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).