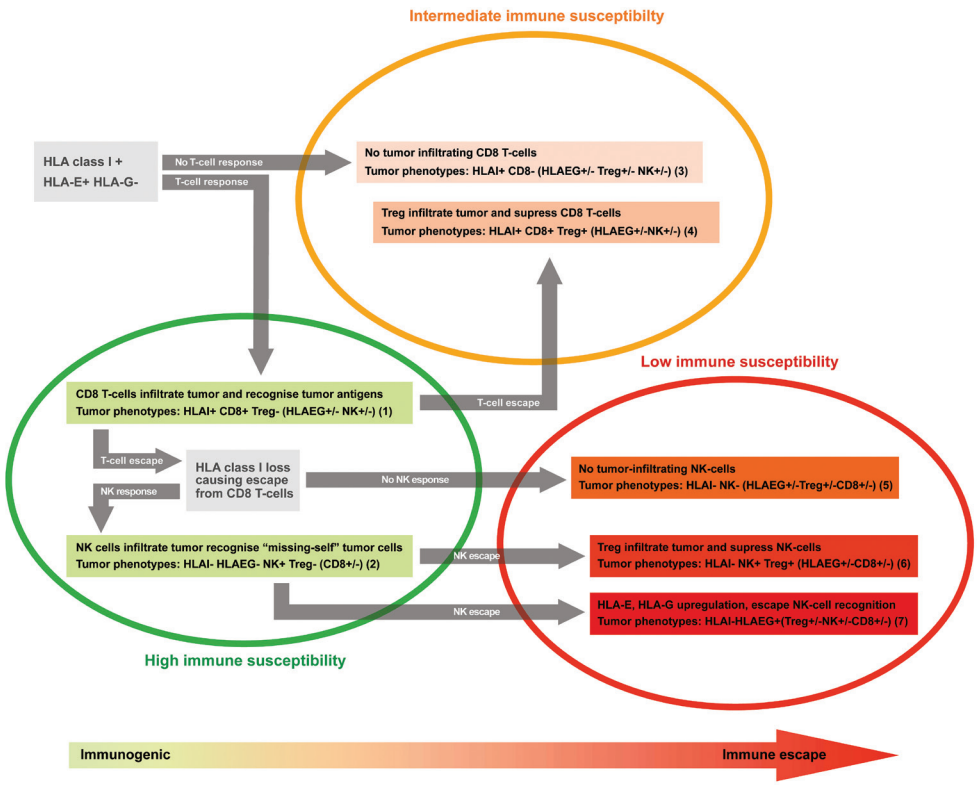

Figure 1 Tumor immune subtypes showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 tumor immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape 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).

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 primarily 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 ratio's and relative risks were 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).

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

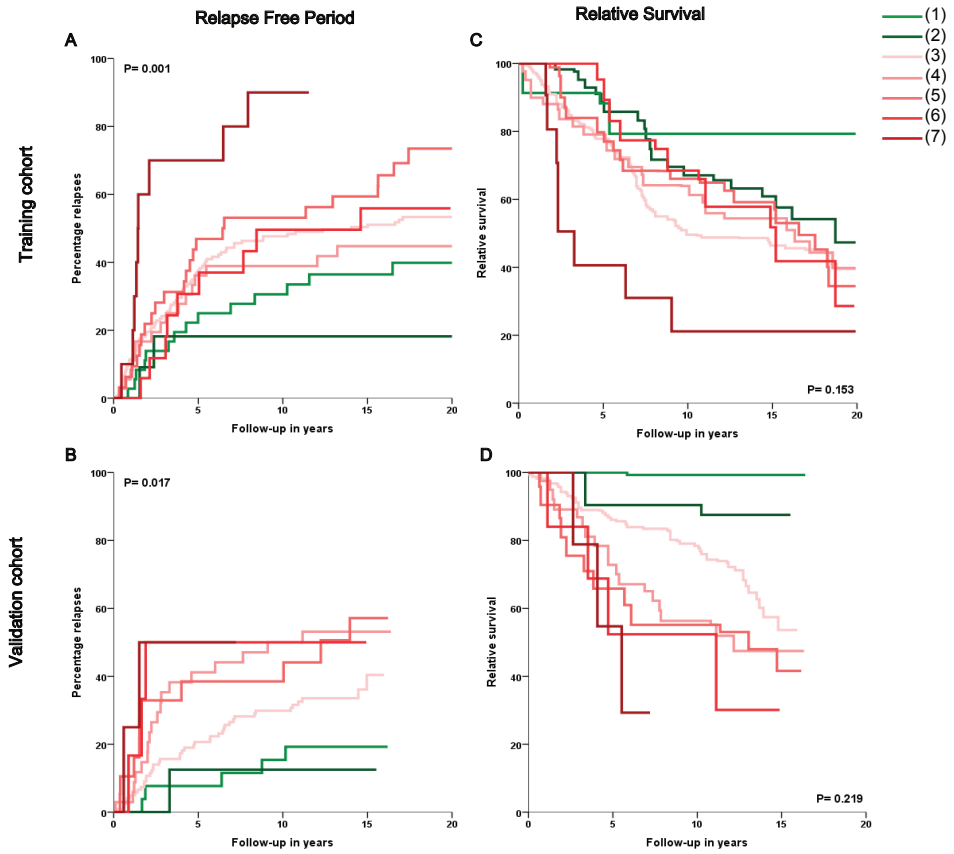

Figure 2 Outcome analyses by tumor immune subtypes for Relapse free period (RFP) **(A, B)** and relative survival (RS) **(C, D)** according to the 7 tumor immune subtypes that are described in the Results section for training cohort patients **(A, C)**, and for validation cohort patients **(B, D)**.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

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 (Figure 1, clustered groups shown by circles).

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	74	1.00		0.354			
40-50	92	0.87	0.58-1.33				
50-60	81	1.24	0.82-1.88				
>60	133	0.95	0.64-1.42				
Grade							
I	53	1.00		0.030	1.00		0.293
II	186	1.38	0.86-2.22		1.30	0.73-2.31	
III	136	1.83	1.13-2.96		1.55	1.55-0.86	
Histological type							
Ductal	345	1.00		0.405			
Other	31	1.23	0.76-2.00				
Tumor stage							
pT1	127	1.00		0.001	1.00		0.045
pT2	198	1.34	0.97-1.86		1.03	0.70-1.51	
pT3/4	45	2.56	1.51-3.69		1.75	1.06-2.88	
Nodal stage							
Negative	199	1.00		<0.001	1.00		<0.001
Positive	171	3.09	2.30-4.16		2.78	1.97-3.92	
ER status							
Negative	133	1.00		0.890			
Positive	229	1.02	0.76-1.38				
PgR status							
Negative	155	1.00		0.765			
Positive	201	1.05	0.78-1.41				
HER2 status							
Negative	271	1.00		0.166			
Positive	32	1.42	0.87-2.32				
Immune phenotyope							
High immune susceptibility	48	1.00		0.005	1.00		<0.001
Intermediate immune susceptibility	186	1.80	1.06-3.05		1.95	1.13-3.39	
Low immune susceptibility	59	2.56	1.44-4.57		2.98	1.62-5.48	

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. **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.

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 respectively. No statistically significant validated association was found between patient and tumor characteristics and tumor immune subtypes classified into 7 groups and into 3 groups.

Relative Survival					
Univariate analysis			Multivariable analysis		
RER	95% CI	P	RER	95% CI	P
1.00		0.048	1.00		0.031
0.79	0.49-1.28		0.60	0.32-1.12	
1.51	0.96-2.38		1.49	0.83-2.65	
1.20	0.71-2.03		1.05	0.54-2.05	
1.00		0.005	1.00		0.023
1.74	0.82-3.68		0.62	0.30-1.30	
2.73	1.29-5.75		1.20	0.60-2.41	
1.00		0.333			
1.34	0.74-2.40				
1.00		<0.001	1.00		0.003
1.84	1.18-2.86		1.90	1.10-3.29	
3.69	2.18-6.24		3.40	1.68-6.89	
1.00		<0.001	1.00		<0.001
2.97	2.04-4.33		2.30	1.48-3.56	
1.00		0.157			
0.77	0.54-1.10				
1.00		0.248			
0.81	0.56-1.16				
1.00		0.004	1.00		0.154
2.03	1.25-3.30		1.59	0.84-3.00	
1.00		0.098	1.00		0.006
1.95	0.98-3.98		3.84	1.62-9.09	
2.02	0.97-4.53		4.26	1.70-10.70	

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

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	63	1.00		0.147			
40-50	83	0.62	0.38-1.03				
50-60	76	0.57	0.33-0.97				
>60	112	0.68	0.42-1.10				
Grade							
I	63	1.00		0.001	1.00		0.433
II	156	1.45	0.82-2.59		1.68	0.68-4.16	
III	108	2.54	1.43-4.52		1.86	0.72-4.79	
Histological type							
Ductal	293	1.00		0.298			
Other	35	1.35	0.77-2.35				
Tumor stage							
pT1	162	1.00		<0.001	1.00		0.171
pT2	130	2.18	1.46-3.23		1.78	0.98-3.26	
pT3/4	32	2.46	1.34-4.51		1.54	0.63-3.77	
Nodal stage							
Negative	182	1.00		<0.001	1.00		0.01
Positive	142	2.81	1.93-4.08		2.06	1.19-3.57	
ER status							
Negative	155	1.00		0.034	1.00		0.889
Positive	164	0.67	0.46-0.97		1.04	0.60-1.82	
PgR status							
Negative	161	1.00		0.006	1.00		0.184
Positive	150	0.59	0.40-0.86		0.68	0.38-1.20	
HER2 status							
Negative	249	1.00		0.002	1.00		0.934
Positive	27	2.36	1.36-4.09		0.97	0.42-2.22	
Immune phenotyope							
High immune susceptibility	34	1.00		0.005	1.00		0.025
Intermediate immune susceptibility	156	2.66	1.15-6.16		2.45	0.87-6.89	
Low immune susceptibility	29	4.72	1.83-12.18		4.73	1.48-15.06	

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. **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.

known clinicopathological parameters are shown in supplementary tables (training cohort Table 1A; validation cohort Table 2A).

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-

Relative Survival					
Univariate analysis			Multivariable analysis		
RER	95% CI	P	RER	95% CI	P
1.00		0.431			
0.58	0.30-1.10				
0.80	0.42-1.53				
0.77	0.35-1.69				
1.00		0.026	1.00		0.603
1.83	0.64-5.28		1.99	0.50-7.99	
3.27	1.16-9.21		1.69	0.40-7.14	
1.00		0.300			
1.46	0.71-3.01				
1.00		0.002	1.00		0.227
2.57	1.34-4.90		1.96	0.85-4.52	
4.30	1.86-9.96		2.30	0.78-6.79	
1.00		<0.001	1.00		0.208
3.09	1.73-5.13		1.59	0.77-3.25	
1.00		0.008	1.00		0.488
0.44	0.24-0.81		0.78	0.39-1.57	
1.00		0.028	1.00		0.232
0.54	0.31-0.93		0.65	0.31-1.38	
1.00		<0.001	1.00		0.232
3.52	1.91-6.49		1.71	0.71-4.10	
1.00		0.089	1.00		0.040
5.31	0.64-31.33		5.47	0.72-41.70	
11.12	1.12-55.41		10.95	1.31-91.63	

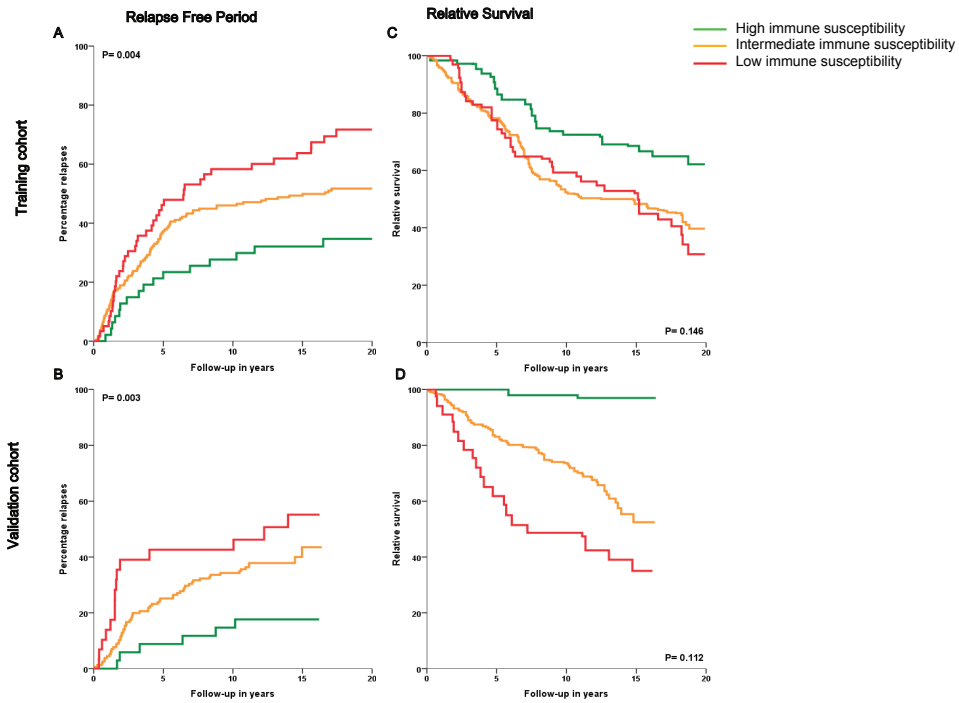

Figure 3 Outcome analyses by tumor immune subtypes for Relapse free period (RFP) **(A, B)** and relative survival (RS) **(C, D)** according to the 3 tumor immune subtypes that are described in the Results section for training cohort patients **(A, C)**, and for validation cohort patients **(B, D)**.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

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 3 A). 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 3 C). Results of outcome analyses in the validation cohort were similar to the results found in the training cohort (RFP p=0.003, Figure 3 B 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 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.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.
2. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007;18(7):1133-1144.
3. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6(10):715-727.
4. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-360.
5. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53(10):904-910.
6. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007;17(6):459-468.
7. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3(11):999-1005.
8. Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. Immunogenetics 2003;54(11):767-775.
9. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A 2001;98(20):11521-11526.
10. Liu F, Lang R, Zhao J et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat 2011.
11. Mahmoud SM, Paish EC, Powe DG et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29(15):1949-1955.
12. de Kruijf EM, Sajet A, van Nes JG et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. J Immunol 2010;185(12):7452-7459.
13. Kleinberg L, Florenes VA, Skrede M et al. Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch 2006;449(1):31-39.
14. Lefebvre S, Antoine M, Uzan S et al. Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. J Pathol 2002;196(3):266-274.
15. Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006;24(34):5373-5380.
16. de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res 2010;16(4):1272-1280.
17. Ladoire S, Arnould L, Apetoh L et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res 2008;14(8):2413-2420.
18. Galon J, Costes A, Sanchez-Cabo F et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313(5795):1960-1964.
19. Ghiringhelli F, Menard C, Terme M et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 2005;202(8):1075-1085.
20. DeNardo DG, Brennan DJ, Rexhepaj E et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov 2011;1(1):54-67.
21. Matkowski R, Gisterek I, Halon A et al. The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. Anticancer Res 2009;29(7):2445-2451.
22. Gobert M, Treilleux I, driss-Vermare N et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res 2009;69(5):2000-2009.
23. Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. Int J Cancer 2005;117(2):248-255.
24. Gudmundsdottir I, Gunnlaugur JJ, Sigurdsson H, Olafsdottir K, Tryggvadottir L, Ogmundsdottir HM. Altered expression of HLA class I antigens in breast cancer: association with prognosis. Int J Cancer 2000;89(6):500-505.
25. Redondo M, Garcia J, Villar E et al. Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. Hum Pathol 2003;34(12):1283-1289.

26. Menon AG, Janssen-Van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest 2004;84(4):493-501.
27. Harris L, Fritsche H, Mennel R et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25(33):5287-5312.
28. Michiels S, Koscielny S, Hill C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. Lancet 2005;365(9458):488-492.
29. van de Vijver M.J., He YD, van't Veer LJ et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347(25):1999-2009.
30. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009;360(8):790-800.

A			(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	0.047
<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	
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																	0.033
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	
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																	0.578
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	
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																	0.305
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	
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																	0.321
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	
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																	0.057
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	
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																	0.131
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	
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	
Her2-status																	0.206
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	
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																	0.714
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	
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																	0.273
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	
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	

Supplementray 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 in the validation cohort of patients **(B)** and well-established prognostic factors using chi-square test.

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.

B			(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	0.794
<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	
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																	0.420
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	
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																	0.109
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	
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																	0.541
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	
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																	0.779
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	
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																	0.411
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	
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	
PgR-status																	0.046
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	
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	
Her2-status																	0.316
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	
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																	0.594
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	
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	

A			High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
	N	%	N	%	N	%	N	%	
Age									0.094
<40	74	19.5	12	25.0	31	16.7	15	25.4	
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									0.138
I	53	14.1	8	17.0	18	9.7	13	22.0	
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									0.534
T1	127	34.3	12	25.0	62	33.9	22	37.9	
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									0.058
Negative	133	36.7	23	47.9	72	38.9	15	25.9	
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	
Her2-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									0.928
MAST-RT	132	34.7	17	35.4	70	37.6	19	32.2	
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	

Supplementary Table 2 Correlations between clustered tumor immune subtypes into 3 groups that are described in the Results section in the training cohort of patients **(A)** and in the validation cohort of patients **(B)** and well-established prognostic factors using chi-square test.

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

B			High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
	N	%	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									0.035
Ductal	293	89.3	30	90.9	140	90.9	20	74.1	
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									0.740
Negative	155	48.6	16	50.0	66	42.6	13	44.8	
Positive	164	51.4	16	50.0	89	57.4	16	55.2	
PgR-status									0.901
Negative	161	51.8	17	53.1	76	48.7	14	50.0	
Positive	150	48.2	15	46.9	80	51.3	14	50.0	
Her2-status									0.691
Overexpression -	249	90.2	21	87.5	127	92.0	23	88.5	
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									0.104
CT alone	49	14.7	3	8.8	24	15.4	9	31.0	
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	

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	74	1.00		0.354			
40-50	92	0.87	0.58-1.33				
50-60	81	1.24	0.82-1.88				
>60	133	0.95	0.64-1.42				
Grade							
I	53	1.00		0.030	1.00		0.384
II	186	1.38	0.86-2.22		1.35	0.76-2.41	
III	136	1.83	1.13-2.96		1.51	0.84-2.73	
Histological type							
Ductal	345	1.00		0.405			
Other	31	1.23	0.76-2.00				
Tumor stage							
pT1	127	1.00		0.001	1.00		0.153
pT2	198	1.34	0.97-1.86		1.01	0.69-1.49	
pT3/4	45	2.56	1.51-3.69		1.57	0.94-2.61	
Nodal stage							
Negative	199	1.00		<0.001	1.00		<0.001
Positive	171	3.09	2.30-4.16		2.81	1.98-3.99	
ER status							
Negative	133	1.00		0.890			
Positive	229	1.02	0.76-1.38				
PgR status							
Negative	155	1.00		0.765			
Positive	201	1.05	0.78-1.41				
HER2 status							
Negative	271	1.00		0.166			
Positive	32	1.42	0.87-2.32				
Immune phenotyope							
(1)	36	1.00		0.002	1.00		0.010
(2)	12	0.43	0.10-1.91		0.53	0.12-2.38	
(3)	149	1.60	0.90-2.82		1.82	1.00-3.32	
(4)	37	1.34	0.65-2.75		1.40	0.67-2.94	
(5)	32	2.15	1.11-4.18		2.45	1.20-4.99	
(6)	17	1.48	0.64-3.41		2.18	0.91-5.22	
(7)	10	5.09	2.19-11.82		4.41	1.83-10.62	

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.

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.

Relative Survival					
Univariate analysis			Multivariable analysis		
HR	95% CI	P	HR	95% CI	P
1.00		0.048	1.00		0.006
0.79	0.49-1.28		0.50	0.27-0.96	
1.51	0.96-2.38		1.52	0.84-2.72	
1.20	0.71-2.03		1.00	0.49-2.04	
1.00		0.005	1.00		0.043
1.74	0.82-3.68		0.59	0.28-1.24	
2.73	1.29-5.75		1.11	0.56-2.23	
1.00		0.333			
1.34	0.74-2.40				
1.00		<0.001	1.00		0.002
1.84	1.18-2.86		2.11	1.21-3.68	
3.69	2.18-6.24		3.62	1.77-7.41	
1.00		<0.001	1.00		<0.001
2.97	2.04-4.33		2.30	1.47-3.60	
1.00		0.157			
0.77	0.54-1.10				
1.00		0.248			
0.81	0.56-1.16				
1.00		0.004	1.00		0.135
2.03	1.25-3.30		1.62	0.86-3.07	
1.00		0.098	1.00		0.002
0.12	0.00-62.27		0.001	0-∞	
1.54	0.80-2.97		3.43	1.41-8.32	
1.26	0.54-2.92		2.40	0.86-6.67	
1.39	0.62-3.13		2.33	0.84-6.51	
1.03	0.33-3.21		4.26	1.28-14.15	
3.68	1.44-9.40		11.84	3.86-36.34	

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	63	1.00		0.147			
40-50	83	0.62	0.38-1.03				
50-60	76	0.57	0.33-0.97				
>60	112	0.68	0.42-1.10				
Grade							
I	63	1.00		0.001	1.00		0.61
II	156	1.45	0.82-2.59		1.55	0.62-3.89	
III	108	2.54	1.43-4.52		1.62	0.61-4.30	
Histological type							
Ductal	293	1.00		0.298			
Other	35	1.35	0.77-2.35				
Tumor stage							
pT1	162	1.00		<0.001	1.00		0.113
pT2	130	2.18	1.46-3.23		1.93	1.04-3.56	
pT3/4	32	2.46	1.34-4.51		1.79	0.73-4.39	
Nodal stage							
Negative	182	1.00		<0.001	1.00		0.014
Positive	142	2.81	1.93-4.08		2.03	1.16-3.56	
ER status							
Negative	155	1.00		0.034	1.00		0.728
Positive	164	0.67	0.46-0.97		1.11	0.62-1.97	
PgR status							
Negative	161	1.00		0.006	1.00		0.243
Positive	150	0.59	0.40-0.86		0.70	0.39-1.27	
HER2 status							
Negative	249	1.00		0.002	1.00		0.815
Positive	27	2.36	1.36-4.09		1.11	0.46-2.66	
Immune phenotyope							
(1)	26	1.00		0.031	1.00		0.055
(2)	8	0.58	0.07-4.94		0.77	0.08-7.67	
(3)	122	2.10	0.83-5.31		2.04	0.61-6.89	
(4)	34	3.45	1.28-9.28		3.06	0.85-10.97	
(5)	19	4.09	1.39-12.01		3.67	0.91-1479	
(6)	6	3.82	0.91-16.02		4.16	0.81-21.44	
(7)	4	5.91	1.14-30.67		13.4	2.12-84.86	

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.

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.

Relative Survival					
Univariate analysis			Multivariable analysis		
HR	95% CI	P	HR	95% CI	P
1.00		0.431			
0.58	0.30-1.10				
0.80	0.42-1.53				
0.77	0.35-1.69				
1.00		0.026			
1.83	0.64-5.28				
3.27	1.16-9.21				
1.00		0.300			
1.46	0.71-3.01				
1.00		0.002			
2.57	1.34-4.90				
4.30	1.86-9.96				
1.00		<0.001			
3.09	1.73-5.13				
1.00		0.008			
0.44	0.24-0.81				
1.00		0.028			
0.54	0.31-0.93				
1.00		<0.001			
3.52	1.91-6.49				
1.00		0.219			
5.2E5	0-∞				
1.5e6	0-∞				
2.5e6	0-∞				
2.6e6	0-∞				
3.7e6	0-∞				
6.5e6	0-∞				

Part II

PROGNOSTIC BIOMARKERS IN ELDERLY BREAST CANCER PATIENTS

Chapter 6

Age interactions in the prognostic value of aldh1 for clinical outcome in breast cancer

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ABSTRACT

Purpose To compare expression and prognostic effect of the breast cancer stem cell marker aldehyde dehydrogenase-1 (ALDH1) in young and elderly breast cancer patients.

Patients and Methods The study population (N = 574) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1994. Median follow-up was 17.9 years (range: 0.1 to 23.5). Tissue microarray slides were immunohistochemically stained for ALDH1 expression and quantified by two independent observers who were blinded to clinical outcome. Assessment of the prognostic effect of ALDH1 expression was stratified according to age and systemic treatment.

Results Complete lack of expression of ALDH1 was found in 40% of tumors. With increasing age more tumors showed complete absence of ALDH1 expression ($P < .001$). In patients aged > 65 years, ALDH1 status was not associated with any clinical outcome. Conversely, in patients aged < 65 years, ALDH1 positivity was an independent risk factor of worse outcome for relapse free period (hazard ratio = 1.71 (95% CI, 1.09 to 2.68); $P = .021$) and relative survival (relative excess risks of death = 2.36 (95% CI, 1.22 to 3.68); $P = .016$). Ten-year relative survival risk was 57% in ALDH1-positive patients compared to 83% in ALDH1-negative patients.

Conclusion ALDH1 expression and its prognostic effect are age-dependent. Our results support the hypothesis that breast cancer biology is different in elderly patients compared to their younger counterparts and emphasizes the importance of taking into consideration age-specific interactions in breast cancer research.

INTRODUCTION

Age at diagnosis of breast cancer is an important independent prognostic factor. Young age is associated with more aggressive tumors with a relatively high risk of distant metastasis and loco-regional recurrence,[1] whereas old age is associated with more indolent tumors.[2, 3] Although tumor characteristics differ considerably between age groups (including hormone receptor and human epidermal growth factor receptor 2 (HER2) status), these tumor characteristics can only account for part of the divergence in survival witnessed between age groups.[2] Little is known about the impact and significance of various prognostic and predictive factors in elderly as compared to their younger counterparts. As is the case with randomized trials, elderly are underrepresented in translational studies on molecular markers.[4, 5] This caveat is especially worrisome since studies show that the relative survival in elderly breast cancer patient is lower, despite more favorable tumor characteristics, which is probably due to the fact that these patients receive less aggressive treatment.[6] Molecular markers could aid to guide therapy in the fit elderly. Moreover, specific age-interactions might underlie pathophysiological processes in the development of primary breast cancer and subsequent local and distant metastases. Therefore, breast cancer researchers should account for age-specific differences.[4]

Recent evidence in tumor biology supports the cancer stem cell theory and may also provide a biological reason for the age-associated survival difference.[7] According to this theory, cancer stem cells, defined as a small subset of tumor cells with stem cell-like features including epithelial-to-mesenchymal transition, have the capacity to self-renewal and differentiation, giving rise to heterogeneous tumor cell population. Various putative markers of breast cancer stem cells have been proposed, including aldehyde dehydrogenase-1 (ALDH1) activity, CD44+/CD24-, CD133, and ITGA6.[7-10] In particular, ALDH1 expression has shown promise as a clinically relevant prognostic marker.[9, 11, 12] Moreover, various studies have shown that the subset of cancer stem cells is relatively resistant to chemo- and radiotherapy.[13, 14] Thereby, the subpopulation of cancer stem cells can provide both an explanation and a therapeutic target for poor-prognostic, treatment-resistant and recurrent breast cancer.

ALDH1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes and thereby confers resistance to alkylating agents.[12, 15] This detoxifying capacity might underlie the longevity of stem cells by protecting against oxidative stress. Moreover, ALDH1 may have a role in early differentiation of stem cells and stem cell proliferation through its role in oxidizing retinol to retinoic acid, a modulator of cell proliferation.[15] ALDH1 expression is associated with unfavorable tumor characteristics in breast cancer, such as high grade, absence of hormone receptor expression, positive HER2 status and the basal-like molecular subtype.[9, 16-18]

To study whether the expression of the breast cancer stem cell marker ALDH1 is associated with age and has an influence on clinical outcome, we analyzed the age-distribution of ALDH1 expression and its prognostic role in young and elderly patients using long-term follow-up data of a cohort of breast cancer patients primarily treated with surgery in our institution.

PATIENT AND METHODS

Study cohort

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1994 with tumor material available (N = 574).[19] 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, locoregional or distant tumor recurrence, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2). All tumors were graded according to current pathological standards by an experienced breast cancer pathologist (V.S.). Median follow-up was 17.9 years (range: 0.01 to 23.5). Approval 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).

Assessment of ALDH1 expression

Mouse antibody against ALDH1 (611195, BD Biosciences) was used for immunohistochemistry. Tissue sections of 4 μm were cut from a previously constructed tissue microarray of formalin-fixed paraffin-embedded tumors of 574 patients from whom tumor material was available.[19] Immunohistochemical staining was performed according to previously described standard protocols.18 Human liver tissue slides served as positive control. Negative controls were human liver tissue slides that did undergo the whole immunohistochemical staining without primary antibodies. Microscopic analysis of ALDH1 was assessed independently by two observers in a blinded manner. Absence and presence of ALDH1 activity was classified as 0% and 1-100% staining of tumor cells, respectively (Figure 1A).[9 11]

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 was used to assess the inter-observer agreement in quantification of ALDH1 expression. The Cohen's

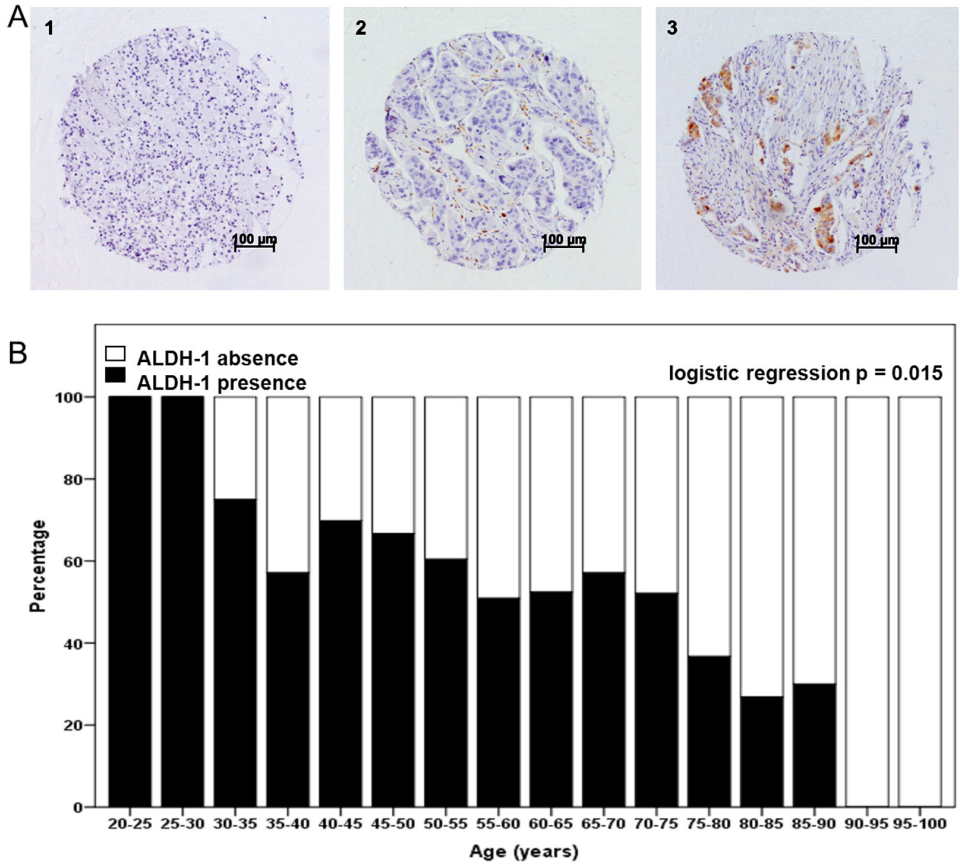

Figure 1 ALDH1 expression and distribution over age groups. **A)** Representative photographs of tissue microarray punches of human breast cancer specimens immunohistochemically stained for ALDH1 with representative examples of strong staining (left panel), intermediate staining (middle panel) and no staining (right panel). Bar represents 100 µm. **B)** ALDH1 status according to age (N = 496). Logistic regression *P*-value is shown.

kappa coefficient was 0.81. The χ^2 test was used to evaluate associations between various clinicopathological parameters and ALDH1 expression. Relapse-free period was defined as the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). Relapse-free period is reported as cumulative incidence function, after accounting for death as competing risk.[20] The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of relapse-free period curves. Cox proportional hazard analysis was used for 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.

Analyses were performed for all patients and stratified for age and systemic treatment. Age of 65 years at time of diagnosis was chosen as the cut-off point for age stratification.[21] An interaction term with age and ALDH1 status was introduced in Cox proportional hazard model to assess the interaction in prognostic effects of ALDH1 status for the age groups. Variables with a *P*-value of < .10 in univariate analysis were entered in multivariable analysis.

Characteristic	Patients < 65 years					Patients > 65 years				
	ALDH1 negative		ALDH1 positive			ALDH1 negative		ALDH1 positive		
	N	%	N	%	P	N	%	N	%	P
Grade					0.02					0.79
I	19	17.3	18	8.5		11	12.9	13	16.2	
II	58	52.7	103	48.6		44	51.8	38	47.5	
III	33	30.0	91	42.9		30	35.3	29	36.2	
Histological type					0.42					0.20
Ductal	103	92.8	191	90.1		72	84.7	73	91.2	
Lobular	8	7.2	21	9.9		13	15.3	7	8.7	
Tumor size					0.34					0.55
T1	45	40.2	74	36.1		27	31.0	24	31.6	
T2	57	50.9	101	49.3		48	55.2	37	48.7	
T3/4	10	8.9	30	14.6		12	13.8	15	19.7	
Nodal status					0.02					0.71
Negative	69	62.2	101	47.9		46	56.1	46	59.0	
Positive	42	37.8	110	52.1		36	43.9	32	41.0	
ER status					0.61					0.01
Negative	43	41.0	91	44.0		16	19.0	30	38.0	
Positive	62	59.0	116	56.0		68	81.0	49	62.0	
PgR status					0.77					0.08
Negative	48	47.1	84	40.6		26	31.3	35	44.9	
Positive	54	52.9	123	59.4		57	68.7	43	55.1	
Her2 status					0.99					0.80
Negative	67	87.0	153	86.9		61	93.8	64	92.8	
Positive	10	13.0	23	13.1		4	6.2	5	7.2	

Table 1. Association of ALDH1 status with clinicopathological charateristics, stratified by age.
Abbreviations N=number of patients; ALDH1=aldehyde dehydrogenase 1; ER=estrogen receptor; PgR=progesterone receptor; HER2=human epidermal growth factor receptor 2.

RESULTS

ALDH1 Expression in Patient Cohort

Immunohistochemical data of ALDH1 expression were available for 496 of the 574 patients (86.4%). Of these patients, 326 (65.7%) were < 65 years at diagnosis and 170 (34.3%) were > 65 years at diagnosis. The Cohen's kappa coefficient for inter-observer agreement was 0.81. Complete lack of expression of ALDH1 of any tumor cell was found in 40.4% of the tumors. The association between ALDH1 status and age is shown in Figure 1B. ALDH1 expression was inversely correlated with age ($P = .0015$) and was significantly higher in patients aged < 65 years (65.3%) than in patients aged > 65 years (48.2%; $P < .001$). The association of ALDH1 expression with classic patient, tumor and treatment characteristics is shown in Table 1. In patients aged < 65 years, ALDH1 expression was significantly correlated with high histological grade and positive nodal status. In patients aged > 65 years, ALDH1 expression was significantly correlated with absence of estrogen-receptor expression.

Impact of ALDH1 on Survival

The association of ALDH1 status with relapse-free period and relative survival is shown in Figures 2 and 3, respectively. Analysis of relapse-free period showed a trend towards

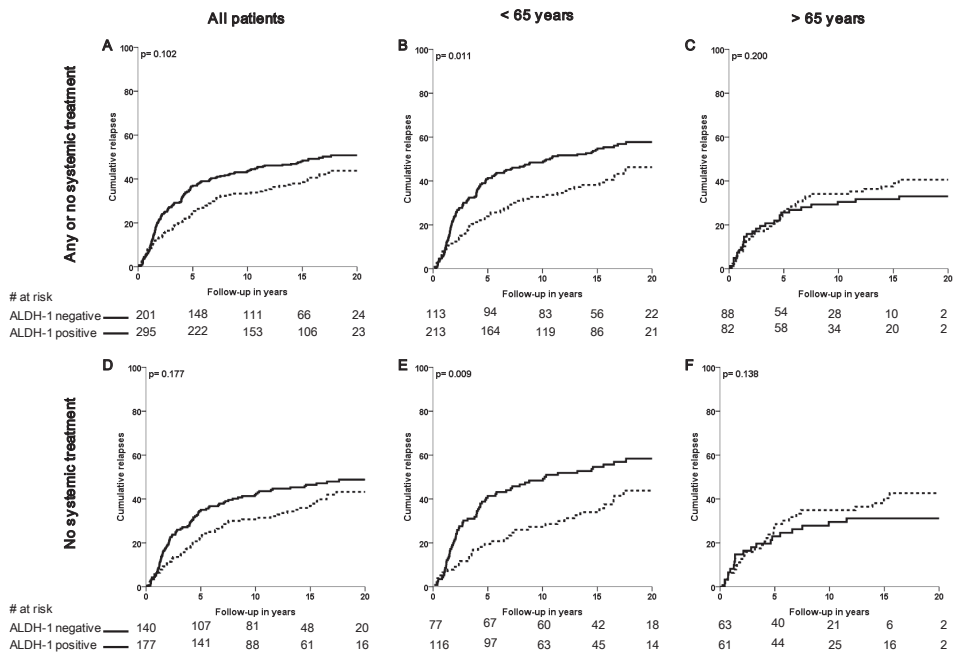

Figure 2. Relapse free period according to ALDH1 status for all patients **(A, D)**, for patients aged < 65 years **(B, E)** and for patients aged > 65 years **(C, F)**; and for patients that received any or no systemic therapy **(A-C)** and for patients that did not receive systemic therapy **(D-F)**. Log-rank *P*-values are shown in each graph.

Characteristic		Patients < 65 years					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age				.74			
<40	33	1					
40-50	98	1.10	0.62-1.96				
50-60	101	1.29	0.73-2.29				
>60	44	1.29	0.66-2.52				
Grade				.03			0.21
I	31	1			1		
II	131	1.45	0.76-2.75		1.06	0.40-2.78	
III	75	2.17	1.12-4.20		1.54	0.58-4.11	
Histological type				.54			
Ductal	223	1					
Other	15	1.24	0.63-2.45				
Tumor stage				<.001			0.05
pT1	137	1			1		
pT2	109	1.66	1.16-2.37		1.60	1.01-2.55	
pT3/4	22	2.73	1.57-4.76		2.04	1.07-3.88	
Nodal stage				<.001			<0.001
Negative	204	1			1		
Positive	69	4.25	3.02-5.99		4.44	2.89-6.82	
ER status				.59			
Negative	87	1					
Positive	126	0.90	0.66-1.32				
PgR status				.78			
Negative	84	1					
Positive	123	0.95	0.64-1.40				
HER2 status				.26			
Negative	143	1					
Positive	19	1.44	0.76-2.71				
ALDH1 status				.01			0.02
Negative	77	1			1		
Positive	116	1.75	1.14-2.68		1.71	1.09-2.68	

Table 2. Univariate and multivariable analysis for relapses free period stratified by age for patients naive to systemic treatment.
Abbreviations N=number of patients; HR=hazard ratio; ER=estrogen receptor; PgR=progesterone receptor; HER2=human epidermal growth factor receptor 2; ALDH1=aldehyde dehydrogenase 1.

a significant association between ALDH1 status and clinical outcome for the whole population (P = .10; Figure 2A, D). In the group of patients aged younger than 65 years, a strong association was found between ALDH1 expression and poor clinical outcome (P = .01; Figure 3B). In the subgroup of younger patients who did not receive any systemic treatment, a comparable association was found (P = .009; Figure 2E). In this group, 52% of patients with ALDH1-positive tumors was relapse-free at 10 years follow-up compared to 72% of patients with ALDH1-negative tumors (absolute

	Patients > 65 years					
	Univariate analysis			Multivariable analysis		
N	HR	95% CI	P	HR	95% CI	P
0						
0						
0						
154						
			.01			.29
24	1			1		
74	1.73	0.66-4.58		1.30	0.48-3.58	
44	3.66	1.39-9.61		2.01	0.70-5.75	
			.53			
124	1					
18	0.74	0.30-1.87				
			.03			.90
59	1			1		
71	2.23	1.22-4.09		1.16	0.56-2.40	
18	2.11	0.82-5.45		1.23	0.42-3.63	
			<.001			<.001
109	1			1		
36	3.94	2.28-6.82		3.33	1.77-6.24	
			.22			
40	1					
95	1.53	0.78-2.98				
			.78			
55	1					
81	1.08	0.61-1.92				
			.05			
105	1					
4	0.05	0.00-23.3				
			.14			
63	1					
61	0.64	0.35-1.16				

difference = 20%). Conversely, in the elderly patients, no association was found between ALDH1 status and clinical outcome (P = .20; Figure 2C, F). Interaction analysis demonstrated a statistically significant difference in the prognostic effect of ALDH1 status in young and elderly patients (P = .007).

Analysis of relative survival showed a similar pattern as for relapse-free period: a strong association between ALDH1-positive tumors and poor relative survival in the younger patient group (Figure 3B, E) and no association between ALDH1 status and relative survival for elderly patients (Figure 3C, F). In the subgroup of younger patients who

Characteristic		Patients < 65 years					
		Univariate analysis			Multivariable analysis		
	N	RER	95% CI	P	RER	95% CI	P
Age				.06			.10
<40	33	1			1		
40-50	98	1.03	0.51-2.08		0.69	0.21-2.24	
50-60	101	1.52	0.76-3.03		1.38	0.46-5.18	
>60	44	2.16	0.99-4.66		1.93	0.56-6.57	
Grade				.03			.95
I	31	1			1		
II	131	2.23	0.80-6.28		1.24	0.31-5.03	
III	75	3.48	1.22-9.94		1.27	0.30-5.45	
Histological type				.80			
Ductal	223	1					
Other	15	1.12	0.46-2.71				
Tumor stage				<.001			.33
pT1	137	1			1		
pT2	109	2.67	1.41-3.64		1.42	0.73-2.76	
pT3/4	22	4.26	2.29-7.94		1.79	0.81-3.95	
Nodal stage				<.001			<.001
Negative	204	1			1		
Positive	69	5.03	3.32-7.62		5.82	3.16-10.7	
ER status				.11			
Negative	87	1					
Positive	126	0.70	0.45-1.09				
PgR status				.25			
Negative	84	1					
Positive	123	0.77	0.49-1.21				
HER2 status				.19			
Negative	143	1					
Positive	19	1.57	0.80-3.09				
ALDH1 status				.008			.02
Negative	77	1			1		
Positive	116	2.12	1.22-3.68		2.36	1.17-4.73	

Table 3. Univariate and multivariable analysis for relative survival stratified by age for patients naive to systemic treatment.
Abbreviations N=number of patients; RER=relative excess risk; ER=estrogen receptor; PgR=progesterone receptor; HER2=human epidermal growth factor receptor 2; ALDH1=aldehyde dehydrogenase 1.

did not receive any systemic treatment, the 10-year relative survival rate was 57% in patients with ALDH1-positive tumors compared to 83% in patients with ALDH1-negative tumors (absolute difference = 26%, $P = .008$; Figure 3E). Interaction analysis demonstrated a statistically significant difference between the prognostic effect of ALDH1 status in young and elderly patients ($P = 0.047$).

Multivariable analyses were conducted for the patient groups that did not receive systemic treatment (276 young patients and 154 elderly patients). ALDH1 status remained an independent prognostic factor in the young patient group for both relapse-

	Patients > 65 years					
	Univariate analysis			Multivariable analysis		
N	RER	95% CI	P	RER	95% CI	P
0						
0						
0						
154						
			.34			
24	1					
74	3.10	0.43-22.4				
44	4.23	0.56-31.8				
			.37			
124	1					
18	1.49	0.62-3.61				
			.01			.09
59	1			1.00		
71	5.47	0.83-35.9		4.26	0.71-25.4	
18	13.2	1.90-92.1		7.90	1.18-52.9	
			.02			.43
109	1			1.00		
36	2.76	1.20-6.35		1.48	0.56-3.92	
			.91			
40	1					
95	0.95	0.42-2.17				
			.58			
55	1					
81	0.80	0.36-1.77				
			.89			
105	1					
4	0.85	0.09-8.40				
			.35			
63	1					
61	0.68	0.30-1.53				

free period (hazard ratio = 1.71; 95% CI, 1.09 to 2.68; *P* = .021; Table 2) and relative survival (relative excess risks of death = 2.36; 95% CI, 1.22 to 3.68; *P* = .016; Table 3).

DISCUSSION

In this study, we demonstrated that the presence of ALDH1 expression is significantly higher in young breast cancer patients than in elderly patients. Moreover, we demonstrated that ALDH1 expression is an independent risk factor for decreased survival in young breast cancer patients, but not in elderly patients.

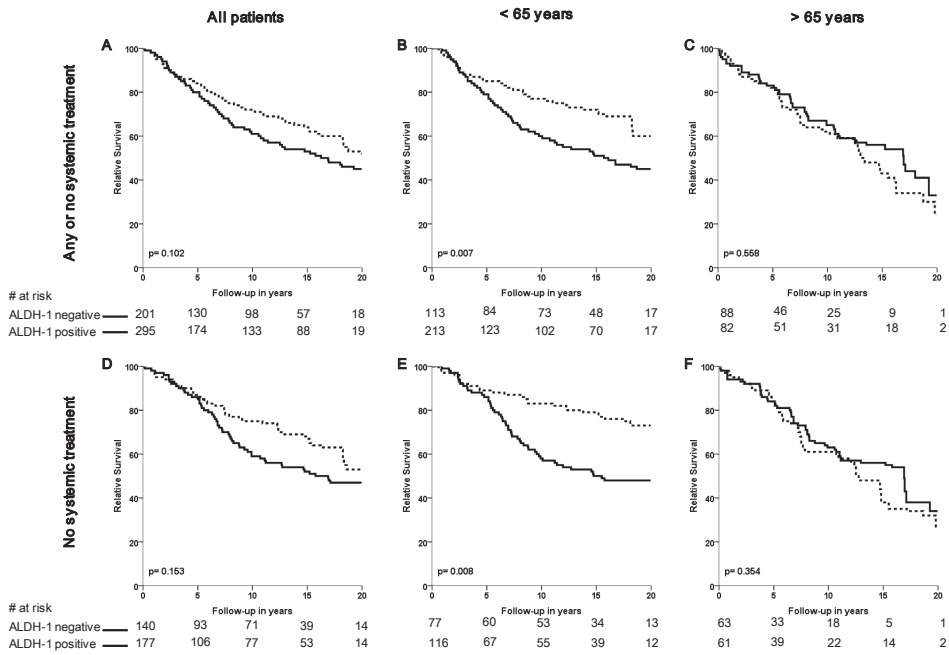

Figure 3. Relative survival according to ALDH1 status for all patients **(A, D)**, for patients aged < 65 years **(B, E)** and for patients aged > 65 years **(C, F)**; and for patients that received any or no systemic therapy **(A-C)** and for patients that did not received systemic therapy **(D-F)**. Log-rank *P*-values are shown in each graph

To the best of our knowledge, we are the first to show that expression of ALDH1 in tumors is age-dependent. A corresponding difference in the number of cancer stem cells might provide an explanation for known differences in survival between young and elderly breast cancer patients. A potential strength of our study is that it includes consecutive patients from one center, not biased by being part of a clinical trial. The age restriction of the majority of clinical trials prohibits inclusion of patients older than 70 year and, indeed, less than 10% of clinical trial participants is older than 65 years.[22] In our study, 34% of patients were 65 years or older at diagnosis of breast cancer. Therefore, our study was not hampered by lack of statistical power to analyse the effect of ALDH1 in the elderly.

We showed that ALDH1 expression has a qualitative age interaction effect. In our study, ALDH1 is a predictor of poor prognosis in young patients, but ALDH1 did not influence clinical outcome in elderly patients. Recently, Zhou and colleagues pooled the available data on the prognostic role of ALDH1 activity in breast cancer.[18] Their meta-analysis demonstrated that ALDH1 activity as assessed by immunohistochemistry was significantly associated with worse overall survival (unadjusted pooled relative risk, 2.83; 95% CI, 2.16 to 3.67; four patient cohorts including 1,158 patients).[18] However, the authors did not stratify for age. In other studies, no interaction was found between

ALDH1 expression and age.[9, 13, 17] However, in these studies, an age of 40 or 50 year was used as a cut-off for age stratification. We used 65 years as a cut-off point as this may better match with the bimodal age distribution of breast cancer, which suggests that breast cancer may be characterized by early- and late-onset tumor types with modes near ages 50 and 70 years.[4, 21] As argued by Anderson *et al.*, these modal ages do not suggest a sharp division of distinctive tumor categories, but rather reflect central tendencies for the age distributions of biologically distinct cancer populations.[4, 23] In line with this bimodal age distribution, a biological explanation of the qualitative age-interaction of the prognostic effect of ALDH1 expression might be that of a changing micro-environment in elderly patients, which may result in hampered signal transduction between tumor stem cells and the micro-environment. Moreover, changes in metabolic processes might limit the role of tumor stem cells in elderly patients. Increasing evidence from the field of epigenetics demonstrates that hypermethylation-induced repression of genes required for stem cell differentiation is linearly associated with age.[24] This suggests that, with increasing age, the role of tumor stem cells becomes more limited. Notwithstanding the need to clarify the underlying mechanism, this new finding on the age-dependent role of ALDH1 activity warrants further validation and underlines the need of age stratification when assessing biomarkers and new therapies for breast cancer patients.

In conclusion, we demonstrated that expression of the putative breast cancer stem cell marker ALDH1 and its prognostic effect are age-dependent in breast cancer patients. We demonstrate, for the first time, the different prognostic impact of a molecular marker in elderly, which suggests that fundamentally different biological mechanisms underlie age-related breast cancer prognosis. Our results support the hypothesis that breast cancer biology of elderly patients and their younger counterparts is distinct and emphasizes the importance of analyzing and reporting age-specific effects in breast cancer research.

REFERENCES

1. Anders CK, Johnson R, Litton J, Phillips M, Bleyer A. Breast cancer before age 40 years. Semin Oncol 2009;36(3):237-249.
2. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. J Natl Cancer Inst 2000;92(7):550-556.
3. Remvikos Y, Magdelenat H, Dutrillaux B. Genetic evolution of breast cancers. III: Age-dependent variations in the correlations between biological indicators of prognosis. Breast Cancer Res Treat 1995;34(1):25-33.
4. Anderson WF, Jatoi I, Sherman ME. Qualitative age interactions in breast cancer studies: mind the gap. J Clin Oncol 2009;27(32):5308-5311.
5. Beadle BM, Woodward WA, Buchholz TA. The impact of age on outcome in early-stage breast cancer. Semin Radiat Oncol 2011;21(1):26-34.
6. Bastiaannet E, Liefers GJ, de Craen AJ et al. Breast cancer in elderly compared to younger patients in The Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. Breast Cancer Res Treat 2010;124(3):801-807.
7. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100(7):3983-3988.
8. Cariati M, Naderi A, Brown JP et al. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. Int J Cancer 2008;122(2):298-304.
9. Ginestier C, Hur MH, Charafe-Jauffret E et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1(5):555-567.
10. Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L. Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. Breast Cancer Res 2008;10(1):R10.
11. Resetkova E, Reis-Filho JS, Jain RK et al. Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. Breast Cancer Res Treat 2010;123(1):97-108.
12. Tanei T, Morimoto K, Shimazu K et al. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. Clin Cancer Res 2009;15(12):4234-4241.
13. Gong C, Yao H, Liu Q et al. Markers of tumor-initiating cells predict chemoresistance in breast cancer. PLoS One 2010;5(12):e15630.
14. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006;98(24):1777-1785.
15. Huang EH, Hynes MJ, Zhang T et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res 2009;69(8):3382-3389.
16. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010;363(20):1938-1948.
17. Nalwoga H, Arnes JB, Wabinga H, Akslen LA. Expression of aldehyde dehydrogenase 1 (ALDH1) is associated with basal-like markers and features of aggressive tumours in African breast cancer. Br J Cancer 2010;102(2):369-375.
18. Zhou L, Jiang Y, Yan T et al. The prognostic role of cancer stem cells in breast cancer: a meta-analysis of published literatures. Breast Cancer Res Treat 2010;122(3):795-801.
19. van Nes JG, de Kruijf EM, Faratian D et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res Treat 2010.
20. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 2007;26(11):2389-2430.
21. Anderson WF, Jatoi I, Devesa SS. Distinct breast cancer incidence and prognostic patterns in the NCI's SEER program: suggesting a possible link between etiology and outcome. Breast Cancer Res Treat 2005;90(2):127-137.
22. Hutchins LF, Unger JM, Crowley JJ, Coltman CA, Jr., Albain KS. Underrepresentation of patients 65 years of age or older in cancer-treatment trials. N Engl J Med 1999;341(27):2061-2067.
23. Anderson WF, Matsuno R. Breast cancer heterogeneity: a mixture of at least two main types? J Natl Cancer Inst 2006;98(14):948-951.
24. Teschendorff AE, Menon U, Gentry-Maharaj A et al. Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. Genome Res 2010;20(4):440-446.

Chapter 7

Comparison of frequencies and prognostic effect of molecular subtypes between young and elderly breast cancer patients

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ABSTRACT

Purpose To compare the distribution and prognostic effect of the breast cancer molecular subtypes in young and elderly breast cancer patients.

Patients and Methods Our study population (n=822) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1996. A total of 142/822 fresh frozen tissues were available with good quality RNA and analyzed by gene expression microarray. Gene expression molecular subtypes were determined by correlation to the expression centroids of 534 "intrinsic" genes. . Sections of a tissue micro array containing formalin-fixed paraffin-embedded tumor tissue of 714/822 patients were immunohistochemically (IHC) stained for Ki67, EGFR, CK5/6. Tumor expression of ER, PR, HER2 was previously determined. IHC molecular subtypes were defined based on expression of these markers: Luminal A: ER+ and/or PR +, HER2- and Ki67-; Luminal B: ER+ and/or PR + and ki67+; ERBB2: ER-, PR- and HER2+; Basal-like: ER-, PR-, HER2- and EGFR+ and/or CK5/6+; Unclassified: ER-, PR-, HER2-, EGFR- and CK5/6-. IHC molecular subtypes were validated against gene expression defined molecular subtypes. Assessment of distribution and prognostic effect of molecular subtypes was stratified to age (<65 versus >=65 years).

Results Validation of molecular subtypes determined by IHC against gene expression revealed a substantial agreement in classification (Cohen's kappa coefficient 0.75). A statistically significant association (p=0.02) was found between molecular subtypes and age, where Luminal tumors were more often found in elderly patients, while ERBB2, basal-like and unclassified subtypes were more often found in young patients. Molecular subtypes showed a prognostic association with outcome in young patients concerning relapse free period (RFP) (p=0.01) and relative survival (RS) (p<0.001). No statistically significant prognostic effect was found for molecular subtypes in elderly patients (RFP p=0.5; RS p=0.1). Additional analyses showed that no molecular subtypes showed a statistically significant difference in outcome for elderly compare to young patients.

Conclusion We have shown that molecular subtypes have a different distribution and prognostic effect in elderly compared to young breast cancer patients, emphasizing the fact that biomarkers may have different distributions and prognostic effects and therefore different implications in elderly compared to their younger counterparts. Our results support the premise that breast cancer clinical behavior is significantly affected by patient age. We suggest that competing risks of death in elderly patients, ER-driven differences and micro-environmental changes in biology are underlying these age-dependent variations in patient prognosis.

INTRODUCTION

Breast cancer is increasingly becoming a disease affecting older women. However, evidence based treatment guidelines specific for this aged breast cancer population are lacking[1]. Decisions regarding breast cancer treatment are based on prognostic and predictive patient and tumor characteristics discovered and analyzed in relatively young patient populations[2-5]. These characteristics have been found to differ considerably between elderly and young breast cancer, i.e. elderly breast cancer patients present more often with tumors positive for hormone receptor expression, no overexpression of human epidermal growth factor receptor 2 (HER2), lower proliferation rates, diploidy, normal p53 expression and bcl-2 overexpression[6-8]. This may be indicative for differences in underlying tumor biology and it has indeed often been suggested that elderly breast cancer is a biologically different tumor type of a more indolent character compared to young breast cancer[7-9]. Moreover, it suggests that biomarkers may show different prognostic and predictive effects in the elderly compared to young breast cancer patients. In addition, due to competing causes of death, life expectancy is significantly shorter in elderly breast cancer patient[10-12]. Therefore, since breast cancer relapses can occur after long periods of time, this further suggests that the impact and significance of prognostic and predictive biomarkers may vary significantly in this patient population. Nevertheless, as this patient population is often underrepresented in translational studies and randomized trials, little is known about the implications on outcome of prognostic and predictive biomarkers in elderly[2, 3].

Gene expression studies have identified several distinct breast cancer subtypes based on gene expression patterns, that showed marked differences in patient prognosis [13-15]. This "intrinsic" classification proposes four different classes of breast tumors: Luminal A and B, which are mostly hormone receptor positive and show high expression of genes characteristic of the luminal epithelial cell layer, including expression of estrogen receptor (ER), GATA3 and genes regulated by these[14, 15]. Compared with Luminal A tumors, Luminal B tumor often express genes associated with high tumor proliferation[14, 15]. The "intrinsic" subtypes further include 2 main subtypes of hormone receptor negative tumors: Basal-like tumors, which typically are triple negative tumor (ER, progesterone receptor (PR), and HER2 negative) and exhibit high expression of genes characteristic of the basal epithelial cell layer such as cytokeratin (CK) 5, 6 and 17 [13] and the ERBB2 tumor subtype, which clusters near the basal-like tumor, are mostly hormone receptor negative and show high overexpression of HER2 and high HER2 gene amplification[14, 15]. Concerning outcome, hormone receptor positive tumors result in the best patient outcome where, compared to Luminal B tumors, Luminal A tumors seem to be the most indolent tumors[14]. Hormone receptor negative "intrinsic" subtypes, ERBB2 and Basal-like tumors have an aggressive natural history, resulting in an unfavorable patient outcome[14]. In a large study on almost 500 breast cancer patients Perou et al. (2000)

found the molecular subtypes, determined with immunohistochemistry (IHC), to be significantly associated with tumor histological grade, lymph node status and patient age, where ERBB2 and Basal-like subtypes showed to correlate with unfavorable tumor characteristics and younger patient age[16]. The distribution and prognostic effect of molecular breast cancer subtypes specific in the elderly breast cancer population compared to younger breast cancer patients is still unknown.

We used immunohistochemical (IHC) surrogates, which we validated against gene expression determined molecular subtypes, to identify breast tumor molecular subtypes in a large cohort of breast cancer patients. The aim was to investigate the distribution and prognostic effect of molecular subtypes of breast cancer in elderly patients compared to their younger counterparts.

PATIENTS AND METHODS

Patients and tumors

The patient population comprised all 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, locoregional/distant tumor recurrence and survival. Expression of ER, PR and HER2 were previously determined using standard immunohistochemistry protocols and semi-automated quantifications[17]. All tumors were graded according to current pathological standards, by an experienced breast cancer pathologist (VS). Approval 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).

Microarray analysis

Fresh frozen tumor material was available of 33% (268/822). Total RNA was isolated by phenol-chloroform extraction (Trizol reagent). The Quality control, RNA labeling, hybridisation and data extraction were performed at ServiceXS (Leiden, The Netherlands). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications starting with 200 ng total RNA. Per sample 750 ng of cRNA was used to hybridise to the HumanHT-

12 v4 Expression BeadChips (Illumina, Inc., San Diego, CA, U.S.A.). Each BeadChip contains twelve arrays. Hybridisation and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the Illumina iScan (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio; Gene Expression software with default settings (no background substraction) and no normalisation. A total of 142 (53%) breast tumor fresh frozen tissues had good quality mRNA and could be analyzed for gene expression. The Illumina HumanHT-12 Oligo Microarray contains 47,231 50–mer oligonucleotide probes representing 39,809 unique genes and transcripts. Labeling of total RNA was performed according to manufacturer's protocol. Hybridization was performed for 16-20 hours at 58 °C and arrays were scanned on a iScan scanner. Images were analyzed and data were extracted using GenomeStudio Software. Robust spline normalization (RSN) and variance stabilizing transformation (VST) were performed using R/Bioconductor Lumi Package[18].

Immunohistochemistry

Mouse antibodies against ki67 (clone MIB-1, Dako, NL), epidermal growth factor receptor (EGFR) (NCL-EGFR, Novocastra, UK) and CK5/6 (clone D5/16 B4, Dako, NL) were used for immunohistochemistry. Tissue sections of 4 μm were cut from a previously constructed tissue microarray (TMA) of formalin-fixed paraffin-embedded tumors of 714 patients from whom tumor material was available[17]. Immunohistochemical staining was performed according to previously described standard protocols[19]. Human tonsil tissue slides served as positive control. Negative controls were human tonsil tissue slides that did undergo the whole immunohistochemical staining without primary antibodies. Microscopic analysis of Ki67, EGFR and CK5/6 was assessed independently by two observers in a blinded manner. Cut-offs for low versus high expression of Ki67, EGFR and CK5/6 were based on the median expression level and were respectively 0%, 10% and 0% positive stained cells. Immunohistochemical staining and quantification of ER, PR and HER2 are described elsewhere (Representative examples of all stainings are shown in Figure 1A)[17].

Determination molecular subtypes

Gene expression subtyping:

The gene expression subtypes were determined as follows: An "intrinsic" gene list consisting of 534 genes represented by 552 clones, was previously selected based on their low variation in expression in successive samples from the same patient's tumor and at the same time, high degree of variation among tumors from different patients[15]. Hierarchical clustering of data from 122 breast tissue samples usingthese intrinsic genes were used to define five subtypes of breast tumors and five correspondning core expression centroids (i.e., average expression profile of the 534 intrinsic genes). Intrinsic

molecular subtypes were assigned to each sample by computing the correlation to each of the five centroids..

IHC subtyping

The IHC profiles have been previously developed by combinations of the following markers: ER, PR, HER2, Ki67, EGFR and CK5/6[16, 20]. We defined the immunohistochemistry molecular subtypes as follows (Figure 1A): Luminal A: ER+ and/or PR +, HER2- and Ki67-; Luminal B: ER+ and/or PR +, HER2- and/or ki67+; ERBB2: HER2+; Basal-like: ER-, PR-, HER2- and EGFR+ and/or CK5/6+; Unclassified: ER-, PR-, HER2-, EGFR- and CK5/6-.

Statistical analysis

Statistical analysis of gene expression data were performed with the software packages MATLAB (Mathworks, Natick, Ma), R/Bioconductor and Spotfire Functional Genomic (Spotfire, Göteborg, Sweden). Intrinsic genes were mapped to the corresponding genes represented on the Illumina HumanHT-12 Microarray platform. Using these mapped genes, we computed the Pearson's correlation coefficient of each sample from this study to each of the five centroids and assigned each sample to the subtype with which it showed the highest correlation.

Statistical analyses of IHC data 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 was used to assess the inter-observer agreement in quantification of Ki67, EGFR and CK5/6 tumor expression. In addition, to assess a measurement of inter-assay agreement in determination of molecular subtype between gene expression and IHC (in order to validate the IHC subtypes with the gene expression subtypes), Cohen's kappa coefficient was used. The χ^2 test was used to evaluate associations between various clinicopathological parameters and molecular subtypes. Relapse-free period (RFP) was defined as the time from date of surgery until an event (locoregional recurrence and/or a distant recurrence, whichever came first). RFP is reported as cumulative incidence function, after accounting for death as competing risk[21]. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of relapse-free period curves. Cox proportional hazard analysis was used for univariate and multivariable analysis for relapse-free period. Relative survival (RS) 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 (RER) of death were estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times.

Analyses were performed for all patients and stratified for age and systemic treatment. Age of 65 years at time of diagnosis was chosen as the cut-off point for age stratification. Variables with a *P*-value of < .10 in univariate analysis were entered in multivariable analysis.

RESULTS

Patient and tumor characteristics

Figure 1 shows a diagram illustrating the various phases of exclusion or loss of patients in this study. Tumor material was available of 86% (714/822) of the patients. Of these patients, 469 (66%) were < 65 years at diagnosis and 245 (34%) were > 65 years at diagnosis. Median age of patients was 58 years (range 23-96 years). Median follow-up of patients alive was 15 years (range 12-23 years). Clinicopathological and treatment characteristics are shown in Table 1.

IHC expression of ER, PR, HER2, ki67, EGFR and CK5/6 in patient cohort

The Cohen's kappa coefficient for inter-observer agreement of Ki67, EGFR and CK5/6 quantification were 0.71, 0.91 and 0.78 respectively. Immunohistochemical data of ER, PR, HER2, Ki67, EGFR and CK5/6 expression was available for respectively 94% (669/714) and 92% (657/714), 76% (545/714), 78% (556/714), 72% (516/714) and 79% (561/714) of all patients (Figure 2). Missing immunohistochemical data was due to lost TMA cores, insufficient tumor tissue present in the core or tissue damage of tumors. High expression of ER, PR, HER2, Ki67, EGFR and CK5/6, were found in 58% (388/669), 55% (358/657), 10% (56/545), 46% (257/556), 58% (301/516), 24% (134/561).

Validation IHC molecular subtypes with gene expression subtypes

Subtyping with IHC was possible in 99% (140/142) of these tumors (Figure 2). We were not able to subtype the Normal-like breast cancers using IHC, therefore this molecular subtype was excluded in analyses, leaving 117 tumors for which both IHC and gene subtyping was successful. With gene expression subtyping, 44% (51/117), 15% (18/117), 15% (17/117), 15% (17/117) and 12% (14/117), were respectively classified as Luminal A, Luminal B, HER2, Basal-like and Unclassified. A total of 17% (20/117) cases were misclassified and 83% (97/117) of cases were classified correctly (Table 2) with IHC subtyping compared to gene expression subtyping. Cohen's kappa coefficient for inter-assay agreement in molecular subtype classification was 0.75, which can be interpreted as a substantial agreement.

Moleular subtypes distribution in patient cohort

Molecular subtypes could be determined with IHC for 77% (551/714) of all patients. Luminal A, Luminal B, ERBB2, Basal-like an Unclassified molecular subtypes were seen

in 46% (255/551), 21% (118/551), 11% (59/551), 16% (86/551), 6% (33/551) of patients respectively. Associations with known clinicopathological parameters are shown in Table 1. Statistically significant correlations were found between unfavorable tumor characteristics and more Luminal B, ERBBR2 and Basal-like subtypes: more ductal

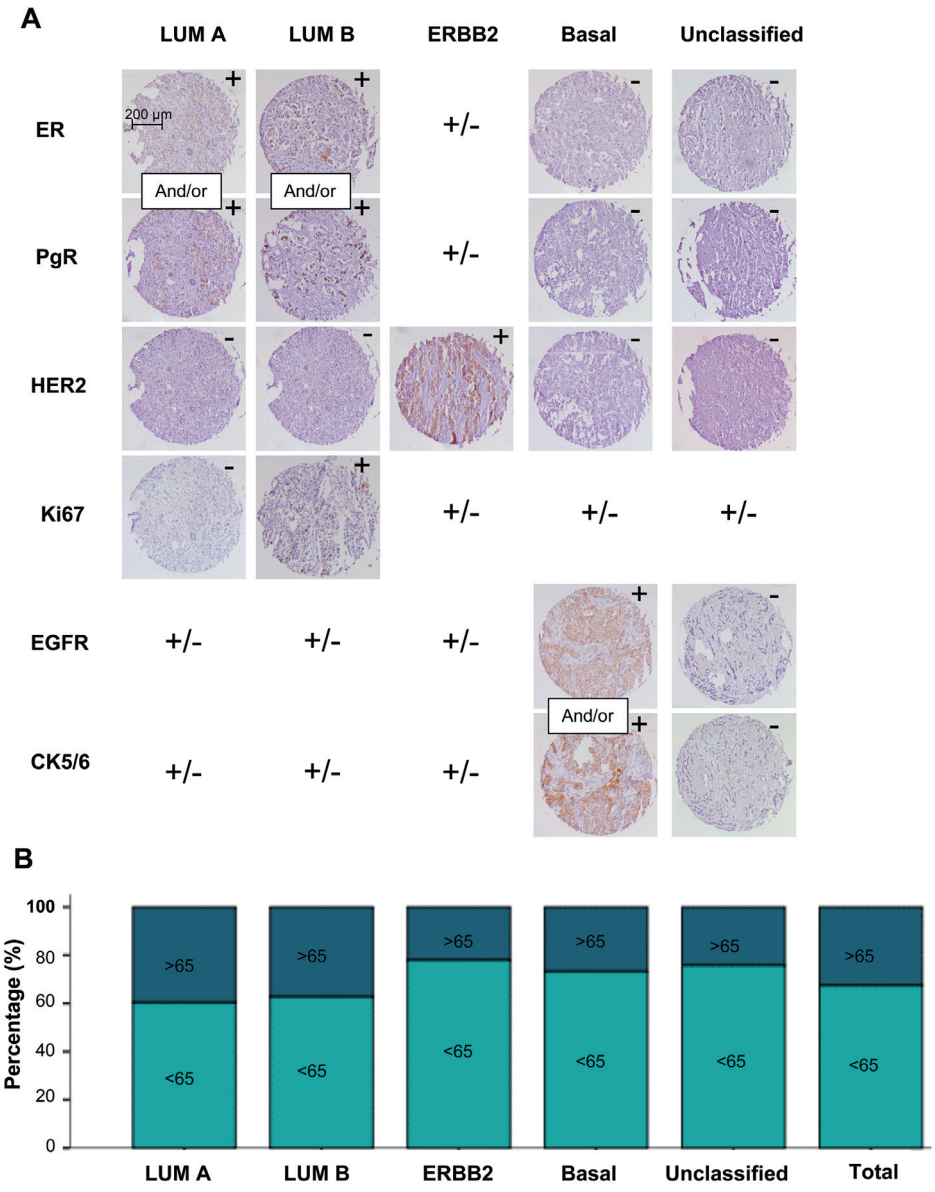

Figure 1 Molecular subtypes immunohistochemical stainings and distribution over age groups
A) Representative photographs of tissue microarray punches of human breast cancer specimens immunohistochemically stained for ER, PR, HER2, ki67, EGFR and CK5/6 and corresponding molecular subtypes. Bar represents 100 µm. **B)** Molecular subtypes according to age (<65 versus >65 years).

	Total		Molecular Subtypes										
			Unclassified		Luminal A		Luminal B		ERBB2		Basal		p-value
	N	%	N	%	N	%	N	%	N	%	N	%	
Age													
<65	361	66	25	76	154	60		63	46	78	63	73	0.02
>=65	189	34	8	24	101	40	74	37	13	22	23	27	
Grade													<0.001
I	116	17	7	23	59	24	9	8	4	7	7	8	
II	342	49	17	55	151	61	51	44	20	34	25	29	
III	224	35	7	23	40	16	57	49	35	59	53	62	
Histological type													0.03
Ductal	638	91	27	87	218	87	109	93	57	97	82	97	
Lobular	66	9	4	13	32	13	8	7	2	4	3	4	
T-status													0.02
T1	289	42	16	50	122	49	38	33	16	28	26	31	
T2	328	47	13	41	104	42	62	54	33	57	49	58	
T3/4	77	11	3	9	23	9	14	12	9	16	10	12	
N-status													0.008
N0	381	55	22	67	146	59	56	50	22	37	40	48	
N1-3	313	45	11	33	101	41	57	50	37	63	44	52	
ER-status													<0.001
Negative	281	42	33	100	45	18	12	10	49	83	86	100	
Positive	388	58	0	0	210	82	106	90	10	17	0	0	
PgR-status													<0.001
Negative	299	46	33	100	58	23	31	26	48	81	86	100	
Positive	358	55	0	0	197	77	87	74	11	19	0	0	
Her2-status													<0.001
Overexpression -	489	90	33	100	255	100	117	100	0	0	86	100	
Overexpression +	56	10	0	0	0	0	0	0	59	100	0	0	
Ki67													<0.001
Negative	299	54	23	74	188	100	0	0	19	39	27	33	
Positive	257	46	8	26	0	0	118	100	30	61	54	67	
CK56													<0.001
Negative	427	76	33	100	156	78	91	82	41	84	38	46	
Positive	134	24	0	0	42	21	20	18	8	16	45	54	
EGFR													<0.001
Negative	215	42	33	100	93	49	28	28	14	33	13	16	
Positive	301	58	0	0	97	51	74	72	29	67	67	84	
Local Therapy													0.3
MAST-RT	285	40	17	52	103	40	49	42	23	39	34	40	
MAST+RT	132	19	5	15	37	15	21	18	18	31	20	23	
BCS-RT	4	1	0	0	1	0	1	1	0	0	0	0	
BCS+RT	293	41	11	33	114	45	47	40	18	31	32	37	
Systemic therapy													0.1
CT alone	127	18	7	21	36	14	23	20	14	24	19	22	
HT alone	113	16	4	12	41	16	20	17	9	15	14	16	
CT&HT	27	4	1	3	6	2	3	3	7	12	7	8	
None	447	63	21	64	172	68	72	61	29	49	46	54	
Total	714	100	33	100	255	100	118	100	59	100	86	100	

Table 1 Correlations between molecular subtypes and well-established prognostic factors using chi-square test (missing data not shown). 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.

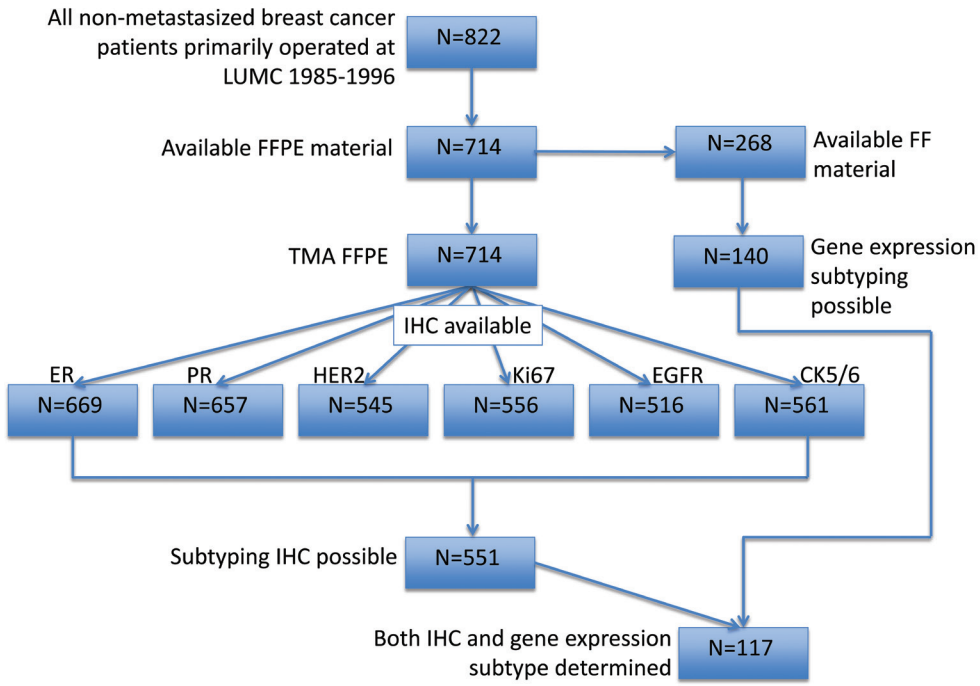

Figure 2 Diagram illustrating patient cohort and various stages of loss of cases due to unavailable tumor material, tumor core or tissue damage of TMA or inadequate mRNA quality as described in the Patients and Methods and Results section.

histological tumor types, higher tumor histological grade, higher tumor stage and more lymph node positivity showed a positive association with more Luminal B, ERBBR2 and Basal-like subtypes. A statistical significant association was found between molecular subtypes and age, where Luminal tumor types were more often found in patients aged >65 years, while ERBBR2, Basal-like an Unclassified molecular subtypes were more often found in patients aged <65 years (p=0.02) (Figure 1B).

Molecular subtypes and age-related prognostic associations with outcome

The association of molecular subtypes with relapse-free period and relative survival are shown in Figure 3 and 4. Analysis of relapse-free period and relative survival showed a significant association molecular subtypes and clinical outcome for the whole population (RFP p=0.003, Figure 3A; RS p<0.001, Figure 4A), where Unclassified tumor subtypes resulted in the most favorable patient outcome, followed by Luminal A subtypes, Luminal B subtypes, Basal-like subtypes and with the worst outcome for patients with ERBBR2 breast cancer subtypes. In patients who did not receive any systemic treatment molecular subtypes showed a similar but weaker prognostic effect (RFP p=0.208, Figure 3D; RS p=0.017, Figure 4D). Explanations to the loss of statistical significance may be due to loss in power due to less patients analyzed and to the fact that patients with Luminal B subtypes showed a worse outcome in patients who did not

		Immunohistochemistry Subtypes					
		Unclassified	Luminal A	Luminal B	ERBB2	Basal	Total
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Gene expression Subtypes	Unclassified	7 (43.8)	5 (7.1)	0 (0)	1 (5.0)	1 (5.3)	14 (12.0)
	Luminal A	1 (6.3)	48 (68.6)	2 (15.4)	0 (0)	0 (0)	51 (43.5)
	Luminal B	1 (6.3)	0 (0)	11 (84.6)	3 (15.0)	3 (15.8)	18 (15.4)
	ERBB2	0 (0)	0 (0)	0 (0)	16 (80)	1 (5.3)	17 (14.5)
	Basal	0 (0)	3 (4.3)	0 (0)	0 (0)	14 (73.7)	17 (14.5)
	Total	9 (100)	56 (100)	13 (100)	20 (100)	19 (100)	117 (100)

Table 2 Correlation between immunohistochemistry and gene expression molecular subtype classification. Abbreviations N number of patients; % percentage;

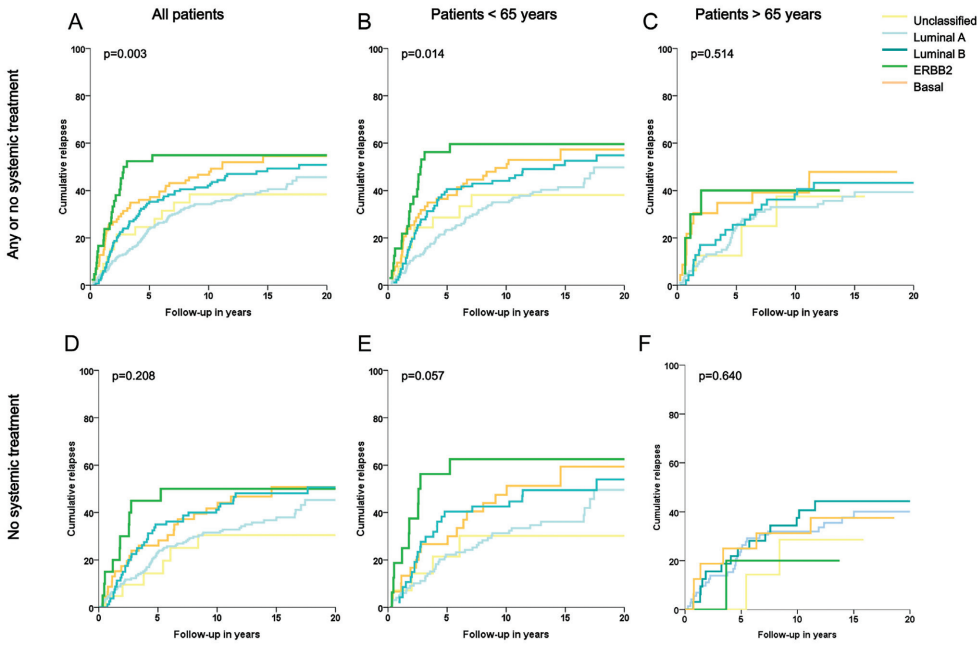

Figure 3 Relapse free period according to molecular subtypes for all patients (A, D), for patients aged < 65 years (B, E) and for patients aged > 65 years (C, F), with no stratification for systemic treatment (A, B, C) and on selected patient population that did not receive any systemic treatment (D, E, F). Log-rank P-values are shown in each graph.

receive systemic treatment, explainable by the fact that these tumors may benefit more from chemotherapy treatment than other tumor subtypes due to the high proliferative tumor character.

Similarly, in the group of patients aged <65 years, a strong association was found between molecular subtypes and clinical outcome in all patients aged <65 (RFP p=0.014, Figure 3B; RS p<0.001 Figure 4B) and patients aged <65 who did not receive any systemic treatment (RFP p= 0.057, Figure 3E; RS p=0.003, Figure 4E). In patients aged >65 years, no significant association was found between molecular subtypes and clinical

A RFP

Characteristic	Patients < 65 years						
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Grade							
I	74	1.00		0.03	1.00		
II	225	1.32	0.87-2.02		1.13	0.67-1.71	0.7
III	164	1.72	1.12-2.66		1.27	0.73-2.22	
Histological type							
Ductal	429	1.00		0.3			
Other	36	1.31	0.82-2.10				
Tumor stage							
pT1	203	1.00		<0.001	1.00		
pT2	210	1.53	1.14-2.05		1.22	0.84-1.77	0.1
pT3/4	44	2.65	1.74-4.04		1.74	1.03-2.96	
Nodal stage							
Negative	249	1.00		<0.001	1.00		<0.001
Positive	213	2.85	2.16-3.76		2.41	1.70-3.40	
Mol subtypes							
Unclassified	25	1.00		0.01	1.00		
Luminal A	154	1.02	0.51-2.06		1.14	0.52-2.50	
Luminal B	74	1.31	0.63-2.72		1.18	0.51-2.72	0.5
ERBB2	46	2.10	0.99-4.46		1.70	0.72-4.03	
Basal	63	1.61	0.77-3.37		1.32	0.57-3.05	

B RS

	N	RER	95% CI	P	RER	95% CI	P
Grade							
I	74	1.00		0.008	1.00		
II	225	1.65	0.94-2.90		1.15	0.60-2.21	0.8
III	164	2.28	1.30-4.02		1.22	0.62-2.40	
Histological type							
Ductal	429	1.00		0.2			
Other	36	1.44	0.86-2.42				
Tumor stage							
pT1	203	1.00		<0.001	1.00		
pT2	210	2.05	1.47-2.88		1.23	0.78-1.93	0.3
pT3/4	44	3.20	2.01-5.08		1.67	0.90-3.12	
Nodal stage							
Negative	249	1.00		<0.001	1.00		<0.001
Positive	213	3.47	2.51-4.80		2.38	1.57-3.60	
Mol subtypes							
Unclassified	25	1.00		<0.001	1.00		
Luminal A	154	0.92	0.39-2.16		1.19	0.44-3.26	0.02
Luminal B	74	1.49	0.62-3.59		1.60	0.56-4.51	
ERBB2	46	3.00	1.24-7.23		2.82	0.99-8.03	
Basal	63	1.97	0.82-4.75		2.02	0.71-5.69	

Table 3 Cox univariate and multivariate analysis for recurrence free period (A) and relative survival (B) for molecular subtypes. Abbreviations N number of patients; HR hazard ratio; RER relative excess risk; 95%CI 95% Confidence Interval;. * NA not applicable; too few patients in life table.

	Patients > 65 years					
	Univariate analysis			Multivariable analysis		
N	HR	95% CI	P	HR	95% CI	P
42	1.00		<0.001	1.00		0.01
117	1.84	0.89-3.80		1.48	0.67-3.26	
80	3.72	1.81-7.64		2.72	1.21-6.12	
209	1.00		0.5			
30	1.23	0.69-2.22				
86	1.00		0.001	1.00		0.3
118	2.34	1.43-3.83		1.28	0.73-2.23	
33	2.68	1.35-5.32		1.83	0.86-3.86	
132	1.00		<0.001	1.00		<0.001
100	3.18	2.06-4.89		2.60	1.64-4.12	
8	1.00		0.5			
101	1.01	0.31-3.28				
44	1.14	0.34-3.87				
13	1.82	0.45-7.27				
23	1.62	0.45-5.81				
N	RER	95% CI	P	RER	95% CI	P
42	1.00		0.02	1.00		0.1
117	4.73	0.22-100.74		8.15	0.05-1309	
80	10.93	0.55-218.77		16.88	0.10-2787	
209	1.00		0.8			
30	1.12	0.46-2.72				
86	1.00		0.02	1.00		0.06
118	5.38	1.38-20.99		1.72	0.62-4.78	
33	13.17	3.25-53.41		3.45	1.18-10.07	
132	1.00		0.005	1.00		0.1
100	3.75	1.50-9.42		1.69	0.85-3.34	
8	1.00		0.07	1.00		0.1
101	0.63	0.12-3.17		1.98	0.18-21.25	
44	0.39	0.05-3.05		1.00	0.10-10.43	
13	2.16	0.39-12.12		2.85	0.25-32.41	
23	1.40	0.26-7.66		1.69	0.16-17.79	

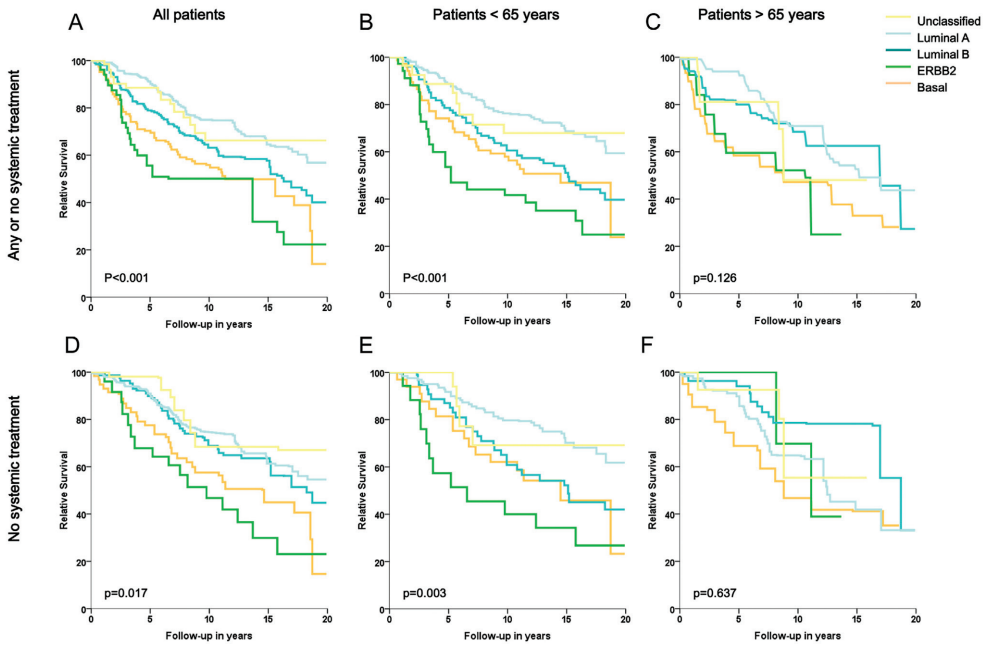

Figure 4 Relative survival according to molecular subtypes for all patients (A, D), for patients aged < 65 years (B, E) and for patients aged > 65 years (C, F), with no stratification for systemic treatment (A, B, C) and on selected patient population that did not receive any systemic treatment (D, E, F). Log-rank P-values are shown in each graph.

outcome in all patients aged >65 (RFP p=0.514, Figure 3C; RS p=0.126, Figure 4C) and neither in patients aged >65 who did not receive any systemic treatment (RFP p=0.640, Figure 3F; RS p=0.637, Figure 4F).

Univariate analyses were performed for molecular subtypes and known clinicopathological parameters: histological tumor grade, histological tumor type, tumor stage, lymph node status (due to their inclusion in molecular subtypes ER, PR, HER2, Ki67, EGFR and CK5/6 expression were not separately analyzed in univariate analysis). Multivariable analyses were performed including variables which had shown to be of influence on patient outcome (univariate p<0.1) on patients who did not receive any systemic treatment and were stratified for age (<65 versus >65 years). In patients aged <65 years, histological grade, tumor stage, lymph node status and molecular subtypes were included in multivariate analysis for RFP and RS. The prognostic effect of molecular subtypes got weaker in both analyses; it remained statistically significant for RS analysis (p=0.02 Table 3B), but did not reach statistical significance in RFP analysis (p=0.5 Table 3A), probably due to their strong associations with tumor histological grade, tumor stage and lymph node status (Table 1). In patients aged >65 years, molecular subtypes did not reach the criteria to be included in multivariable analysis for RFP (Univariate p=0.9, Table 3A) and lost statistical significance when included in multivariable RS analysis (Univariate p=0.7, Table 3B).

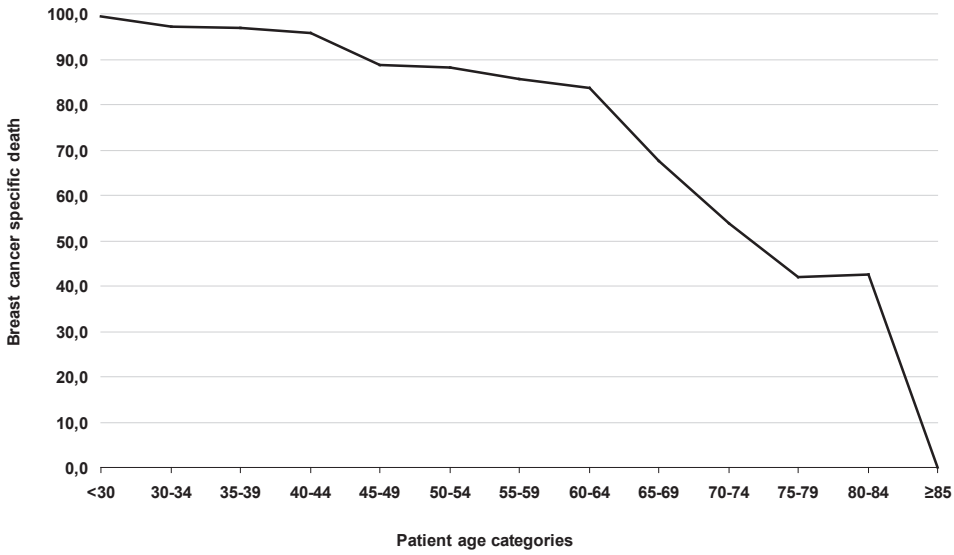

Figure 5 Breast cancer specific death per age category calculated by the percentage of observed death (O%) minus the expected death based on the general population according to age and time period (E%) divided by the total observed death (O%) per age category for the cohort breast cancer patients used in this study. Death cancer specific death calculation: (O%-E%/E%).

DISCUSSION

In this study, we used IHC surrogates, which we validated against gene expression determined molecular subtypes, to identify breast tumor molecular subtypes in a large cohort of breast cancer patients. We demonstrated that the distribution of molecular subtypes between elderly and young patients was statistically significantly different, where elderly patients more frequently had less aggressive Luminal A and Luminal B tumor subtypes. Moreover, both RFP and RS outcome analyses showed molecular subtypes to be a statistically significant prognostic factor in young, but not in elderly breast cancer patients.

We have shown that the distribution of molecular subtypes differed between elderly and young breast cancer patients, where we defined elderly breast cancer patients as patients aged 65 years or older according to World Health Organization definition (www.who.int). With this cut-off point, elderly breast cancer patients showed more often Luminal A and Luminal B molecular subtypes less often ERBB2, basal and unclassified molecular subtypes. This is in line with a previous study by Perou et al., who investigated the associations of molecular subtypes with patient clinical data, demographic data and survival[16]. Though they did not specifically look at elderly breast cancer patients, in this study on almost 500 breast cancer patients, molecular subtypes as assessed by immunohistochemstry, were statistically significantly associated with age,

where Luminal A and Luminal B tumor were more often found in older aged patients[16]. In addition our data are also concordant to previous studies that showed more ER and/or PR positivity and less overexpression of EGFR, HER2 and ki67 in tumors of elderly breast cancer patients[22, 23].

In addition to differing distributions in molecular subtypes, we found a different prognostic effect for molecular subtypes in elderly breast cancer patients compared to their younger counterparts. In the period analyzed adjuvant systemic treatment changed, where not all hormone receptor positive patients received adjuvant endocrine therapy and trastuzumab was not yet introduced. In addition, breast cancer patients received different adjuvant therapy according to their age, where elderly received less aggressive treatment regimens. Considering these differences in adjuvant therapy regimens between analyzed patients and in order to analyze a true prognostic effect, we stratified our analyses and selected patients who did not receive any adjuvant treatment, hereby filtering out any predictive adjuvant therapy effect. In the whole breast cancer cohort and in young breast cancer patients molecular subtypes showed to be statistically significant prognostic factors for RFP and RS. These prognostic effects weakened in multivariable analyses, however this could well be explained by correction for tumor histological grade, tumor stage and lymph node status and the strong associations of molecular subtypes with these unfavorable tumor characteristics. Importantly, molecular subtypes did not show any statistically significant effect on patient outcome in elderly breast cancer patients in this study. Further underlying differences in tumor biology might explain this fading prognostic effect. These underlying biological differences may result in the molecular subtypes to behave differently and have a different effect on tumor progression in elderly breast cancer patients, which may be reflected in a differing prognostic effect of the same molecular subtype in elderly compared to young patients. Indeed, as shown priory by others, elderly breast cancer tumors are of a more indolent and less aggressive and proliferative character (Eppenberger-Castori *et al.*, 2002; Nixon *et al.*, 1994; Thomas and Leonard, 2009). However, this contradicts the fact that increased breast cancer specific mortality is seen with ageing, where elderly breast cancer patients were found to decease more often due to breast cancer regardless of a higher risk of mortality from other causes [24]. Joining these paradoxal findings together, an explanation might be sought in differences in the tumor microenvironment in elderly breast cancer patients compared to young breast cancer patients. With increasing age, there appears to be a progressive accumulation of cellular and molecular alterations leading to tissue dysfunction [25, 26]. This may apply to the tumor micro-environment, thereby facilitating tumor progression. Evidence has shown that an age-related decline of functional innate and adaptive immunity leads to a reduced ability to respond to infection and vaccinations [27]. This phenomenon, known as immunosenescence, is characterized by a decreased output of naïve T cells, altered cytokine production and inoptimale functioning of T cells, B cells and NK cells [27-30]. There has been increasing

evidence that immunosenescence might promote cancer progression in elderly breast cancer patients, which would explain the worse breast cancer specific outcome of these patients [31]. If tumors become less aggressive with increasing age, but this is simmulataneously accompanied by an even faster deteriorating host defence, i.e. tumor micro-environment, this altogether can result in more tumor progression and finally lead to worse patient outcome.

Another explanation for the finding that molecular subtypes are not statistically significant prognostic indicators in elderly breast cancer may be the competing risks of death in elderly patients. Elderly breast cancer patients compared to their younger counterparts have shown in absolute sense to develop more relapses [24], however proportionally due to higher risk of dying earlier and from other causes they show less breast cancer relapses and breast cancer specific deaths [10-12]. In fact, as shown by Figure 5 the approximated breast cancer specific decease declines as the patients age increases. Only about 60% of elderly breast cancer patients die as a consequence of breast cancer, compared to almost 100% of young patients. This has major implications on the impact and value of prognostic biomarkers in elderly breast cancer patients. Prognostic biomarkers, identifying patients with low versus high risk of breast cancer progression and breast cancer related death will show limited to no prognostic effect in the 40% of elderly patients which have a short-term prognosis due to breast cancer un-related causes, especially in those who are considered frail. These elderly patients are also unlikely to benefit from systemic treatment, since their cause of death will be other than due to breast cancer. Therefore, the clinical value of prognostic biomarkers, which aid at distinguishing between patients who might and might not benefit from systemic treatment, is also limited in this patient population. Breast cancer prognostic biomarkers can only have a prognostic value in elderly patients whose life expectation will be long enough for the cancer to progress and cause patient death, which are the patients reflected by the 60% of elderly breast cancer patients dying as a consequence of breast cancer. It is only in these fit enough patients that prognostic biomarkers may show differences in outcome between elderly breast cancer patients and may aid clinical decision making on systemic treatment. In order to improve tailored treatment in elderly with the aid of prognostic biomarkers, the first step would therefore be to identify these fit elderly patients.

The identification of breast cancer molecular subtypes has proven breast cancer to be a heterogeneous group of diseases, needing different approaches to systemic treatment administration. This molecular taxonomy and its impact on patient clinical outcome have been extensively investigated in breast cancer. However, as is the case for most translational studies and randomized clinical trials, these studies included relatively young patients. To the best of our knowledge, we are the first to have shown that molecular subtypes have a different distribution and prognostic effect in elderly compared to young breast cancer patients, highlighting the fact that the prognostic effect

and clinical value as found for biomarkers in translational studies and randomized trials, cannot simply be extrapolated to elderly breast cancer patients. Our results support the premise that breast cancer clinical behavior is significantly affected by patient age. We suggest that competing risks of death in elderly patients, ER-driven differences and micro-environmental changes in biology are underlying these age-dependent variations in patient prognosis.

REFERENCES

1. Petrakis IE, Paraskakis S. Breast cancer in the elderly. Arch Gerontol Geriatr 2010;50(2):179-184.
2. Anderson WF, Jatoi I, Sherman ME. Qualitative age interactions in breast cancer studies: mind the gap. J Clin Oncol 2009;27(32):5308-5311.
3. Beadle BM, Woodward WA, Buchholz TA. The impact of age on outcome in early-stage breast cancer. Semin Radiat Oncol 2011;21(1):26-34.
4. Early breast cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365(9472):1687-1717.
5. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007;18(7):1133-1144.
6. Daidone MG, Coradini D, Martelli G, Veneroni S. Primary breast cancer in elderly women: biological profile and relation with clinical outcome. Crit Rev Oncol Hematol 2003;45(3):313-325.
7. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. J Natl Cancer Inst 2000;92(7):550-556.
8. Remvikos Y, Magdelenat H, Dutrillaux B. Genetic evolution of breast cancers. III: Age-dependent variations in the correlations between biological indicators of prognosis. Breast Cancer Res Treat 1995;34(1):25-33.
9. Thomas GA, Leonard RC. How age affects the biology of breast cancer. Clin Oncol (R Coll Radiol) 2009;21(2):81-85.
10. Wildiers H, Kunkler I, Biganzoli L et al. Management of breast cancer in elderly individuals: recommendations of the International Society of Geriatric Oncology. Lancet Oncol 2007;8(12):1101-1115.
11. Bastiaannet E, Liefers GJ, de Craen AJ et al. Breast cancer in elderly compared to younger patients in The Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. Breast Cancer Res Treat 2010;124(3):801-807.
12. Bastiaannet E, Portielje JE, van d, V et al. Lack of survival gain for elderly women with breast cancer. Oncologist 2011;16(4):415-423.
13. Perou CM, Sorlie T, Eisen MB et al. Molecular portraits of human breast tumours. Nature 2000;406(6797):747-752.
14. Sorlie T, Perou CM, Tibshirani R et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-10874.
15. Sorlie T, Tibshirani R, Parker J et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003;100(14):8418-8423.
16. Carey LA, Perou CM, Livasy CA et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006;295(21):2492-2502.
17. van Nes JG, de Kruijf EM, Faratian D et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res Treat 2010.
18. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics 2008;24(13):1547-1548.
19. de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res 2010;16(4):1272-1280.
20. Nielsen TO, Hsu FD, Jensen K et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 2004;10(16):5367-5374.
21. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 2007;26(11):2389-2430.
22. Eppenberger-Castori S, Moore DH, Jr., Thor AD et al. Age-associated biomarker profiles of human breast cancer. Int J Biochem Cell Biol 2002;34(11):1318-1330.
23. Nixon AJ, Neuberg D, Hayes DF et al. Relationship of patient age to pathologic features of the tumor and prognosis for patients with stage I or II breast cancer. J Clin Oncol 1994;12(5):888-894.
24. van de WW, Markopoulos C, van d, V et al. Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. JAMA 2012;307(6):590-597.
25. Teschendorff AE, Menon U, Gentry-Maharaj A et al. Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. Genome Res 2010;20(4):440-446.
26. Naumova E, Ivanova M, Pawelec G. Immunogenetics of ageing. Int J Immunogenet 2011;38(5):373-381.
27. Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. Immunology 2007;120(4):435-446.
28. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev 2005;205:257-268.

29. Campos C, Pera A, Sanchez-Correa B et al. Effect of age and CMV on NK cell subpopulations. Exp Gerontol 2014.
30. Gayoso I, Sanchez-Correa B, Campos C et al. Immunosenescence of human natural killer cells. J Innate Immun 2011;3(4):337-343.
31. Fulop T, Kotb R, Fortin CF, Pawelec G, de AF, Larbi A. Potential role of immunosenescence in cancer development. Ann N Y Acad Sci 2010;1197:158-165.

Chapter 8

Comparison of distribution and prognostic effect of adaptive tumor immune subtypes between young and elderly breast cancer patients

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Submitted

ABSTRACT

Purpose: As demonstrated recently, tumor immune subtypes, representative for various tumor immune control and host immune escape phases, are a strong prognostic factor for breast cancer outcome. With ageing, immunosenescence occurs, which might impair tumor immune surveillance.

Experimental Design: All non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1996 were included (n=714). Tumor immune subtypes were previously categorized in groups of increasing immune susceptibility using quantifications of immunohistochemically stained tumor infiltration of CD8+ cells, natural killer cells, T regulatory cells, and tumor expression of HLA class I and HLA EG. Associations between immune markers and age at diagnosis (<65 versus >=65 years) and outcome analyses according to age were performed.

Results: A statistically significant association was found between less HLA-EG upregulation and patients aged >=65 years ($p=0.015$). In addition, though not significant, less low immune susceptible tumors were seen in these older patients. In patients aged <65 tumor, higher immune susceptibility resulted in statistically significant favorable patient outcome independently of known clinicopathological parameters (RFP $p<0.001$, RS $p<0.001$). In patients aged ≥65, immune subtypes showed no statistically significant association with outcome.

Conclusions: Less immune susceptible tumors were found in elderly breast cancer patients, supporting the idea of immunosenescence potential role in cancer progression. In addition, contrary to the results found in patients aged <65 years, no statistically significant association was found between tumor immune subtypes and patient outcome in patients aged >65 years. A better understanding of processes of immunosenescence and tumor progression and future possibilities in immune manipulations and vaccinations might lead to more tailored treatment of elderly breast cancer patients.

INTRODUCTION

Breast cancer is the leading contributor to cancer incidence and cancer mortality in women worldwide, with 1.383.500 new cases in 2008 (1). Nearly one third of these breast cancer patients are 65 years or older (2). As breast cancer incidence increases with increasing age, changing demographics and continuously increasing life expectancy will further enlarge the number of elderly women confronted with breast cancer. A recent report observed that regardless of a higher risk of mortality from other causes and independent of known tumor and patient characteristics, mortality from breast cancer increased with age (3) Cancer immune surveillance and immunosenescence at increasing age, may contribute to an explanation of this finding.

There has been strong evidence that the host's immune system is able to control tumor progression (4). On the other hand, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from such immune recognition (5). Various interactions underlie this balance between tumor immune control and escape (Figure 1A). Cytotoxic T-lymphocytes (CTL) are capable of recognising 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 (6). However, this makes them prone to natural killer (NK) cell recognition (7). 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 (7-9). Another known tumor escape mechanism is the attraction and induction of immune suppressive regulatory T cells (Treg) in the tumor microenvironment (10). There is evidence for a variety of these immune reactions in breast cancer, where it has been shown that breast cancer is highly immunogenic (11, 12), but also capable of evading immune recognition. (13, 13-21) This emphasizes the importance of taking into account the various interactions which exist between the tumor and the immune system. We recently defined tumor immune subtypes, based on the above mentioned immunological interactions, which were shown to be a highly discriminative prognostic biomarker with solid underlying biological rationale (22).

With increasing age, there appears to be a progressive accumulation of cellular and molecular alterations leading to tissue dysfunction. This equally applies to the immune system, where the age-related decline of functional innate and adaptive immunity leads to a reduced ability to respond to infection, vaccinations or cancer (23, 24). Though the exact mechanisms of immunosenescence are not fully understood, various phenomena may be explanatory for the decline in functioning of the immune system with age. With increasing age thymic involution leads a decreased output of naïve T cells, which subsequently leads to a reduction of peripheral T cell diversity, changes in

phenotype, altered cytokine production, modification in immune responses (25, 26). A decline in production of immune cells with increasing age is seen caused by changes in bone marrow constitution and decline in function of haematopoietic stem cells (27, 28). Another contributor to immunosenescence are the deficiencies in functioning of secondary lymphoid organs, causing less migration of immune cells to the spleen and therefore less antigenic stimulation (29). Both the innate and adaptive immune system appear to be affected by immunosenescence, where amongst many others deficiencies in numbers and inoptimal functioning of CD4+ T cells, CTL, B cells and NK cells are found (24, 30-32).

There has been increasing evidence that age associated immunosenescence might contribute to cancer development and progression (33). This raised the question whether age at diagnosis affects the interplay between the balance in cancer immune surveillance and tumor immune escape and consequently its effects on tumor progression and patient outcome in breast cancer patients. We priory determined tumor immune subtypes, which reflect tumor-immune interactions and represent a strong, validated, independent prognostic factor in breast cancer patients (22). We evaluated the distribution and prognostic effect of these tumor immune subtypes in elderly patients versus their younger counterparts.

MATERIALS AND METHODS

Patients and tumors

The patient population has been priory described in detail (22) and comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1996. 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, locoregional/distant tumor recurrence, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR), Ki67, and human epidermal growth factor receptor 2 (HER2) (34). All tumors were graded according to current pathological standards, by an experienced breast cancer pathologist. Approval 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).

Immunohistochemistry and quantification of immunostaining

Immunohistochemistry and quantifications of immune markers used to construct tumor immune subtypes were previously performed as previously described in detail (13, 17,

35). Formalin-fixed paraffin-embedded tumor material was immunohistochemically stained according to standard protocols. Mouse antibody against CD8, PEN5 and Foxp3 were used for recognition of respectively CTL, NK cell and Treg infiltration (13, 35). Stainings for classical HLA class I were performed using the mouse monoclonal antibodies HCA2 and HC10 (13). Non-classical HLA class I molecules using mouse monoclonal antibodies against HLA-E and HLA-G (17).
Expression of classical HLA class I, combined HLA-E and –G expression, CTL infiltration, PEN5 infiltration and Treg infiltration were categorized respectively as loss versus expression, no expression versus expression, high versus low infiltration and absent infiltration versus present infiltration (35).

Tumor immune subtypes

Categorization of tumor immune subtypes, representing adaptive immune susceptibility of tumors was previously described (35). Briefly, different stages of immune surveillance and tumor immune escape were classified using combinations of CTL infiltration, NK-cell infiltration, Treg infiltration, classical HLA class I tumor expression and HLA-EG tumor expression. Tumors were first classified according to their immune susceptiblity resulting in the tumor immune subtypes which consisted of three clustered groups: "High immune susceptibility", "Intermediate immune susceptibility" and "Low immune susceptibility".

Statistical analysis

Statistical analyses were performed using the statistical packages SPSS (version 20.0 for Windows, Spps Inc, Chicago, IL, USA) and Stata (version 10.0 for Windows, StataCorp, College Station, TX, USA). The χ^2 test was used to evaluate associations between various clinicopathological parameters and tumor immune subtypes. Relapse-free period was defined as the time from date of surgery until an event (locoregional recurrence and/or a distant recurrence, whichever came first). Relapse-free period is 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 relapse-free period curves. Cox proportional hazard analysis was used for 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. Analyses were stratified by age at diagnosis (<65 years versus ≥65 years). Multivariable analyses were adjusted for histological grade, histological type, T stage, N stage, estrogen receptor expression, progesterone receptor expression, HER2 status, local therapy and systemic therapy.

RESULTS

Patient and tumor characteristics

Of the total patient population (n=714), 469 (66%) were < 65 years at diagnosis and 245 (34%) were ≥ 65 years at diagnosis. Median age of patients was 58 years (range= 23-96 years). Clinicopathological and treatment characteristics of patients with available data for analysis are shown in Table 1. More detailed patient and tumor characteristics are described elsewhere (35).

Expression of immune markers by age

As priory described, immunohistochemical data of CTL infiltration, NK cell infiltration, Treg infiltration, classical HLA class I expression and HLA-EG expression were available for respectively 85% (607/714) and 91% (650/714), 95% (679/714), 83% (594/714) and 73% (519/714). Missing immunohistochemical data was due to tissue damage and unsuccessful staining of tumors. Cohen's kappa coefficient for inter-observer agreement of all these markers was determined previously and gave substantial to almost perfect agreements (13, 17, 35). The association between these markers and age is shown in Figure 1. Only HLA-EG expression showed a statistically significant inverse correlation with age (p=0.015), where expression of HLA-EG was more frequently found in patients aged <65 (27%) compared to patients aged ≥65 (17%). No statistically significant associations were found between patients aged ≥65 and patients aged <65 in frequency of high infiltration of CTL (31% versus 27%; p=0.253), present infiltration of PEN5 (51% versus 53%; p=0.599), present infiltration of Treg (45% versus 45%; p=0.991) or expression of classical HLA class I (77% versus 80%; p=0.282).

Tumor immune subtypes distribution by age

Tumor immune subtypes could be determined for patients with data available for all immune markers; 72% (512/714) of patients. Tumor immune subtypes showed the following distribution: "High immune susceptibility" 16% of patients (82/512), "Intermediate immune susceptibility" 67% (342/512), "Low immune susceptibility" 17% (88/512). Associations with known clinicopathological parameters are shown in Table 1. No statistically significant association was found between tumor immune subtypes and age (<65 versus ≥65; p=0.381), though low immune susceptible tumors were shown to occur more often in patients aged <65 (Figure 2).

Tumor immune subtypes and prognostic associations with outcome

The age-specific association of tumor immune subtypes with relapse-free period and relative survival are shown in Figure 3. In the group of patients aged <65 years, a strong association was found between immune subtypes and clinical outcome. Lower immune susceptibility, resulted in more relapses over time compared to higher immune susceptible tumors (RFP p<0.001, Figure 3 B; RS p< 0.001 Figure 3 E). Though a

similar trend was noticed in patients aged >=65 years, this was not statistically significant (RFP p= 0.147, Figure 3C; RS p=0.45 Figure 3F) Multivariable analyses were stratified for age and demonstrated that immune subtypes remained a statistically significant

	Total population analyzed		Immune susceptibility						
			High		Intermediate		Low		p-value
	N	%	N	%	N	%	N	%	
Age	341	67			222	65	64	73	0.381
<65	171	33	55	67	120	35	24	37	
>=65			27	33					
Grade									0.306
I	78	15	16	20	45	13	17	20	
II	246	49	33	41	173	51	40	47	
III	182	36	31	39	122	36	29	34	
Histological type									0.255
Ductal	460	91	71	89	314	92	75	87	
Lobular	46	9	9	11	26	8	11	13	
T-status									0.829
T1	192	38	31	38	131	39	30	36	
T2	242	48	42	52	159	48	41	49	
T3/4	66	13	8	10	45	13	13	16	
N-status									0.316
N0	267	54	48	60	178	54	41	48	
N1-3	231	46	32	40	155	46	44	52	
ER-status									0.093
Negative	205	40	39	49	138	41	28	32	
Positive	302	60	41	51	202	59	59	68	
PgR-status									0.460
Negative	231	46	41	51	155	46	35	42	
Positive	274	54	39	49	186	55	49	58	
Her2-status									0.352
Overexpression -	390	90	58	89	260	89	72	95	
Overexpression +	42	10	7	11	31	11	4	5	
Local Therapy									0.905
MAST-RT	207	40	34	42	139	41	34	39	
MAST+RT	99	19	17	21	62	18	20	23	
BCS	206	41	31	38	141	41	34	39	
Systemic therapy									0.622
CT alone	100	20	15	18	68	20	17	19	
ET alone	76	15	13	16	52	15	11	13	
CT&ET	21	4	1	1	18	5	2	2	
None	315	62	53	65	204	60	58	66	
Total	512	100	82	100	342	100	88	100	

Table 1 Correlations between molecular subtypes and well-established prognostic factors using chi-square test (missing data not shown).

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.

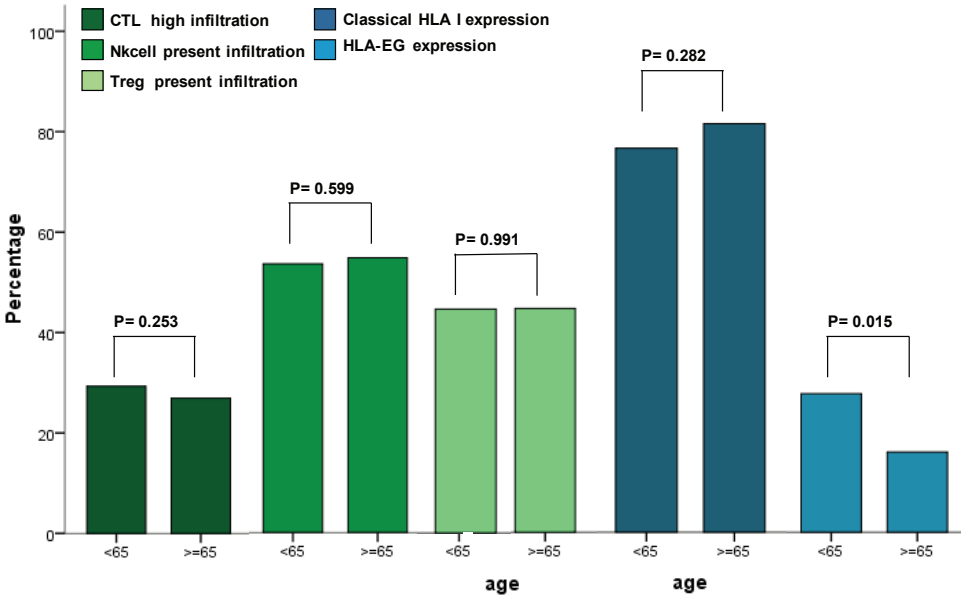

Figure 1 CTL, NK cell and Treg infiltration and classical HLA class I and HLA-E and HLA-G expression Histograms depicting distributions of high infiltration and high expression of these markers amongst age groups <65 and >=65 years are shown. Statistically significant less HLA-EG expression was found in patients aged >=65 years old.

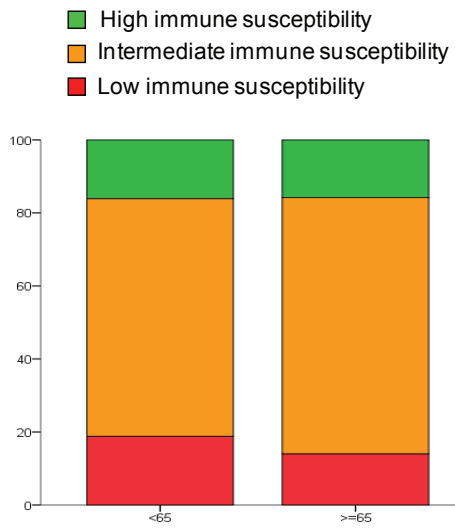

Figure 2 Tumor immune subtypes Bar chart depicting the distribution of immune subtypes according to patients aged <65 years and >=65 years. No statistically significant differences were found.

independent prognostic factor in young patients (RFP p<0.001, Table 2; RS p<0.001, Table 3). In patients aged ≥65 years, no statistical association was found in multivariable analyses between immune subtypes and clinical outcome (RFP p=0.15, Table 2; RS p= 0.45, Table 3).

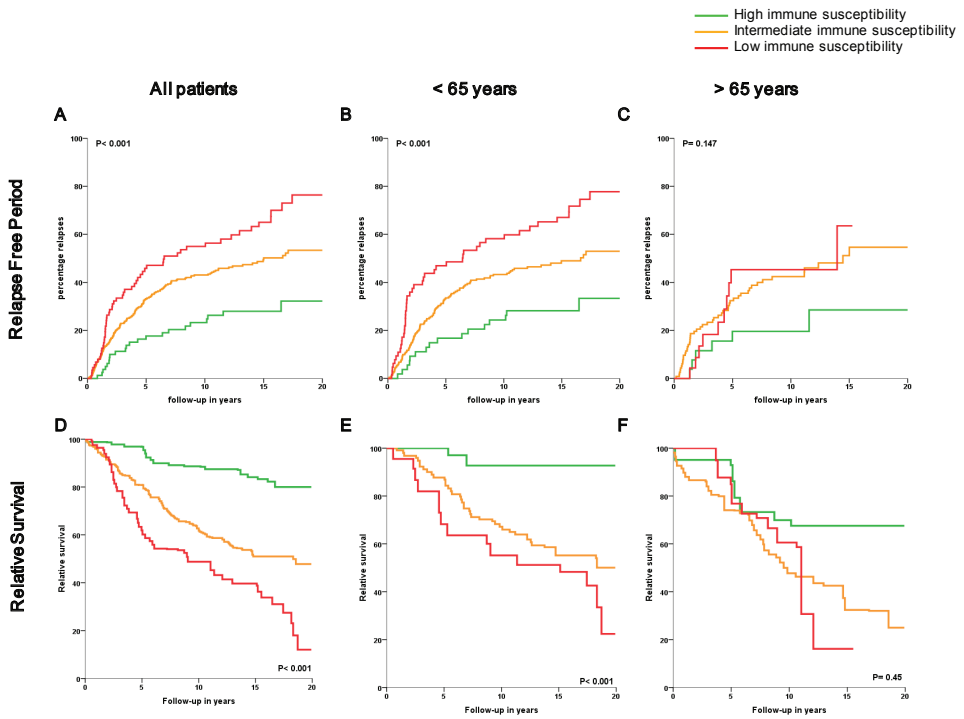

Figure 3 Kaplan Meier outcome analyses by tumor immune subtypes for Relapse free period (RFP) (A, B, C) and relative survival (RS) (D, E, F) according to the tumor immune subtypes. Tumor immune subtypes representing low immune susceptible resulted in a statistically significant unfavourable patient outcome concerning RFP and RS in patients aged <65 years. No statistically significant differences in outcome were seen in patients aged >=65 years. Log-rank P-values are shown in each graph.

DISCUSSION

In this study, we evaluated the distribution and impact on tumor progression and patient outcome of anti-tumor immune response and tumor immune evasion in elderly breast cancer patients compared to their younger counterparts. We compared previously determined numbers of infiltrating CTL, NK cells, Tregs, expression of classical HLA class I and HLA-E and –G and tumor immune subtypes, representing cancer immune susceptibility, between these two patient populations. Our results showed no differences in number of infiltrating CTL, NK cells or Treg, but a trend towards less classical HLA class I downregulation and statistically significant less HLA-E expression or HLA-G upregulation of tumors. These differences were also reflected, though not statistically significant, in less "low immune susceptible" tumors in patients aged >=65. Moreover, both RFP and RS outcome analyses showed tumor immune subtypes to be a statistically significant prognostic factor in young, but not in elderly breast cancer patients.

Characteristic		Patients < 65 years					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Grade							
I	74	1.00		0.03	1.00		0.40
II	225	1.32	0.86-2.02		1.24	0.66-2.34	
III	164	1.72	1.12-2.66		1.50	0.79-2.86	
Histological type							
Ductal	429	1.00		0.27	1.00		0.35
Other	36	1.31	0.82-2.10		1.37	0.71-2.63	
Tumor stage							
pT1	203	1.00		<0.001	1.00		0.13
pT2	210	1.53	1.14-2.05		1.38	0.89-2.12	
pT3/4	44	2.65	1.74-4.04		1.94	1.00-3.78	
Nodal stage							
Negative	249	1.00		<0.001	1.00		<0.001
Positive	213	2.85	2.16-3.76		3.46	2.18-5.50	
ER status							
Negative	216	1.00		0.61	1.00		0.44
Positive	236	0.93	0.71-1.22		0.85	0.56-1.29	
PgR status							
Negative	217	1.00		0.61	1.00		0.99
Positive	222	0.93	0.71-1.22		1.00	0.67-1.50	
HER2 status							
Negative	333	1.00		0.005	1.00		0.52
Positive	46	1.80	1.19-2.72		1.20	0.69-2.07	
Local Therapy							
MAST-RT	138	1.00		<0.001	1.00		0.56
MAST+RT	91	1.97	1.38-2.81		1.08	0.62-1.89	
BCS	24	0.78	0.56-1.07		0.84	0.54-1.29	
Systemic Therapy							
CT alone	120	1.00		0.037	1.00		0.003
ET alone	47	1.42	0.90-2.23		1.60	0.88-2.89	
CT&ET	25	0.74	0.38-1.44		0.37	0.14-0.98	
None	277	0.79	0.58-1.08		1.76	1.12-2.75	
Immune susceptibility							
High	55	1.00		<0.001	1.00		<0.001
Intermediate	222	1.93	1.14-3.26		2.77	1.46-5.24	
Low	64	3.51	1.98-6.19		4.61	2.32-9.18	

Table 2 Cox univariate and multivariable analyses for relapse free period stratified by patients aged <65 versus patients aged >=65 years.

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; MAST mastectomy; RT radiotherapy; BCS breast conservative surgery; ET endocrine therapy; CT chemotherapy.

Patients > 65 years						
	Univariate analysis			Multivariable analysis		
N	HR	95% CI	P	HR	95% CI	P
42	1.00		<0.001	1.00		0.019
117	1.84	0.89-3.80		1.93	0.63-5.97	
80	3.72	1.81-7.64		4.08	1.32-12.59	
209	1.00		0.49	1.00		0.19
30	1.23	0.69-2.22		2.17	0.68-6.90	
86	1.00		0.001	1.00		0.77
118	2.34	1.43-3.83		0.75	0.34-1.65	
33	2.68	1.35-5.32		0.78	0.25-2.47	
132	1.00		<0.001	1.00		0.004
100	3.18	2.06-4.89		2.76	1.39-5.49	
72	1.00		0.43	1.00		0.005
157	0.83	0.53-1.31		3.08	1.41-6.74	
99	1.00		0.18	1.00		0.02
129	0.75	0.49-1.14		0.43	0.22-0.85	
187	1.00		0.25	1.00		0.59
13	1.63	0.71-3.75		1.44	0.37-5.57	
147	1.00		<0.001	1.00		0.20
41	2.48	1.55-3.97		1.66	0.74-3.77	
57	0.49	0.27-0.90		0.56	0.22-1.41	
7	1.00		0.17	1.00		0.13
66	1.44	0.35-6.05		0.45	0.09-2.28	
2	3.09	0.43-21.99		4.77	0.34-67.55	
170	0.98	0.24-4.00		0.70	0.14-3.59	
27	1.00		0.16	1.00		0.15
120	2.24	0.96-5.23		2.62	0.98-7.01	
24	2.35	0.85-6.48		2.60	0.81-8.35	

Characteristic		Patients < 65 years					
		Univariate analysis			Multivariable analysis		
	N	RER	95% CI	P	RER	95% CI	P
Grade							
I	74	1.00		0.007	1.00		0.33
II	225	1.76	0.96-3.2		1.10	0.53-2.30	
III	164	2.46	1.34-4.50		1.53	0.72-3.26	
Histological type							
Ductal	429	1.00		0.09	1.00		0.26
Other	36	1.55	0.93-2.60		1.51	0.73-3.11	
Tumor stage							
pT1	203	1.00		<0.001	1.00		0.08
pT2	210	2.07	1.46-2.94		1.11	0.66-1.86	
pT3/4	44	3.46	2.16-5.54		2.34	1.06-5.16	
Nodal stage							
Negative	249	1.00		<0.001	1.00		<0.001
Positive	213	3.51	2.51-4.89		3.12	1.79-5.44	
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Positive	236	0.89	0.60-1.14		0.94	0.58-1.53	
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Negative	333	1.00		<0.001	1.00		0.14
Positive	46	2.56	1.67-3.90		1.55	0.86-2.79	
Local Therapy							
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MAST+RT	91	1.84	1.25-2.71		1.12	0.59-2.11	
BCS	24	2.20	0.44-7.23		0.81	0.47-1.38	
Systemic Therapy							
CT alone	120	1.00		0.05	1.00		0.005
ET alone	47	1.54	0.93-2.54		1.82	0.91-3.62	
CT&ET	25	0.66	0.28-1.57		0.20	0.06-0.72	
None	277	0.84	0.59-1.20		1.46	0.87-2.43	
Immune susceptibility							
High	55	1.00		<0.001	1.00		0.001
Intermediate	222	2.55	1.22-5.31		3.93	1.68-9.18	
Low	64	4.01	1.24-8.74		5.53	2.28-13.40	

Table 3 Cox univariate and multivariable analyses for relative survival stratified by patients aged <65 versus patients aged >=65 years.

Abbreviations N number of patients; RER hazard ratio; 95%CI 95% Confidence Interval; 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.

Patients > 65 years						
	Univariate analysis			Multivariable analysis		
N	RER	95% CI	P	RER	95% CI	P
42	1.00		0.07	1.00		0.57
117	3.82	0.46-31.80		3.09	0.16-60.7	
80	6.67	0.83-53.37		4.11	0.22-76.8	
209	1.00		0.42	1.00		0.86
30	1.39	0.62-3.09		1.18	0.18-7.87	
86	1.00		0.001	1.00		0.51
118	3.27	1.20-8.92		1.50	0.29-7.74	
33	8.73	3.08-24.77		2.69	0.46-15.8	
132	1.00		0.008	1.00		0.29
100	3.02	1.34-6.82		1.65	0.66-4.12	
72	1.00		0.027	1.00		0.57
157	0.45	0.22-0.91		0.64	0.14-2.99	
99	1.00		0.13	1.00		0.71
129	0.53	0.23-1.21		1.40	0.24-8.08	
187	1.00		0.02	1.00		0.07
13	3.10	1.24-7.76		3.19	0.91-11.2	
147	1.00		0.003	1.00		0.95
41	3.45	1.61-7.38		0.90	0.25-3.28	
57	2.53	1.01-1.95		0.80	0.18-3.59	
7	1.00		0.70	1.00		0.75
66	0.74	0.22-2.48		0.81	0.23-3.01	
2	0.74	0.05-10.01		0.79	0.10-11.2	
170	0.54	0.17-1.68		0.66	0.21-1.99	
27	1.00		0.45	1.00		0.45
120	2.85	0.46-17.74		3.57	0.42-30.3	
24	1.99	0.22-17.92		3.85	0.46-32.3 0.16-60.7	

The immune system plays an important role in the battle of the host against cancer development and progression (4). With aging, there are well-known alterations occurring in the immune response affecting both innate and adaptive immunity. It has been suggested that this process of immunosenescence might contribute to cancer development and progression, however this relation is nowadays still poorly understood (36). Previous studies have found differences in T cell and NK cell compartments between young and old people. T cells, especially CD8+ T cells, more often show poor proliferation, resistance to apoptosis and functional abnormalities, leading to a shift from so-called "truly naïve" T cells to "exhausted senescent" T cells. This reduced availability of naïve cells and T cell disfunctionalities are thought to explain the reduced ability of the elderly to respond to new antigens, including tumor associated antigens (37). NK cells also have shown to have decreased cytotoxicity and decreased IL-2 production in elderly patients (36). We found no statistically significant differences in number of infiltrating CTL or NK cells between breast cancer patients aged >=65 years versus aged <65 years. These results however do not contradict the theory of immunosenescence. As pointed out by previous studies, immunosenescence seems to be identified by a disfunctioning in immune recognition and cytotoxicity of CTL or NK cells, rather than by a non-capability of migration and infiltration in inflamed or carcinogeneous environments (36, 37). Like most retrospective immunohistochemical cohort studies, we were limited by the fact that we could not measure this direct functioning of tumor immune recognition and cytotoxicity. However, since data were present for both immune factors and tumor response, we were able to study interaction between the tumor and immune system, by which we could indirectly conclude on the function of the immune system.

During advancing oncogenesis, tumor immune recognition and attack by the immune system, causes immunoselection of target cancer cells, whom on their turn evolve variants able to resist immune attack. This results in the appearance of new tumor cells variants in order to maintain a state of equilibrium between the immune system and the tumor. The immune system must now exert new powerful selective pressures on the tumor cells, which will evolve again new variants able to resist this immune response, which finally leads to tumor immune escape (4, 5). It therefore is likely that a compromised immune system, as seen with aging, may lead to a left skewed shift in this tumor-immune equilibrium, where less tumor immune attack correlates with lower stages of tumor immune escape variant phenotypes (33, 36). Our results showed a statistically significant difference in HLA-EG upregulation and, though not statistically significant, a difference in classical HLA class I expression of tumors between elderly and younger breast cancer patients; less HLA class I downregulation and less HLA-EG upregulation were found in patients aged >=65 years. These results suggest that tumors in elderly patients have less need to downregulate expression of classical HLA class I and upregulate expression of HLA-EG, because less immune selective pressure is given by respectively CTL and NK cells. Our results strongly suggest a decreased

need for immune escape strategies in higher aged patients compared to their younger counterparts and are therefore in line with the left skewed tumor-immune equilibrium theory. Moreover, this theory is supported by the differences seen in distribution of tumor immune subtypes between patients aged <65 years compared to aged >=65 years; though not significant, tumors with low immune susceptibility are seen less in patients aged >=65 years.

Another method by which we indirectly measured the efficacy of tumor immune surveillance between elderly breast cancer patients and their younger counterparts were the associations of tumor immune subtypes with outcome. These subtypes were defined based on tumor susceptibility for cellular immune responses using expression of key factors in these responses: high CTL infiltration, presence of NK cells, and Tregs and tumor expression of classical HLA class I and HLA-E and -G. Outcome analyses of the immune subtypes in patients aged <65 years revealed strong associations with patient outcome where tumors defined as being highly susceptible to immune system attack showed a favourable outcome for breast cancer patients compared to patients with tumors defined as having a low immune susceptible profile. Though a trend towards similar outcomes was found in patients aged >=65 years, no such statistically significant association could be found. The fact that elderly breast cancer patients have comparable outcomes independently of the immune susceptibility of tumors is again highly suggestive for a less effective immune system in elderly patients and supports the hypothesis of immunosenescence and its contribution to cancer progression.

The phenomenon of immunosensence in humans is still hypothetical, but has previously been described in animal models where less immune responses were found after immunotherapy in old animals compared to young animals (38). The effects of immunosenesence on cancer development and progression have been suggested before, but to the best of our knowledge, we are the first to have found such age specific interactions between the immune system and cancer progression in a clinical dataset. These results might add to an explanation on the previously observed increase in breast cancer specific mortality with age by our group (3). While prior studies show tumors in elderly breast cancer patients to be of equal or of less malignant biological character than tumors found in their younger counterparts (39, 40), elderly breast cancer patients were in our study found to decease more often due to breast cancer regardless of a higher risk of mortality from other causes and independent of known tumor and patient characteristics. These contradictive findings might be explained by processes like immunosenescence and changes in tumor microenvironment, where it might not so much be the increased malignancy of tumor as the weakening of host defense against cancer determining tumor progression and therefore patient outcome. Future research is needed to confirm our results and to further unravel the complex interactions between immunosenescence, tumor progression and response to therapy. A better understanding

of these processes and future possibilities of immune manipulations and vaccinations might lead to more tailored treatment of elderly breast cancer patients.

In line with emerging evidence on immunosenescence in elderly and its hypothesized effects on tumor development and progression, we found less tumor immune escape variants and a fading prognostic effect of tumor immune subtypes in elderly breast cancer patients. To our knowledge we are the first to study the age-specific impact of the immune response and subsequent tumor immune evasion on tumor progression and patient outcome in a clinical set of breast cancer patients. Evidence based tailored treatment is highly necessitated in elderly breast cancer patients. Age-specific malfunctioning of the immune system in tumor control and it's implications on patient prognosis and response to treatments might aid in therapeutic decisions making for this specific breast cancer population. In addition, these data might contribute to the development of immune manipulations and cancer vaccinations.

REFERENCES

(1) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.

(2) Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007;18:581-92.

(3) van de WW, Markopoulos C, van d, V, Seynaeve C, Hasenburg A, Rea D, et al. Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. JAMA 2012;307:590-7.

(4) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6:715-27.

(5) Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-60.

(6) Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53:904-10.

(7) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007;17:459-68.

(8) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3:999-1005.

(9) Marin R, Ruiz-Cabello F, Pedrinaci S, Mendez R, Jimenez P, Geraghty DE, et al. Analysis of HLA-E expression in human tumors. Immunogenetics 2003;54:767-75.

(10) Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A 2001;98:11521-6.

(11) Liu F, Lang R, Zhao J, Zhang X, Pringle GA, Fan Y, et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat 2011.

(12) Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29:1949-55.

(13) de Kruijf EM, van Nes JG, Sajet A, Tummers QR, Putter H, Osanto S, et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res 2010;16:1272-80.

(14) Gudmundsdottir I, Gunnlaugur JJ, Sigurdsson H, Olafsdottir K, Tryggvadottir L, Ogmundsdottir HM. Altered expression of HLA class I antigens in breast cancer: association with prognosis. Int J Cancer 2000;89:500-5.

(15) Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. Int J Cancer 2005;117:248-55.

(16) Redondo M, Garcia J, Villar E, Rodrigo I, Perea-Milla E, Serrano A, et al. Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. Hum Pathol 2003;34:1283-9.

(17) de Kruijf EM, Sajet A, van Nes JG, Natanov R, Putter H, Smit VT, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. J Immunol 2010;185:7452-9.

(18) Kleinberg L, Florenes VA, Skrede M, Dong HP, Nielsen S, McMaster MT, et al. Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch 2006;449:31-9.

(19) Lefebvre S, Antoine M, Uzan S, McMaster M, Dausset J, Carosella ED, et al. Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. J Pathol 2002;196:266-74.

(20) Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006;24:5373-80.

(21) Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res 2008;14:2413-20.

(22) de Kruijf EM, Engels CC, van de Water W, Bastiaannet E, Smit VT, van de Velde CJ, et al. Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients. Breast Cancer Res Treat 2013;142:355-64.

(23) Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med 2010;16:238-46.

(24) Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. Immunology 2007;120:435-46.

(25) Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. Trends Immunol 2009;30:366-73.

(26) Shanley DP, Aw D, Manley NR, Palmer DB. An evolutionary perspective on the mechanisms of immunosenescence. Trends Immunol 2009;30:374-81.

(27) Beerman I, Maloney WJ, Weissmann IL, Rossi DJ. Stem cells and the aging hematopoietic system. Curr Opin Immunol 2010;22:500-6.

(28) Woolthuis CM, de HG, Huls G. Aging of hematopoietic stem cells: Intrinsic changes or micro-environmental effects? Curr Opin Immunol 2011;23:512-7.

(29) Lefebvre JS, Maue AC, Eaton SM, Lanthier PA, Tighe M, Haynes L. The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. Aging Cell 2012;11:732-40.

(30) Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev 2005;205:257-68.

(31) Campos C, Pera A, Sanchez-Correa B, Alonso C, Lopez-Fernandez I, Morgado S, et al. Effect of age and CMV on NK cell subpopulations. Exp Gerontol 2014.

(32) Gayoso I, Sanchez-Correa B, Campos C, Alonso C, Pera A, Casado JG, et al. Immunosenescence of human natural killer cells. J Innate Immun 2011;3:337-43.

(33) Fulop T, Kotb R, Fortin CF, Pawelec G, de AF, Larbi A. Potential role of immunosenescence in cancer development. Ann N Y Acad Sci 2010;1197:158-65.

(34) van Nes JG, de Kruijf EM, Faratian D, van d, V, Putter H, Falconer C, et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res Treat 2010.

(35) de Kruijf EM, Engels CC, van de Water W, Bastiaannet E, Smit VT, van de Velde CJ, et al. Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients. Breast Cancer Res Treat 2013;142:355-64.

(36) Fulop T, Larbi A, Kotb R, de AF, Pawelec G. Aging, immunity, and cancer. Discov Med 2011;11:537-50.

(37) Larbi A, Fulop T. From "truly Naive" to "exhausted senescent" T cells: When markers predict functionality. Cytometry A 2013.

(38) Pawelec G, Lustgarten J, Ruby C, Gravekamp C. Impact of aging on cancer immunity and immunotherapy. Cancer Immunol Immunother 2009;58:1907-8.

(39) Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. J Natl Cancer Inst 2000;92:550-6.

(40) Thomas GA, Leonard RC. How age affects the biology of breast cancer. Clin Oncol (R Coll Radiol) 2009;21:81-5.

Chapter 9

Summary and general discussion

SUMMARY

The research in this thesis has used a well described retrospective breast cancer cohort including all women with non-metastasized breast cancer who were primarily operated in the Leiden University Medical Center between 1985 and 1996. This cohort has been used for all studies, which made comparisons and combinations of various markers possible. Elderly breast cancer patients were considered patients aged 65 years or more, according to the world health organization definition (www.who.int).
The main results found in this thesis were that (1) we found strong independent prognostic effects for biomarkers involved in immunosurveillance and tumor immune escape, especially when accounted for various interactions between these markers and (2) key prognostic biomarkers differed in their distribution and prognostic effect in elderly breast cancer patients compared to their younger counterparts.

Part I: Prognostic biomarkers in the interactions between the host's immune system and breast cancer

In the first part of this thesis we investigated the expression and prognostic effect of various crucial immunological markers and their interactions in breast cancer patients.
In **Chapter 2** frequent down regulation or loss of expression of classical HLA class I and high tumor infiltration of Treg were seen in tumors of breast cancer patients. Prior studies indicate that breast cancer is immunogenic and induces tumor associated antigen (TAA)-specific CTL[1]. Our finding of HLA class I down regulation in more than half of all tumors, which was concordant with results found in previous studies [2-4], therefore implies a common phenomenon in breast cancer of selective outgrow of these cells which were able to escape from immune destruction[5]. The frequent presence of immunosuppressive Treg in the tumor microenvironment supports the hypothesis that tumors may attract these immune-suppressing cells in order to evade attack from effector T cells. These data are strongly suggestive for immune escape mechanisms in breast cancer tumors [5]. Further supporting this was the specific prognostic effects found for HLA class I and Treg among chemotherapy-treated patients. Cyclophosphamide, which was included in all chemotherapy regimens of our studied population, is known to positively influence host immune responses against cancer through selective elimination of Treg [6-8]. Ceasing of Treg, reduces immunosuppressive effects, resulting in an enhanced expansion and function of responding of CTL [6-8]. This restored CTL functioning leads to an increased anti-tumor response and therefore might lead to a better patient outcome. We explain the specific prognostic effect found for HLA class I and Treg among chemotherapy-treated patients by this restored CTL functioning, which logically specifically takes place in patients with Treg presence in the tumor microenvironment before chemotherapy administration and in patients whose tumors

had not lost expression of HLA class I. This hypothesis was further supported by a previous study that found a decline in absolute numbers of tumor infiltrating Treg after preoperative chemotherapy, which was associated with a pathological complete response in combination with presence of CTL infiltration [9]. Additional to the fact that our results strongly support the immunoediting hypothesis and add to the current knowledge of the interactions between breast cancer and the immune system, HLA class I and Treg are one of the few predictive factors for chemotherapy response in breast cancer and these markers could therefore be applied in response prediction to chemotherapy in breast cancer patients [10]. Contrary to some previous reports however, no unfavorable prognostic effect was found for classical HLA class I down regulation or loss [3, 4, 11]. An explanation to this might be the increased susceptibility to NK cell recognition and attack of cells with loss of HLA class I expression. The following stages of in tumor immune escape after classical HLA class I down regulation or loss are therefore focused on the escape of NK cell recognition.

Non-classical HLA class I molecules, HLA-E and HLA-G, play an important role in controlling auto-immune NK cell reactions. Under normal circumstances, expression of the HLA-E molecule is found in most tissues that express classical HLA class I or HLA-G molecules and is thought to provide an important "self-signal" to the immune system by accommodating and presenting peptide fragments from leader sequences of these molecules [12, 13]. HLA-G expression, on the other hand, has very restricted tissue expression and has been mostly found in extravillous trophoblastic cells, where it mediates semi-allograft immunotolerance during pregnancy [14]. Expression of HLA-E and HLA-G on the cell surface can respectively bind with the inhibitory receptors CD94/NKG2A and KIR2DL4/p49 of NK cells, and thereby cause inhibition of their proliferation and cytotoxic effector functions [15, 16].Tumors may acquire or up regulate expression of HLA-E and HLA-G as protective property against immune recognition and elimination by NK cells [12]. HLA-E is regularly expressed in various healthy tissues and correlates with expression of classical HLA class I molecules. This physiological correlation with classical HLA class I molecules has been found to be disturbed in tumors, suggesting that malignant cells which escape T cell immune recognition by down regulation of classical HLA class I expression, may further escape immune recognition by up regulation of HLA-E [17]. In addition, expression of HLA-G protects against "missing self" recognition of NK. Expression of this molecule, which is rarely found in healthy tissues, is frequently observed in pathological conditions such as in tumors [18, 19]. Both HLA-E and HLA-G expression showed an association with a worse clinical outcome in various tumor types [20-27]. In **Chapter 3** we showed that HLA-E and HLA-G expression were of independent statistically significant similar influence on outcome of breast cancer patients with high discriminative power, however, specifically in tumors devoid of classical HLA class I expression. This suggests that specifically in tumors devoid of classical HLA class I expression, up regulation of HLA-E and HLA-G expression counteracts the resulting NK cell susceptibility, leading to immune

escape of tumor cells. Supportive for a specific NK cell inhibition of the non-classical HLA class I molecules, for both HLA-E and HLA-G an inverse correlation was found with NK cell infiltrate in a colorectal cancer and gastric cancer study respectively [28, 29], while other studies demonstrated that overexpression of HLA-E and HLA-G directly inhibited NK-mediated cell lysis [29].

Aside from non-classical HLA class I, the activating receptor NK cell lectin-like receptor gene 2D (NKG2D) ligands have great influence on NK cell recognition of pathological cells. NKG2D ligands bind to the NKG2D receptors on NK cells, NKT cells, $\gamma\delta^+$ T cells and CD8+ T cells and provide a stimulatory activating response[30]. Ligands which bind NKG2D receptors comprise major histocompatibility complex class I chain-related proteins A and B (MIC-AB) and unique long 16 (UL16) binding proteins 1-6 (ULBP1-6)[31, 32]. Expression of these ligands may be induced upon infection and other inducers of cellular stress, such as malignant transformation, and may initiate an immune response by binding to the NKG2D receptors on NK and T cells [33]. In **Chapter 4** we show that NKG2D ligands are frequently high expressed in breast tumors and that a statistically significant association exists between expression levels of these ligands and that this expression influences patient's prognosis. We were able to statistically prove that high expression levels of MIC-AB, ULBP-2, and mostly a combination of high expression of both markers, resulted in a beneficial relapse free period with high discriminative power, comparable to results found in previous studies on colorectal cancer [34, 35]. Altogether, these results indicate a cooperation of NKG2D ligands with each other and further add to the hypothesis that low expression of these ligands is a result of selective pressure by the immune system that results in cancer immune evasion or immunoediting. Additional analyses were performed in our study with two different variables that represented combined number of highly co-expressed ligands and amount of co-expression of all ligands. The results of these analyses revealed no patterns of any cooperative functioning between all ligands, as both variables showed no consistent and significant relationship with clinical outcome of disease. Supported by the results found in previous studies [30, 34-37] this shows that each NKG2D ligand analyzed separately does not show equal effects on clinical outcome, that different ligands show varying prognostic effects in different tumors and that a simple additive effect of all NKG2D ligands cannot be assumed. This indicates the complexity of NKG2D ligands functioning and emphasizes again the importance of interactions between various immune markers.

The above mentioned results show that loss of classical HLA class I, up regulation of classical HLA-E and HLA-G expression, induction of Treg in the tumor microenvironment and low expression of certain NKG2D ligands are frequent events in breast cancer, supporting the hypothesis that breast tumors are capable of evading immune recognition. In addition, they highlight the importance of accounting for these interactions within and between the immune system and breast tumors and therefore studying combinations of markers of immune surveillance together with markers of

tumor immune escape. This lead to the construction of tumor immune subtypes based on tumor susceptibility for cellular immune responses (**Chapter 5**) (Figure 1).

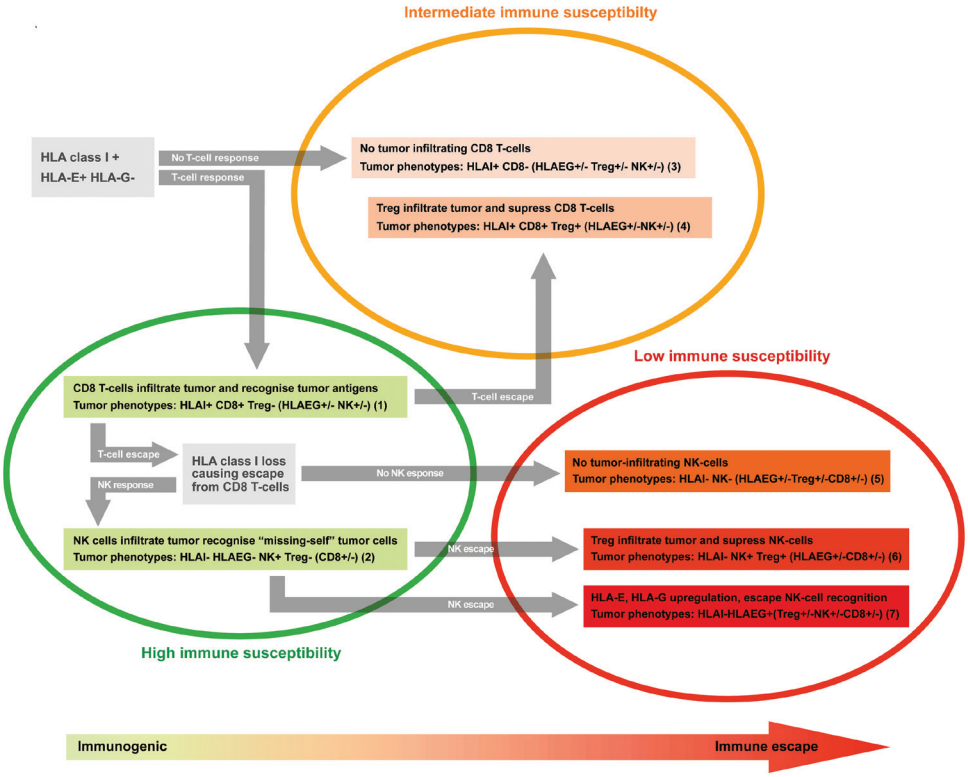


Figure 1 Tumor immune subtypes showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 tumor immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape 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).

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. The study showed 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 this 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. While most studies focus on the effect of single parameters and thereby many contradictory results have been published on immune biomarkers, we showed that it is combinations of these markers which are able to reflect the multifaceted interaction between immune cells and tumor cells, thereby filtering out understating or confounding impacts of immunosurveillance and therefore predict outcome with high stratification capacity. The results found for the tumor immune subtypes are not only concordant with prior evidence on tumor immune biology in breast cancer[38, 39], but additionally join together the conclusions of prior studies by linking single tumor-immune markers to functional tumor-immune interaction.

Part II: Prognostic biomarkers in elderly breast cancer patients

In the second part of this thesis we studied differences in the distribution and effect on outcome of prognostic biomarkers in elderly breast cancer patients.

First, in **Chapter 6** we demonstrated that the presence of ALDH1, a representative marker for cancer stem cells, expression is significantly higher in young breast cancer patients than in elderly patients and demonstrated that ALDH1 expression is an independent risk factor for decreased survival in young breast cancer patients, but not in elderly patients. Cancer stem cells, defined as a small subset of tumor cells with stem cell-like features, including epithelial-to-mesenchymal transition, have the capacity of self-renewal and differentiation; giving rise to a heterogeneous tumor cell population[40]. A biological explanation of the qualitative age-interaction of the prognostic effect of ALDH1 expression might be that of a changing micro-environment in elderly patients, which may result in hampered signal transduction between tumor stem cells and the micro-environment. Moreover, changes in metabolic processes might limit the role of tumor stem cells in elderly patients. Increasing evidence from the field of epigenetics demonstrates that hypermethylation-induced repression of genes required for stem cell differentiation is linearly associated with age.[41] This suggests that, with increasing age, the role of tumor stem cells becomes more limited.

In **Chapter 7**, using gene expression validated IHC surrogates of molecular subtypes, we demonstrated that elderly breast cancer patient tumors show a different distribution of molecular subtypes where were more Luminal A and Luminal B subtypes are found compared to their younger counterparts. These data are concordant to previous studies that showed more ER and/or PR positivity and less overexpression of EGFR, HER2 and ki67 in tumors of elderly breast cancer patients [42-44]. Results also showed no statistically

significant association for molecular subtypes and patient outcome in elderly breast cancer patients in contrary to young breast cancer patients. We sought an explanation to this finding in competing risks of death in elderly breast cancer patients; elderly breast cancer patients compared to their younger counterparts have shown in absolute sense to develop more relapses [45], however proportionally due to higher risk of dying earlier and from other causes they show less breast cancer relapses and breast cancer specific deaths [46-48]. Only about 60% of elderly breast cancer patients die as a consequence of breast cancer, compared to almost 100% of young patients. It is important to realize that this has major implications on the impact and value of prognostic biomarkers in elderly breast cancer patients. Prognostic biomarkers, identifying patients with low versus high risk of breast cancer progression and breast cancer related death will show limited to no prognostic effect in the 40% of elderly patients which have a short-term prognosis due to breast cancer un-related causes, especially in those who are considered frail. These elderly patients are also unlikely to benefit from systemic treatment, since their cause of death will be other than due to breast cancer. Therefore, the clinical value of prognostic biomarkers, which aid at distinguishing between patients who might and might not benefit from systemic treatment, is also limited in this patient population.

As described in the first part of this thesis, the immune system plays an important role in the battle of the host against cancer development and progression. With aging, there are well-known alterations occurring in the immune response affecting both innate and adaptive immunity. It has been suggested that this process of immunosenescence might contribute to cancer development and progression, however this relation is nowadays still poorly understood [49]. In **Chapter 8** we evaluated the distribution and impact on patient outcome of anti-tumor immune response and tumor immune evasion in elderly breast cancer patients compared to their younger counterparts. This showed no differences in number of infiltrating CTL, NK cells or Treg, but a trend towards less classical HLA class I down regulation and statistically significant less HLA-E or HLA-G up regulation of tumors. These differences were also reflected, though not statistically significant, in less "low immune susceptible" tumors in elderly breast cancer patients. These results suggest that tumors in elderly patients have less need to down regulate expression of classical HLA class I and up regulate expression of HLA-EG, because of less immune selective pressure is given by respectively CTL and NK cells. Our results strongly suggest a decreased need for immune escape strategies in higher aged patients compared to their younger counterparts suggesting a left skewed tumor-immune equilibrium. During advancing oncogenesis, tumor immune recognition and attack by the immune system, causes immunoselection of target cancer cells, whom on their turn evolve variants able to resist immune attack. This results in the appearance of new tumor cells variants in order to maintain a state of equilibrium between the immune system and the tumor. The immune system must now exert new powerful selective pressures on the tumor cells, which will evolve again new variants able to resist this immune response, finally leading to tumor immune escape [38, 50]. It therefore is likely

that a comprised immune system, as seen with aging, may lead to a left skewed shift in this tumor-immune equilibrium, where less tumor immune attack correlates with lower stages of tumor immune escape variant phenotypes [49, 49]. Comparable numbers of infiltrating CTL of NK cells between tumors of elderly and young breast cancer patients does not contradict the theory of immunosenescence, since immunosenescence seems to be identified by a disfunctioning in immune recognition and cytotoxicity of CTL or NK cells, rather than by a non-capability of migration and infiltration in inflamed or carcinogeneous environments [49]. Comparable to ALDH1 and molecular subtypes, again no statistically significant association was found between tumor immune subtypes and outcome in elderly breast cancer patients, contrary to their younger counterparts. The fact that elderly breast cancer patients have comparable outcomes independently of the immune susceptibility of tumors is again highly suggestive for a less effective immune system in elderly patients and supports the hypothesis of immunosenescence and its contribution to cancer progression.

Conclusions and future perspectives

Not only did our results provide further evidence supporting an immunoediting hypothesis; we also found various biomarkers with the ability to stratify breast cancer patients according to their predicted prognostic outcome with high discriminative power, especially when accounted for the various interactions between these markers. Differences in distributions and prognostic effect of these tumor immune subtypes in elderly breast cancer patients compared to their younger counterparts, provided further prove for immunoediting and immunosenescence theories. Differences in distributions and prognostic effects of stem cells marker ALDH-1 and molecular subtypes in these elderly breast cancer patients further suggest that it is underlying biological differences in the micro-environmental changes which might influence differences in tumor behavior. We also consider competing risk of death in elderly to be an important factor in fading prognostic effects with increasing age. We finally conclude that biomarkers need validation in elderly breast cancer patients, since results from young patients cannot be simply extrapolated to this patients group.

Immunoediting and immunosenescence hypothesis:

The concept that the immune system can recognize and eliminate primary developing tumors has existed for more than a 100 years [51]. Clearer insight of interactions between the immune system and malignant tumors during the first years of the 21st century gave rise the "cancer immunoediting" hypothesis, characterized by three phases: elimination, equilibrium and escape [38]. Tumor cells can be successfully eradicated by the immune system during the elimination phase. On the other hand, some tumor cells may be capable of escaping from these first line mechanisms of host tumor immune elimination and enter the next phase of cancer immunoediting; equilibrium. During this phase it

is suggested that there is a constant interaction between the tumor, which consists of rapidly mutating and genetically unstable cells, and the immune system. Many tumor cells, susceptible for immune recognition and attack, are eradicated by the immune system, but new tumor cell variants arise which have increased resistance to immune attack. Following these interactions, the tumor constitution is constantly shaped by the immune system. Following the equilibrium phase, tumors can transit to the final escape phase of cancer immunoediting [38]. During this final phase the balance of the battle between the immune system and the malignant tumor becomes favorable to the latter. Following this balance shift, the tumor growth proceeds and tumors become clinically detectable. Further development of significant immune escape mechanisms by the tumor are suggested to result in further tumor progression and spread. Tumor immune escape mechanisms are various and complex, but briefly characterized by: classical HLA class I down regulation, HLA-E and HLA-G up regulation, down regulation of stress molecules NKG2D ligands and attraction of immunosuppressive regulatory T cells in the tumor microenvironment. Evidence supporting the cancer immunoediting theory has been described in various mice studies [38, 52-57]. Human data is limited. Though our results on the distribution and prognostic effect do not prove the existence of the immunoediting theory in human breast cancer, it is strongly supportive for this hypothesis, where we show higher immune susceptible tumors to be associated with a beneficial patient outcome while on the other hand more tumor immune escape variants result in a worse patient prognosis. Importantly, our results highlight the importance of considering various factors in investigating the interplay between tumors and the immune system, accounting for the significant complexity and interactions in these processes.

With increasing age, the immune system declines in functional innate and adaptive immunity leading to a reduced ability to respond to infection and vaccinations [58-61]. There has been increasing evidence that age associated immunosenescence might contribute to cancer development and progression [49]. The phenomenon of immunosenescence and its possible effects on cancer development and progression in humans is still hypothetical and especially our results on less immune escape variants and fading prognostic effect with increasing age in elderly breast cancer patients provides evidence for this.

It appears that unravelling the complex interactions of the immune system in tumor development and progression leads to valuable new insights in the tumor biology. These new insights have a high potential to lead to prognostic or predictive biomarkers and might form the basis of new immunotherapeutical strategies. Future research providing new evidence for the immunoediting and immunosenescence hypotheses is needed.

Tumor immune subtypes as prognostic biomarker in breast cancer:

Many prognostic biomarkers 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[62]. 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[63, 64]. 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[65]. One can imagine that such methods contain a high proportion of "noise" due to the involvement of unknown chance in the statistical construction and the optimalisation of the factor during creation and that these prognostic factors almost definitely lose discriminative power due to these reasons. On the other hand, bottom-up analyses, where a biological factor is correlated to patient outcome has a high chance of not showing any prognostic association and therefore a high chance of failure. In addition, it generally shows lower discriminative power than the prior mentioned technique, since it is not optimized on prognostic outcome of patients. However, when a prognostic biomarker is found with this method it provides reliability since it is based on well-founded biological facts. In the first part of this thesis we provided various prognostic biomarkers using bottom-up analyses based on well-founded biological hypotheses on breast cancer immunoediting. The final tumor immune subtypes that were constructed lead to an independent prognostic variable with high discriminative power, comparable to ones found in gene expression prognostic arrays. In addition, independent validation of this biomarker lead to similar results. Moreover, as shown by the predictive effect of HLA class I and Treg on chemotherapy response and as suggested before in literature reports, treatment response is in part regulated by the immune microenvironment, which gives the tumor immune subtypes potential for a predictive effect on existing adjuvant systemic treatment next to potential for development of immunotherapies [66]. Such predictive effects of tumor immune subtypes are not yet investigated and therefore this still remains speculative. A drawback of tumor immune subtypes is the fact that it is still an elaborative test, where many stainings and quantifications need to be performed in order to get the subtype. In addition, it does not show prognostic significance in elderly breast cancer patients. This might in part be due to immunosenescence as explained above and, as explained below, due to competing causes of death in the elderly population.

Biological differences in elderly versus young breast cancer:

Patient, tumor and treatment characteristics have been found to differ considerably between elderly and young breast cancer, [67-69]. This may be indicative for differences in underlying tumor biology and it has indeed often been suggested that elderly breast cancer is a biologically different tumor type of a more indolent character compared to young breast cancer [68-70]. This hypothesis contradicts the fact that increased breast cancer specific mortality is seen with increasing age [45]; elderly breast cancer patients were found to decease more often due to breast cancer regardless of a higher risk of mortality from other causes and independent of known tumor and patient characteristics. In our

studies we did find differences in distributions of key biomarkers, where less aggressive phenotypes with low numbers of ALDH-1, luminal molecular subtypes and less tumor immune escape were seen in elderly breast cancer patients compared to their younger counterparts. However, an explanation to the contradictory findings can be sought in the interactions between tumor and its microenvironment (Figure 2).

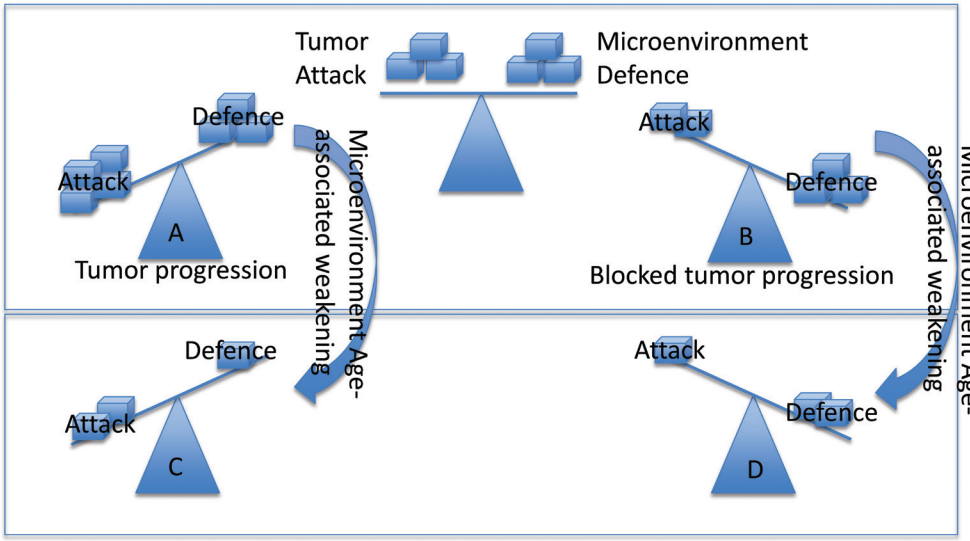


Figure 2 schematically representing the balance between tumor aggressiveness and host microenvironmental defence against the tumor. The number of cubusses represents the weight or strength of the attack, or the progression and invasion of the tumor (left on the balance scale) and the microenvironmental defence against this attack (right on the balance scale). The final equilibrium resulting from this balance results in either: (1) the tumor attack dominates (shown in (A) and (C)), resulting in tumor progression or (2) host microenvironmental defence dominates (shown in (B) and (D)), resulting in a blocked tumor progression. Situations (C) and (D) show the hypothetical situations in elderly patients, where the tumor host microenvironment has weakened compared to the microenivronment in younger patients as shown in (A) and (B).

The outcome of the balance between the tumor and the microenvironment results in changes in patients' outcome, where high tumor aggressiveness should result in more tumor progression and therefore a worse outcome of patients (A), whereas a low aggressive tumor should results in a blocked tumor progression by a well-functioning microenvironment and therefore a beneficial outcome of patients (B). However, usually not the case in young breast cancer patients, but a relevant factor which should be taken into account in elderly breast cancer patients, is the fact that host's defenses against tumors might have deteriorated due to ageing. Herein the suggestion that elderly breast tumors are of a more indolent character and therefore should lead to a favorable outcome compared to younger breast tumors are depicted in Figure 2 by situation B, in case of a host defense comparable to young patients, or D, where the host defense has deteriorated, but the tumor aggressiveness is reduced even more. The combination of a less aggressive tumor with an even more lowered host defense results in tumor progression and therefore a worse patient outcome (C). Less aggressive tumors combined

with the finding of a worsened breast cancer specific outcome with increasing age can be explained by the fact that host defenses deteriorate faster than the aggressiveness of tumors. Changes in the surroundings of the tumor, i.e. the tumor microenvironment, with increasing age seems to be the bridge between the contradictory findings of less aggressive tumor types but worse patient outcomes in elderly breast cancer patients. In this scenario it is not so much the increased malignancy of tumors as it is the weakening of the host defense against cancer, in determining tumor progression and therefore patient outcome. As explained, a changing micro-environment might lead to a hampered signal transduction between tumor stem cells and the micro-environment in elderly breast cancer patients, causing differences in prognostic effects of ALDH1 compared to young breast cancer patients. Moreover, the differences in distribution and prognostic effect for tumor immune subtypes suggest processes like immunosenescence to influence tumorigenesis in elderly breast cancer patients.

Prognostic biomarkers in elderly breast cancer patients:

None of the key biomarkers we investigated showed a statistically significant prognostic effect in elderly breast cancer patients. A combination of underlying differences in tumor biology and behavior, loss of statistical power due to differences in distribution of the biomarker subcategories and competing causes of death in elderly breast cancer patients might explain the fading prognostic significance of biomarker. The fact remains that the clinical value of prognostic biomarkers, which aid at distinguishing between patients who might and might not benefit from adjuvant systemic treatment, is limited in the elderly breast cancer population. Therefore we conclude that validation of biomarkers in elderly is required, since possible differences in the tumor microenvironment and in addition competing causes of death in elderly might results in a significant differing prognostic value and which therefore significantly interferes with patient treatment modalities. Importantly, considering competing causes of death in elderly breast cancer patients, breast cancer prognostic biomarkers can only have a prognostic value in elderly patients whose life expectation will be long enough for the cancer to progress and cause patient death. It is only in these fit enough patients that prognostic biomarkers may show differences in outcome between elderly breast cancer patients and may aid clinical decision making on systemic treatment. In order to improve tailored treatment in elderly with the aid of prognostic biomarkers, the first step would therefore be to identify these fit elderly patients.

Reference List

1. Sotiropoulou,P.A. *et al.* Cytotoxic T-cell precursor frequencies to HER-2 (369-377) in patients with HER-2/neu-positive epithelial tumours. *Br. J. Cancer* **89**, 1055-1061 (2003).
2. Cabrera,T. *et al.* High frequency of altered HLA class I phenotypes in invasive breast carcinomas. *Hum. Immunol.* **50**, 127-134 (1996).
3. Gudmundsdottir,I. *et al.* Altered expression of HLA class I antigens in breast cancer: association with prognosis. *Int. J. Cancer* **89**, 500-505 (2000).
4. Madjd,Z., Spendlove,I., Pinder,S.E., Ellis,I.O., & Durrant,L.G. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. *Int. J. Cancer* **117**, 248-255 (2005).
5. Ferrone,S. & Whiteside,T.L. Tumor microenvironment and immune escape. *Surg. Oncol. Clin. N. Am.* **16**, 755-74, viii (2007).
6. Dudley,M.E. *et al.* Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* **298**, 850-854 (2002).
7. Ghiringhelli,F. *et al.* CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur. J. Immunol.* **34**, 336-344 (2004).
8. Proietti,E. *et al.* Importance of cyclophosphamide-induced bystander effect on T cells for a successful tumor eradication in response to adoptive immunotherapy in mice. *J. Clin. Invest* **101**, 429-441 (1998).
9. Ladoire,S. *et al.* Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin. Cancer Res.* **14**, 2413-2420 (2008).
10. Goldhirsch,A. *et al.* Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. *J. Clin. Oncol.* **21**, 3357-3365 (2003).
11. Redondo,M. *et al.* Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. *Hum. Pathol.* **34**, 1283-1289 (2003).
12. Algarra,I., Garcia-Lora,A., Cabrera,T., Ruiz-Cabello,F., & Garrido,F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol. Immunother.* **53**, 904-910 (2004).
13. Wei,X.H. & Orr,H.T. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Hum. Immunol.* **29**, 131-142 (1990).
14. Kovats,S. *et al.* A class I antigen, HLA-G, expressed in human trophoblasts. *Science* **248**, 220-223 (1990).
15. Braud,V.M. *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795-799 (1998).
16. Rajagopalan,S. & Long,E.O. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* **189**, 1093-1100 (1999).
17. Palmisano,G.L. *et al.* HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum. Immunol.* **66**, 1-12 (2005).
18. Wischhusen,J., Waschbisch,A., & Wiendl,H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin. Cancer Biol.* **17**, 459-468 (2007).
19. Menier,C. *et al.* Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule. *Tissue Antigens* **75**, 201-206 (2010).
20. Ye,S.R. *et al.* Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer. *Mod. Pathol.* **20**, 375-383 (2007).
21. Yie,S.M. *et al.* Expression of human leukocyte antigen G (HLA-G) correlates with poor prognosis in gastric carcinoma. *Ann. Surg. Oncol.* **14**, 2721-2729 (2007).
22. Yie,S.M. *et al.* Expression of HLA-G is associated with prognosis in esophageal squamous cell carcinoma. *Am. J. Clin. Pathol.* **128**, 1002-1009 (2007).
23. Yie,S.M. *et al.* Expression of human leucocyte antigen G (HLA-G) is associated with prognosis in non-small cell lung cancer. *Lung Cancer* **58**, 267-274 (2007).
24. Bianchini,M. *et al.* Comparative study of gene expression by cDNA microarray in human colorectal cancer tissues and normal mucosa. *Int. J. Oncol.* **29**, 83-94 (2006).
25. Malmberg,K.J. *et al.* IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J. Clin. Invest* **110**, 1515-1523 (2002).
26. Marin,R. *et al.* Analysis of HLA-E expression in human tumors. *Immunogenetics* **54**, 767-775 (2003).
27. Rouas-Freiss,N., Moreau,P., Ferrone,S., & Carosella,E.D. HLA-G proteins in cancer: do they provide tumor cells with an escape mechanism? *Cancer Res.* **65**, 10139-10144 (2005).
28. Ishigami,S. *et al.* HLA-G expression in gastric cancer. *Anticancer Res.* **26**, 2467-2472 (2006).
29. Levy,E.M. *et al.* Human leukocyte antigen-E protein is overexpressed in primary human colorectal cancer. *Int. J. Oncol.* **32**, 633-641 (2008).

30. Champsaur,M. & Lanier,L.L. Effect of NKG2D ligand expression on host immune responses. *Immunol. Rev.* **235**, 267-285 (2010).
31. Bauer,S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727-729 (1999).
32. Cosman,D. *et al.* ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity.* **14**, 123-133 (2001).
33. Stern-Ginossar,N. & Mandelboim,O. An integrated view of the regulation of NKG2D ligands. *Immunology* **128**, 1-6 (2009).
34. McGilvray,R.W. *et al.* NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. *Clin. Cancer Res.* **15**, 6993-7002 (2009).
35. McGivary,R.W. *et al.* ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. *Int. J. Cancer*(2010).
36. Groh,V., Wu,J., Yee,C., & Spies,T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* **419**, 734-738 (2002).
37. Li,K. *et al.* Clinical significance of the NKG2D ligands, MICA/B and ULBP2 in ovarian cancer: high expression of ULBP2 is an indicator of poor prognosis. *Cancer Immunol. Immunother.* **58**, 641-652 (2009).
38. Dunn,G.P., Old,L.J., & Schreiber,R.D. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* **22**, 329-360 (2004).
39. Galon,J. *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
40. Al-Hajj,M., Wicha,M.S., Benito-Hernandez,A., Morrison,S.J., & Clarke,M.F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A* **100**, 3983-3988 (2003).
41. Teschendorff,A.E. *et al.* Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. *Genome Res.* **20**, 440-446 (2010).
42. Eppenberger-Castori,S. *et al.* Age-associated biomarker profiles of human breast cancer. *Int. J. Biochem. Cell Biol.* **34**, 1318-1330 (2002).
43. Nixon,A.J. *et al.* Relationship of patient age to pathologic features of the tumor and prognosis for patients with stage I or II breast cancer. *J. Clin. Oncol.* **12**, 888-894 (1994).
44. Carey,L.A. *et al.* Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* **295**, 2492-2502 (2006).
45. van de,W.W. *et al.* Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. *JAMA* **307**, 590-597 (2012).
46. Wildiers,H. *et al.* Management of breast cancer in elderly individuals: recommendations of the International Society of Geriatric Oncology. *Lancet Oncol.* **8**, 1101-1115 (2007).
47. Bastiaannet,E. *et al.* Breast cancer in elderly compared to younger patients in The Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. *Breast Cancer Res. Treat.* **124**, 801-807 (2010).
48. Bastiaannet,E. *et al.* Lack of survival gain for elderly women with breast cancer. *Oncologist.* **16**, 415-423 (2011).
49. Fulop,T. *et al.* Potential role of immunosenescence in cancer development. *Ann. N. Y. Acad. Sci.* **1197**, 158-165 (2010).
50. Zitvogel,L., Tesniere,A., & Kroemer,G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6**, 715-727 (2006).
51. Ehrlich P. Ueber den jetzigen Stand der Karzinomforschung. 5, 273-290. 1909. Ned. Tijdschr. Geneeskd.
52. Engel,A.M. *et al.* Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *APMIS* **104**, 629-639 (1996).
53. Smyth,M.J., Godfrey,D.I., & Trapani,J.A. A fresh look at tumor immunosurveillance and immunotherapy. *Nat. Immunol.* **2**, 293-299 (2001).
54. Shankaran,V. *et al.* IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107-1111 (2001).
55. Smyth,M.J. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* **191**, 661-668 (2000).
56. Hayakawa,Y., Rovero,S., Forni,G., & Smyth,M.J. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc. Natl. Acad. Sci. U. S. A* **100**, 9464-9469 (2003).
57. Gao,Y. *et al.* Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J. Exp. Med.* **198**, 433-442 (2003).
58. Aw,D., Silva,A.B., & Palmer,D.B. Immunosenescence: emerging challenges for an ageing population. *Immunology* **120**, 435-446 (2007).
59. Pawelec,G. *et al.* Human immunosenescence: is it infectious? *Immunol. Rev.* **205**, 257-268 (2005).

60. Campos,C. *et al.* Effect of age and CMV on NK cell subpopulations. *Exp. Gerontol.*(2014).
61. Gayoso,I. *et al.* Immunosenescence of human natural killer cells. *J. Innate. Immun.* **3**, 337-343 (2011).
62. Harris,L. *et al.* American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J. Clin. Oncol.* **25**, 5287-5312 (2007).
63. Michiels,S., Koscielny,S., & Hill,C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. *Lancet* **365**, 488-492 (2005).
64. van de Vijver M.J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med.* **347**, 1999-2009 (2002).
65. Sotiriou,C. & Pusztai,L. Gene-expression signatures in breast cancer. *N. Engl. J. Med.* **360**, 790-800 (2009).
66. DeNardo,D.G. *et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* **1**, 54-67 (2011).
67. Daidone,M.G., Coradini,D., Martelli,G., & Veneroni,S. Primary breast cancer in elderly women: biological profile and relation with clinical outcome. *Crit Rev. Oncol. Hematol.* **45**, 313-325 (2003).
68. Diab,S.G., Elledge,R.M., & Clark,G.M. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J. Natl. Cancer Inst.* **92**, 550-556 (2000).
69. Remvikos,Y., Magdelenat,H., & Dutrillaux,B. Genetic evolution of breast cancers. III: Age-dependent variations in the correlations between biological indicators of prognosis. *Breast Cancer Res. Treat.* **34**, 25-33 (1995).
70. Thomas,G.A. & Leonard,R.C. How age affects the biology of breast cancer. *Clin. Oncol. (R. Coll. Radiol.)* **21**, 81-85 (2009).

Chapter 10

Nederlandse samenvatting

SAMENVATTING

Introductie

Borstkanker is een van de meest gediagnostiseerde type kanker en de hoofddoodsoorzaak door kanker in vrouwen in de Westerse Wereld [1]. De behandeling bestaat uit locoregionale behandeling door middel van chirurgie al dan niet gevolgd door radiotherapie en systemische therapie zoals chemotherapie, endocriene therapie en immunotherapie. Systemische therapie wordt gegeven ter voorkoming van afstandmetastasen. Prognostische en predictieve factoren identificeren patiënten op respectievelijk hun voorspelde prognose en reactie op therapie en helpen hiermee bij klinische beslissingen omtrent systemische behandeling van borstkankerpatiënten.
Huidige prognostische factoren zoals leeftijd, menopausale status, tumorgrootte, lymfeklierstatus, histologische graad, hormoonreceptorstatus en HER2-overexpressie resulteren nog niet in optimale behandeling van borstkankerpatiënten [2]. Om over- en onder-behandeling te beperken zijn er daarom nieuwe en meer accurate prognostische factoren vereist.

Er is bewijs dat het adaptieve immuunsysteem tumorprogressie kan controleren [3]. Echter, tumoren kunnen, door hun intrinsieke genetische instabiele karakter, eigenschappen ontwikkelen om te ontsnappen aan dergelijke immuunherkenning en eliminatie [4]. Helaas is er weinig bekend over deze tumor-immuun-interacties in borstkanker. Daarbij kunnen deze interacties invloed hebben op tumorprogressie en zijn daarmee potentiële prognostische biomarkers.

Door de vergrijzing wordt borstkanker steeds meer een ziekte die oudere vrouwen aangrijpt [5]. Deze patiëntenpopulatie verschilt van de jongere borstkankerpatiëntenpopulatie in meerdere aspecten, waaronder co-morbiditeit en hiermee de balans van toxiciteit versus effectiviteit van behandeling, kortere levensverwachting en patiënt voorkeuren voor behandeling [6, 7]. Daarbij zijn er aanwijzingen dat tumoren van oudere borstkanker patiënten biologisch anders zijn: meer indolent, minder agressief en minder progressief [8]. Momenteel zijn behandelingsrichtlijnen voor borstkanker over het algemeen gebaseerd op onderzoek op een relatieve jongere patiëntenpopulatie en mist translationeel onderzoek geheel binnen deze populatie. Behandeling van oudere borstkanker patiënten is daarom nu niet evidence-based en translationeel onderzoek in deze patiëntenpopulatie is nodig.

Doel van het proefschrift

Dit proefschrift is in twee delen opgesplitst. **Deel I** beschrijft biomarkers gebaseerd op immuun-anti-tumorbescherming en tumor-immuun-ontsnapping in borstkanker en hun prognostische effect. **Deel II** beschrijft de distributie en prognostische effecten van essentiële prognostische biomarkers in oudere borstkanker patiënten.

Het onderzoek in dit proefschrift heeft gebruik gemaakt van een groot goed omschreven retrospectief borstkankercohort waarin alle vrouwen werden geselecteerd met niet-gemetastaseerde borstkanker die primair in het Leidsch Universitair Medisch Centrum werden geopereerd tussen 1985 en 1996. Dit cohort is voor alle studies gebruikt waardoor vergelijkingen en combinaties van markers mogelijk werd. Oudere borstkankerpatiënten werden gedefinieerd als patiënten , die de leeftijd van 65 jaar of ouder bereikt hebben, volgens de definities van de World Health Organisation (www.who.int).

Resultaten beschreven in dit proefschrift

Deel I van dit proefschrift beschrijft de interacties die er plaats vinden tussen borstkanker en het immuun systeem en de potentiele prognostische markers hierin. Er is veel bewijs dat het adaptieve immuun systeem tumorprogressie kan controleren [3]. Daarentegen is ook beschreven dat tumoren, door hun intrinsieke genetische instabiele karakter, eigenschappen kunnen ontwikkelen om te ontsnappen aan dergelijke immuunherkenning en eliminatie [4].

Humaan-leukocyt-antigen (HLA) klasse I kan tumor-geassocieerde-antigenen presenteren op de celmembraan van maligne cellen aan cytotoxische-T-lymfocyten (CTL). Om aan deze immuunherkenning te ontsnappen kunnen maligne cellen hun HLA klasse I expressie verliezen [9]. Daarnaast kunnen regulatoire-T-cellen (Treg) in de tumoromgeving een immuunsuppressief effect uitvoeren op CTL, waardoor aan de immuun-anti-tumorbescherming ontsnapt kan worden [10]. **Hoofdstuk 2** beschrijft dat verlies van HLA klasse I-expressie en aanwezigheid van Treg beide zeer frequent zijn in borstkankertumoren, wat suggereert dat immuun ontsnappingsmechanismen hierbij vaak voorkomen. Tevens werd een specifieke associatie gevonden voor HLA klasse I-expressieverlies en de aanwezigheid van Treg met een slechtere prognose in met chemotherapie behandelde patiënten. Dit kan verklaard worden door de gerichte eliminatie van Treg door cyclophosphamide-bevattende-chemotherapie danwel door een sneller herstel van de CTL-functie na deze behandeling. Hierdoor valt het immunosuppressieve effect op CTL, die voor de adjuvante therapie overheerste, weg waardoor deze CTL tumorcellen met HLA klasse I-expressie weer kunnen herkennen en elimineren.

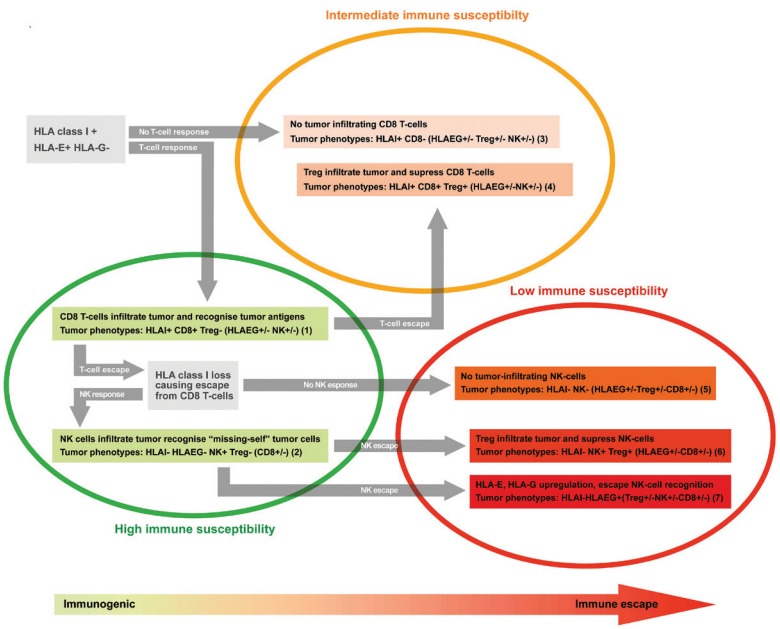
Als gevolg van HLA klasse I-expressieverlies kunnen maligne cellen door Natural Killer (NK-)cellen worden herkentd, waardoor volgende stappen in tumorimmuun ontsnapping gefocust zijn op ontwijking van NK-cel-aanval.

De niet-klassieke HLA-G moleculen komen specifiek tot expressie op trofoblast cellen waar het betrokken is bij immunotolerantie tegen de foetus gedurende de zwangerschap [11]. HLA-E komt tot expressie in bijna alle gezonde cellen, waar het eiwit fragmenten van klassiek HLA klasse I of HLA-G tot expressie brengt[12]. Beide niet-klassieke HLA-moleculen binden aan inhibitoire receptoren op NK-cellen, waarmee ze een belangrijk "zelf-signaal" geven. Maligne cellen kunnen HLA-G en HLA-E expressie verkrijgen of upreguleren. In **hoofdstuk 3** wordt beschreven dat expressie van deze moleculen in

borstkankertumoren resulteert in een slechtere prognose van patiënten, maar dan wel specifiek binnen patiënten wiens tumoren al HLA klasse I-expressie hadden verloren. Dit onderbouwt de hypothese van hun rol binnen "zelf-signalen" en NK-cel-inhibitie.

Activerende NK-cel-lectin-like-receptor-gen-2D (NKG2D) liganden hebben naast niet-klassieke HLA-moleculen ook invloed op NK-cel-herkenning en -eliminatie van pathologische cellen. Expressie van NKG2D-liganden wordt geïnduceerd in cellen onder stress, zoals infectie of maligne transformatie, waarop ze binden aan NKG2D-receptoren op onder andere NK-cellen en een activerende stimulerende respons wordt opgewekt, waardoor deze cellen onder stress geëlimineerd kunnen worden door het immuun systeem [13]. **Hoofdstuk 4** beschrijft dat voornamelijk de combinatie van expressie van twee NKG2D-liganden, MIC-AB en ULBP-2, resulteerde in een beter prognose van borstkankerpatiënten.

Gezien eerdere resultaten het belang hebben aangetoond van de complexiteit en interacties van het immuunsysteem met borstkanker (hoofdstuk 2-4), werd in **hoofdstuk 5** een biomarker gecreëerd op basis van de essentiële markers van immuun-anti-tumorbescherming en immuun ontsnapping hieraan (Figuur 1). Deze prognostische



Figuur 1: Deze figuur toont een schematisch overzicht van de verschillende fases van tumor-immuun ontsnapping. De tumorimmuun subtypes die dit weergeven zijn opgedeeld in 7 groepen, waarbij oplopend de gradaties van (1) tot (7) een hoge immuun vatbaarheid (groen) tot hogere immuun ontsnapping fases tonen (rood). Door middel van combinaties van gegevens van CTL infiltratie, NK-cell infiltratie, Treg infiltratie, klassieke HLA klasse I-expressie en HLA-EG-expressie werden de groepen gedefinieerd. Tumorimmuun subtypes werden geclusterd door groepen te combineren van de 7 afzonderlijke klasses; weergegeven door de omcirkelingen in de figuur: cluster (1) en (2) (groene cirkel), cluster (3) en (4) (oranje cirkel) en cluster (5), (6) en (7) (rode cirkel).

biomarker is zeer statistisch significant, waarbij patiënten met tumoren die werden beschouwd als laag immuun-vatbaar een slechtere prognose hadden dan patiënten met tumoren die als hoog immuun-vatbaar werden beschouwd. Dit prognostische effect was onafhankelijk van bekende prognostische factoren en werd afzonderlijk gevalideerd in een onafhankelijk validatie cohort. Hierbij werd het hoog discriminatieve vermogen nogmaals bevestigd. Tevens werd hierdoor het belang van de interacties binnen en tussen het immuunsysteem en borstkanker weer benadrukt.

Het tweede deel van het proefschrift beschrijft de verschillen in de verdeling en het prognostisch effect van verschillende biomarkers in oudere borstkankerpatiënten.
Kanker-stamcellen zijn gedefinieerd als een kleine subset van de tumor met specieke stamcel-karaketeristieken zoals epitheliale-naar-mesenchymale transitie, de capaciteit tot zelfvernieuwing en -differentiatie [14]. In **hoofdstuk 6** hebben we aangetoond dat de expressie van ALDH-1, een respresentatieve marker voor stamcellen, significant hoger is in jongere borstkankerpatiënten vergeleken met oudere borstkankerpatiënten. Daarbij was ALDH-1-expressie een onafhankelijke risicofactor voor een slechtere prognose in jongere borstkankerpatiënten, maar niet in ouderen. Een verklaring voor de specifieke leeftijd-interactie van het prognostische effect van ALDH-1 kan de verandering van de tumor-micro-omgeving in oudere borstkankerpatiënten zijn, waarbij signalen tussen stamcellen en deze omgeving of metabole processen niet optimaal verlopen en de tumor-stamcellen gelimiteerd functioneren [15].

Borstkanker wordt onderverdeeld in verschillende biologische/moleculaire typen op basis van genexpressie studies[16], deze zijn: Luminal-A, Luminal-B, Basal-like, ERBB2 en unclassified. Deze groepen hebben allen een verschillende prognose. In **hoofdstuk 7** werden de moleculaire subtypes gevalideerd in oudere borstkankerpatiënten. Aangetoond werd dat de subtypes Luminal-A en Luminal-B vaker werden gezien in oudere borstkankerpatiënten. In tegenstelling tot de jongere borstkankerpatiënten, werd geen associatie gezien tussen deze subtypes en prognose. Dit zou verklaard kunnen worden door het competitieve risico op overlijden in ouderen patiënten, waarbij patiënten vaker overlijden met borstkanker in plaats van aan borstkanker. Gezien 60% van deze oudere borstkankerpatiënten overlijdt aan borstkanker is hierdoor het prognostische effect van alle biomarkers gelimiteerd en verzwakt.

In het eerste deel van dit proefschrift werd het belang van het immuunsysteem in de ontwikkeling en progressie van een maligniteit aangetoond. Ouder worden resulteert in een verminderd functioneren van het adaptieve immuunsysteem en daarom is er de suggestie dat deze processen van "immunosenescence" mogelijk bijdragen aan het ontstaan en de progressie van kanker [17]. **Hoofdstuk 8** laat zien dat er geen verschillen zijn in infiltrerende CTL, NK-cellen of Tregs in de oudere populatie vergeleken met de jongere populatie. Er werd een trend gezien naar minder klassieke HLA klasse

I-afschakeling en daarbij statistisch significant minder HLA-E en HLA-G-expressie in deze oudere populatie. Ook werden er minder laag immuun-vatbare-tumoren gezien in deze patiënten. Deze resultaten suggereren een verzwakte immuun-selectieve-druk op borstkankertumoren op hogere leeftijd, waardoor deze minder immuun-ontsnappingsmechanismen nodig hebben. Tevens werd geen statistisch significante associatie met prognose gevonden voor tumorimmuun subtypes in oudere borstkankerpatiënten, in tegenstelling tot de jongere populatie. De vergelijkbare prognoses in oudere borstkankerpatiënten van de verschillende tumorimmuun subtypes suggereert dat het immuunsysteem minder goed functioneert in deze patiënten en ondersteunt verder de immunosensescence hypothese en de rol hiervan in tumor ontwikkeling en progressie.

Conclusies en toekomstperspectieven

Immunoediting en immunosenescense hypothesen:

Het concept dat het immuunsysteem tumoren kan herkennen en elimineren bestaat al meer dan 100 jaar [18]. In het begin van de 21ste eeuw werd inzicht in de interacties van het immuunsysteem met maligne tumoren duidelijker toen de drie fasen van interactie: "eliminatie", "equilibrium" en "escape" onder de "kanker immunoediting" hypothese werden beschreven [4]. Tumorcellen kunnen succesvol geëlimineerd worden door het immuunsysteem gedurende de eliminatie-fase. Als tumorcellen weten te ontsnappen aan deze eerste immuunaanval, begint de volgende fase: equilibrium. Gedurende deze fase is er een constante interactie tussen het immuunsysteem en de tumor, die uit snel muterende en genetisch instabiele cellen bestaat. Tumorcellen die vatbaar zijn voor immuunherkenning en -aanval worden in deze fase geëlimineerd, maar tevens ontstaan er nieuwe tumorvarianten die eigenschappen hebben ontwikkeld om hieraan te ontsnappen. Deze constante interacties leiden ertoe dat de tumoropmaak constant gevormd wordt door het immuunsysteem. Indien tumorcellen genoeg immuun-ontsnappingsmechanismen hebben ontwikkeld, kunnen zij uiteindelijk de finale "escape"-fase bereiken. Deze laatste fase is gekarakteriseerd door de winst van de maligne tumor in de balans met het immuunsysteem, waar de groei van tumoren door kan zetten en zij klinisch detecteerbaar worden. Verdere ontwikkeling van significante immuun-ontsnappingsmechanismen die hierop volgen bevorderen de verdere progressie van de tumoren. Deze ontsnappingsmechanismen zijn complex en bestaan in het kort uit: klassieke HLA klasse I-afschakeling, HLA-E en HLA-G-upregulatie, downregulatie van stressmoleculen NKG2D-liganden en aantrekking van immunosuppressieve Treg [4]. Meerdere muisstudies hebben veel bewijs en onderbouwing aangeleverd voor de immunoediting-theorie, maar data van humane studies ontbreekt [19]. Hoewel onze resultaten geen absoluut bewijs zijn voor een immunoediting-hypothese, suggereren onze data wel sterk dat een dergelijk fenomeen zich afspeelt in borstkanker.

Wij hebben aangetoond dat hoog immuun-vatbare-tumoren geassocieerd zijn met een goede prognose van patiënten, terwijl immuun-ontsnappingsvarianten resulteerden in een slechte prognose. Daarbij is het belangrijk dat onze resultaten aantonen dat de verschillende factoren, die betrokken zijn in het samenspel van de tumor met het immuunsysteem, interacties met elkaar vertonen, waarmee het belang van pathway-analyse wordt benadrukt.

Met toenemende leeftijd neemt de effectiviteit van het immuunsysteem af, waardoor minder goed gereageerd kan worden op infecties en vaccinaties [17]. Er wordt gesuggereerd dat deze immunosenescence-mechanismen mogelijk samenhangen met kanker-ontwikkeling en -progressie. Een dergelijk fenomeen is puur hypothetisch, maar de lagere frequentie in immuunontsnappingsvarianten in ouderen en het verdwenen prognostische effect van immuun subtypes in deze populatie, zoals gevonden in onze studie, zijn in overeenstemming hiermee.

Uit onze resultaten blijkt dat het ontrafelen van de complexe interacties van het immuunsysteem in tumorontwikkeling en -progressie tot waardevolle nieuwe inzichten in tumorbiologie brengt. Deze nieuwe inzichten hebben een hoog potentieel om te leiden tot prognostische en predictieve biomarkers en kunnen mogelijk de basis vormen van toekomstige immunotherapeutische behandelingen. Toekomstig onderzoek zal immunoediting en immunosenescence hypothesen in humane modellen moeten bewijzen.

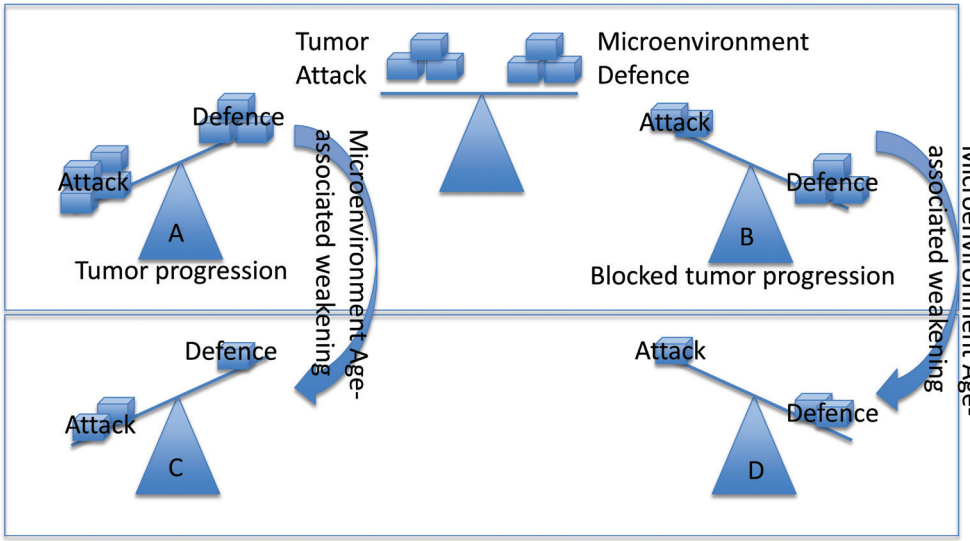
Tumorimmuun subtypes als prognostische biomarker in borstkanker:

Momenteel zijn er veel prognostische biomarkers in borstkanker. De ASCO richtlijnen hebben het gebruik voor in de klinische praktijk geadviseerd voor urokinases plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1) en gen-expressieprofielen gedetecteerd met multiparameter gen-expressie-arrays [20]. De klinische waarde van microarray-gebaseerde prognostische methoden, zoals de MammaPrint, een 70-genen expressieprofiel, en Oncotype DX, een 21-genen expressieprofiel wordt momenteel nog onderzocht in fase drie studes[21, 22]. Een belangrijk punt van kritiek is dat deze genenprints zijn geconstrueerd door top-down-analyses, waardoor het onduidelijk is wat voor tumortypes worden gerepresenteerd door de verschillende patiënt-risico-groepen en het zeer lastig is genprofielen biologisch te onderbouwen[23]. Daarnaast bevat deze methode veel "ruis" door de grote kans op toeval in de statistische constructie en optimalisatie van dergelijke parameters. Hierdoor is er een zeer hoge kans op een verlies aan discrimintatief vermogen bij de validatie. Bij bottom-up-analyses wordt een biologische factor gecorreleerd aan patiënt prognose waaroor er een veel hogere kans is op het vinden van lagere discriminatieve vermogens of het niet vinden van prognostische associaties. Als een prognostische biomaker wordt gevonden met deze laatste methode geeft dit een bepaalde mate van betrouwbaarheid en controleerbaarheid

door de onderliggende biologische rationale. In het eerste deel van dit proefschrift worden verschillende prognostische biomarkers beschreven die met de bottom-up-analyses gevonden zijn en gebaseerd zijn op biologische hypotheses en theorieën van borstkanker-immunoediting. De uiteindelijke tumorimmuun subtypes hebben geleid tot een onafhankelijke statistisch significante prognostische variabele met een hoog onderscheidend vermogen, vergelijkbaar met die van genexpressie-profielen. Daarbij gaf onafhankelijke validatie van de tumorimmuun subtypes vergelijkbare resultaten. Het predictieve effect van HLA klasse I en Treg op chemotherapie respons, zoals eerder beschreven en bewezen in ons onderzoek, geeft een indicatie van het belang en het effect van het immuunsysteem op therapierespons. Hiermee zijn de tumorimmuun subtypes potentiele predictieve biomarkers voor bestaande systemische therapie als ook natuurlijk potentiele immunotherapieën [24]. Dergelijke effecten op therapierespons zijn nog niet onderzocht en blijven daarom speculatief. Een huidig nadeel aan de tumorimmuun subtypes, waardoor zij nog niet geïmplementeerd kunnen worden in klinische praktijk, is de bewerkelijkheid van de bepaling waarin meerdere immunohistochemische kleuringen en handmatige kwantificaties plaats moeten vinden. Daarbij zijn de tumorimmuun subtypes niet prognostisch in oudere borstkanker patiënten. Dit kan gedeeltelijk worden verklaard, zoals eerder omschreven, door immunosenescence of door de competitieve risico's op overlijden in oudere borstkanker patiënten.

Biologische verschillen in borstkanker in ouderen in vergelijk met jongeren:
Er zijn verschillen beschreven in patiënt, tumor en behandelingskarakteristieken tussen oudere en jongere borstkanker patiënten [8, 25]. Dit kan komen door verschillen in onderliggende tumorbiologie. Het is daarom vaak gesuggereerd dat borstkanker in ouderen patiënten een biologisch verschillend tumortype is met een meer indolent en minder agressief en progressief karakter vergeleken met jongere patiënten. Deze hypothese spreekt recente resultaten echter tegen waarin bij toenemende leeftijd een slechtere borstkanker specifieke overleving werd gevonden [26]; oudere borstkankerpatiënten overleden vaker aan borstkanker ongeacht het hogere risico op mortaliteit door andere oorzaken en onafhankelijk van bekende tumor- en patiëntkarakteristieken. Onze studies toonden verschillen in distributie van essentiële biomarkers, waar minder agressieve fenotypes (minder ALDH-1 positiviteit, luminal moleculaire subtypes en minder tumorimmuun ontsnappingsvarianten) werden gevonden in oudere in vergelijk met jongere borstkankerpatiënten. Een verklaring in de tegenstrijdige resultaten kan worden gevonden in de interacties tussen tumoren en hun micro-omgeving (Figuur 2).

De uitkomst van de balans tussen tumoren en hun micro-omgeving, zoals weergegeven in Figuur 2, resulteert in de prognose van patiënten: meer tumor agressiviteit resulteert in meer tumor progressie en daardoor in een verwachte slechtere prognose voor patiënten (A), lage tumor agressiviteit zou moeten resulteren in een geblokkeerde tumor progressie door een goed functionerende beschermende micro-omgeving waardoor een



Figuur 2: Deze figuur geeft schematisch de balans tussen de agressiviteit van de aanval van de tumor ten opzichte van de mate van verdediging van de tumor micro-omgeving hiertegen. Het aantal kubussen in de figuur geeft de "zwaarte" of sterkte weer van de aanval, en daarmee de progressie en invasie, van de tumor (links in de balans) en de verdediging van de micro-omgeving hiertegen (rechts in de balans). De uiteindelijke balans geeft het eindproduct hiervan weer waarbij: (1) de winst aan de aanval van de tumor kan vallen (weergegeven in (A) en (C)), resulterend in tumor progressie of (2) de winst aan de verdediging van de micro-omgeving kan vallen (weergegeven in (B) en (D)), resulterend in een geblokkeerde tumor progressie. (C) en (D) geven hypothetische situaties in oudere patiënten weer, waar de tumor micro-omgeving duidelijk verzwakt is ten op zichte van de micro-omgeving in jongere patiënten zoals weergegeven in (A) en (B).

betere prognose van patiënten verwacht is (B). Echter, hoewel dit vaak niet het geval is in jongere patiënten, speelt in oudere patiënten het minder goed functioneren van de verdedigingsmechanismen tegen tumoren een belangrijke rol. De situatie waarin gesuggereerd wordt dat tumoren in oudere borstkankerpatiënten een meer indolent karakter hebben en daardoor zouden moeten lijden tot een betere prognose vergeleken met jongere patiënten wordt weergegeven door situatie B of D in Figuur 2, waarbij respectievelijk de micro-omgeving en verdedigingsmechanismen even sterk worden geacht als in jongere patiënten (B) en waarin de verdedingsmechanismen door de leeftijd minder goed functionerend zijn, maar de tumor agressiviteit vanwege de leeftijdstoename nog meer is afgenomen (D). De combinatie van een minder agressieve tumor met een sterk verminderde verdediging van de micro-omgeving resulteert in tumorprogressie en daardoor in een verwachte slechtere patiënt-prognose (C). Deze laatste situatie, waarin de micro-omgeving een sterkere achteruitgang vertoont dan de tumoragressiviteit, kan verklaren waarom minder agressieve tumoren gecombineerd met een verslechterde patiënt-prognose gevonden worden in oudere borstkankerpatiënten. Veranderingen in de omgeving van de tumor, de tumor-micro-omgeving, lijkt de brug te vormen tussen de tegenstrijdige resultaten van een minder agressieve tumor met een slechtere patiënt prognose in oudere borstkankerpatiënten. In dit scenario is het niet zo zeer de toegenomen

maligniteit van tumoren, maar de verzwakking in verdedigingsmechanismen die de tumor progressie en daarmee de prognose van de patiënten bepalen. Veranderingen in de micro-omgeving kan leiden tot een verslechtering van signalering met tumor-stamcellen, waardoor een verminderde functie van deze cellen gezien kan worden en verschillen in prognostische effecten van ALDH-1 verklaard kunnen worden in oudere borstkankerpatiënten vergeleken met jongeren. Daarbij zijn de verschillen in distributie en prognostisch effect van de tumorimmuun subtypes suggestief voor onderliggende verandering in de micro-omgeving door processen als immunosenesecence.

Prognostische biomarkers in oudere borstkanker patiënten:

Geen van de biomarkers die onderzocht zijn in dit proefschrift hebben een statistisch significant prognostisch effect in oudere borstkankerpatiënten. Dit zou verklaard kunnen worden door een combinatie van onderliggende verschillen in tumorbiologie en -gedrag, verzwakking van statistische power door verschillen in distributie van biomarkers in subcategorieën en competitieve risico's op overlijden. Geen van deze verklaringen kan door de resultaten van dit proefschrift worden uitgesloten danwel worden bevestigd. Het feit blijft dat de klinische waarde van prognostische biomarkers, gelimiteerd is in de oudere borstkankerpopulatie. We concluderen daarom ook dat validatie van biomarkers in ouderen een vereiste is, gezien de mogelijke verschillen in micro-omgeving en daarbij competitieve overlijdensrisico's invloed kunnen hebben op prognostische effecten; wat uiteindelijk significant interfereert met patiënt behandelingsmodaliteiten. Het is echter belangrijk dat prognostische biomarkers alleen een prognostisch effect kunnen hebben in patiënten wiens levensverwachting lang genoeg is en wiens competitieve overlijdensrisico daarom laag is. . Het is alleen in deze "fitte" oudere borstkankerpatiënten dat biomarkers verschillen in prognose kunnen weergeven en hiermee kunnen helpen in klinische beslissingen omtrent systemische therapie. Het identificeren van deze patiënten zou daarom een eerste stap zijn in de creatie van evidence-based behandeling op maat van oudere borstkankerpatiënten.

Reference List

1. Jemal,A. *et al.* Cancer statistics, 2008. *CA Cancer J. Clin.* **58**, 71-96 (2008).
2. Goldhirsch,A. *et al.* Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. *Ann. Oncol.* **20**, 1319-1329 (2009).
3. Zitvogel,L., Tesniere,A., & Kroemer,G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6**, 715-727 (2006).
4. Dunn,G.P., Old,L.J., & Schreiber,R.D. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* **22**, 329-360 (2004).
5. Petrakis,I.E. & Paraskakis,S. Breast cancer in the elderly. *Arch. Gerontol. Geriatr.* **50**, 179-184 (2010).
6. Albrand,G. & Terret,C. Early breast cancer in the elderly: assessment and management considerations. *Drugs Aging* **25**, 35-45 (2008).
7. Wildiers,H. *et al.* Management of breast cancer in elderly individuals: recommendations of the International Society of Geriatric Oncology. *Lancet Oncol.* **8**, 1101-1115 (2007).
8. Diab,S.G., Elledge,R.M., & Clark,G.M. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J. Natl. Cancer Inst.* **92**, 550-556 (2000).
9. Algarra,I., Garcia-Lora,A., Cabrera,T., Ruiz-Cabello,F., & Garrido,F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol. Immunother.* **53**, 904-910 (2004).
10. Cerwenka,A., Baron,J.L., & Lanier,L.L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. U. S. A* **98**, 11521-11526 (2001).
11. Kovats,S. *et al.* A class I antigen, HLA-G, expressed in human trophoblasts. *Science* **248**, 220-223 (1990).
12. Wei,X.H. & Orr,H.T. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Hum. Immunol.* **29**, 131-142 (1990).
13. Champsaur,M. & Lanier,L.L. Effect of NKG2D ligand expression on host immune responses. *Immunol. Rev.* **235**, 267-285 (2010).
14. Al-Hajj,M., Wicha,M.S., Benito-Hernandez,A., Morrison,S.J., & Clarke,M.F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A* **100**, 3983-3988 (2003).
15. Teschendorff,A.E. *et al.* Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. *Genome Res.* **20**, 440-446 (2010).
16. Perou,C.M. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747-752 (2000).
17. Fulop,T. *et al.* Potential role of immunosenescence in cancer development. *Ann. N. Y. Acad. Sci.* **1197**, 158-165 (2010).
18. Ehrlich P. Ueber den jetzigen Stand der Karzinomforschung. 5, 273-290. 1909. *Ned. Tijdschr. Geneeskd.*
19. Dunn,G.P., Old,L.J., & Schreiber,R.D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity.* **21**, 137-148 (2004).
20. Harris,L. *et al.* American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J. Clin. Oncol.* **25**, 5287-5312 (2007).
21. Michiels,S., Koscielny,S., & Hill,C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. *Lancet* **365**, 488-492 (2005).
22. van de Vijver M.J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med.* **347**, 1999-2009 (2002).
23. Sotiriou,C. & Pusztai,L. Gene-expression signatures in breast cancer. *N. Engl. J. Med.* **360**, 790-800 (2009).
24. DeNardo,D.G. *et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* **1**, 54-67 (2011).
25. Remvikos,Y., Magdelenat,H., & Dutrillaux,B. Genetic evolution of breast cancers. III: Age-dependent variations in the correlations between biological indicators of prognosis. *Breast Cancer Res. Treat.* **34**, 25-33 (1995).
26. van de Water W. *et al.* Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. *JAMA* **307**, 590-597 (2012).

Appendices

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LIST OF PUBLICATIONS

E.M. de Kruijf, J.G.H. van Nes, A. Sajet, Q.R.J.G. Tummers, H. Putter, S. Osanto, F.M. Speetjens, V.T.H.B.M. Smit, G.J. Liefers, C.J.H. van de Velde, P.J.K. Kuppen; The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *(Clin Cancer Res. 2010 Feb)*

E.M. de Kruijf, J.G.H. van Nes, C.J.H.. van de Velde, H. Putter, V.T.H.B.M Smit, G.J. Liefers, P.J.K. Kuppen, R.A.E.M. Tollenaar, W.E. Mesker; Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. *(Breast Cancer Res Treat. 2010 Apr)*

van Nes J.G.H., **E.M. de Kruijf**, Faratian D., van de Velde C.J.H., Putter H., Falconer C., Smit V.T.H.B.M., Kay C, van de Vijver M.J., Kuppen P.J.K., Bartlett J.M.; COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. *(Breast Cancer Res Treat. 2010 Apr)*

E.M. de Kruijf, A. Sajet, J.G.H. van Nes, R. Natanov, H. Putter, V.T.H.B.M. Smit, G.J. Liefers, P.J. van den Elsen, C.J.H. van de Velde, P.J.K. Kuppen; HLA-E and HLA-G expression in classical HLA class I negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *(Journal of Immunology 2010 Dec)*

van Hoesel A.Q., van de Velde C.J.H., Kuppen P.J.K., Putter H., **E.M. de Kruijf**, van Nes J.G.H., Giuliano A.E., Hoon D.S.; Primary tumor classification according to methylation pattern is prognostic in patients with early stage ER-negative breast cancer. (Breast Cancer Research and Treatment 2012 Feb)

P.E.A. Huijts, M. Van Dongen, M.C.M. de Goeij, A. Van Moolenbroek, F. Blanken, M.P.G. Vreeswijk, **E.M. de Kruijf**, W.E. Mesker, E.W. van Zwet, R.A.E.M. Tollenaar, V.T.H.B.M. Smit, C.J. van Asperen, P. Devilee; Allele-specific regulation of FGFR2 expression is cell type-dependent and may increase breast cancer risk through a paracrine stimulus involving FGF10. *(Breast Cancer Research 2011 Jul)*

E.M. de Kruijf, van Nes JG, Putter H, Faratian D, Munro A, Campbell F, Smit VT, Liefers GJ, Kuppen PJ, van de Velde CJ, Bartlett JM.; Co-expression of SNAIL and TWIST determines prognosis in estrogen receptor-positive early breast cancer patients. *(Breast Cancer Research and Treatment 2012 May)*

E.M. de Kruijf, Sajet A, van Nes JG, Putter H, Smit VT, Eagle RA, Jafferji I, Trowsdale J, Liefers GJ, van de Velde CJ, Kuppen PJ.; NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study. *(BMC Cancer 2012 Jan)*

E.M. de Kruijf, Mieog JS, Bastiaannet E, Kuppen PJ, Sajet A, de Craen AJ, Smit VT, van de Velde CJ, Liefers GJ.; Age determines the prognostic role of the cancer stem cell marker aldehyde dehydrogenase-1 in breast cancer. *(BMC Cancer 2012 Jan)*

van Hoesel AQ, van de Velde CJ, Kuppen PJ, Liefers GJ, Putter H, Sato Y, Elashoff DA, Turner RR, Shamonki JM, **E.M. de Kruijf**, van Nes JG, Giuliano AE, Hoon DS.; Hypomethylation of LINE-1 in primary tumor has poor prognosis in young breast cancer patients: a retrospective cohort study. *(Breast Cancer Research and Treatment 2012 Aug)*

E.M. de Kruijf, T.J.A. Dekker, L.J.A.C. Hawinkels, H. Putter, V.T.H.B.M. Smit, J.R. Kroep, J.W.R. Nortier , P.J.K. Kuppen, C.J.H. van de Velde, P. ten Dijke, R.A.E.M. Tollenaar, W.E. Mesker ; The prognostic role of TGF-β signaling pathway in breast cancer patients *(Annals of Oncology 2012 Sept)*

E.M. de Kruijf, S. Saadatmand, A. Sajet, N.G. Dekker-Ensink, J.G.H. van Nes, H. Putter, V.T.H.B.M. Smit, C.J.H. van de Velde, G.J. Liefers, P.J.K. Kuppen; Expression of Cell Adhesion Molecules Predicts Prognosis in Breast Cancer *(British journal of Surgery 2013 Jan)*

B. Kocaturk, YW van den Berg, C Tieken, JS Mieog, **E.M. de Kruijf**, CC Engels, MA van der Ent, PJ Kuppen, CJ van de Velde, W Ruf, PH Reitsma, S Osanto, GJ Liefers, VY Bogdanov, HH Versteeg; Alternatively spliced tissue factor promotes breast cancer growth in a beta1 integrin-dependent manner *(Proc Natl Acad Sci 2013 Jul)*

L Zhang, F Zhou, A Garcia de Vinuesa, **E.M. de Kruijf**, WE Mesker, L Hui, Y Drabsch, Y Li, A Bauer, A Rousseau, KA Sheppard, C Mickanin, PJ Kuppen, CX Lu, P ten Dijke; TRAF4 promotes TGF-beta receptor signaling and drives breast cancer metastasis *(Mol Cell 2013 sept)*

E.M. de Kruijf, C.C. Engels, W. van de Water, E. Bastiaannet, V.T.H.B.M. Smit, C.J.H. van de Velde, G.J. Liefers, P.J.K. Kuppen; Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients *(Breast Cancer Res Treat 2013 Nov)*

CC Engels, F Ruberta, **E.M. de Kruijf**, GW van Pelt, VT Smit, GJ Liefers, T Matsushima, M Shibayama, H Ishihara, CJ van de Velde, PJ Kuppen; The prognostic value of apoptotic and proliferative markers in breast cancern *(Breast Cancer Res Treat 2013 Nov)*

E.M. de Kruijf, E. Bastiaannet, F. Rubertá, A.J.M. de Craen, P.J.K. Kuppen, V.TH.B.M Smit, C.J.H. van de Velde, G.J. Liefers; Comparison of frequencies and prognostic effect of molecular subtypes between young and elderly breast cancer patients *(Mol Onc 2014 Jan)*

E.M. de Kruijf, W. van de Water, E. Bastiaannet, C.C. Engels, A.J.M. de Craen, P.J.K. Kuppen, V.T.H.B.M. Smit, C.J.H. van de Velde, G.J. Liefers; Comparison of prognostic effect of adaptive tumor immune response between young and elderly breast cancer patients *(Submitted Clin Can Res 2014 Sept)*

C.C. Engels, D.B. Fontein, P.J.K. Kuppen, E.M. de Kruijf, V.T.H.B.M. Smit, J.W. Nortier, G.J. Liefers, C.J.H. van de Velde, E. Bastiaannet; Immunological subtypes in breast cancer are prognostic for invasive ductal but not for invasive lobular breast carcinoma *(Br J Cancer. 2014 Jul)*

R.S. Derr, A.Q. van Hoesel, A. Benard, I.J. Goossens-Beumer, A. Sajet, N.G. Dekker-Ensink, **E.M. de Kruijf**, E. Bastiaannet, V.T.H.B.M. Smit, C.J.H. van de Velde, P.J.K. Kuppen; High nuclear expression levels of histone-modifying enzymes LSD1, HDAC2 and SIRT1 in tumor cells correlate with decreased survival and increased relapse in breast cancer patients *(BMC Cancer 2014 Aug)*

W.E. Mesker, T.J.A. Dekker, **E.M. de Kruijf**, C.C. Engels, G.W. van Pelt, V.T.H.B.M. Smit, R.A.E.M. Tollenaar; Comment on "The prognostic significance of tumour-stroma ratio in oestrogen receptor-positive breast cancer" *(Accepted for publication BJCancer 2014 Sept)*

E.M. de Kruijf, Peter J.K.Kuppen; Considerations on the prognostic and predictive significance of HLA-G in breast cancer *(Accepted for publication Br Ca Management 2014 Aug)*

CURRICULUM VITAE

Esther de Kruijf was born on February 23rd 1985 in Angera, Italy. She was raised partially in Italy and in the Netherlands, where she obtained her Baccalaureat from the European School of Bergen in 2003. In the same year she commenced studying medicine at the University of Leiden. During her medical training she conducted research as an internship under the guidance of dr. P.J.K. Kuppen. During her internship she became enthusiast for research on the interactions of the immune system and breast cancer and elderly breast cancer. After completing her preclinical training in medicine in 2007 she therefore started as a PhD-student at the surgical department at the Leiden University Medical Center under the supervision of prof. Dr. C.J.H. van de Velde, dr. G.J. Liefers and dr. P.J.K. Kuppen. This research resulted in the current thesis. In December 2011 she started het clinical rotations and obtained het medical degree in February 2014. She will start her residency in general surgery in Lugano Switserland in January 2016.

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