The Use of Immunological Biomarkers to Improve Individualization of Postoperative Radiotherapy in Breast Cancer

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To my grandmother and other breast cancer victims

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ABSTRACT

Radiotherapy (RT) forms the cornerstone of most curative breast cancer treatment due to its well-established risk-reducing effect on local recurrences at the population level. However, there is heterogeneity regarding treatment benefits at the individual level, and research currently aims to better tailor treatment decisions based on tumor biology. This thesis aimed to investigate if immunological biomarkers from the primary tumor can be used to predict the benefit from RT at the individual level and improve treatment individualization.

Study I: Tumor blocks were collected from the randomized SweBCG91RT cohort, and tumor-infiltrating lymphocytes (TILs) were assessed on whole sections. High levels of TILs were associated with a reduced risk of local recurrence (HR 0.61, CI 95% 0.39-0.96, p=0.033) and a non-significant benefit from RT (HR 0.58, CI 95% 0.28-1.19, p=0.138).

Study II: We evaluated CD8+ and FOXP3+ T cells on tissue microarrays (TMAs) to further characterize the lymphocytic immune infiltrate. We found that unirradiated patients with high levels of CD8 and low levels of FOXP3 had a reduced risk of a local recurrence (HR: 0.41, CI 95% 0.19-0.86, p= 0.018 compared to patients without an immune infiltrate

(HR 1.0, reference) and appeared to derive a reduced benefit from RT (HR 0.60, CI 95% 0.18-2.0, p=0.41) compared to patients with immune-depleted tumors (HR 0.37, CI 95% 0.24-0.57, p<0.001). Gene expression analyses of anti- and protumoral immune cells yielded similar results.

Studies III: We hypothesized that tumor-intrinsic factors modulate an immune infiltrate's biological implications and that immunological biomarkers' predictions would be improved by incorporating this information. We used gene expression analysis to develop two models capturing tumor-intrinsic and immunological characteristics, respectively. We found that the integration of these two dimensions improved the identification of patients with differing RT benefits. We refer to the unpublished manuscript for additional details.

Study IV: Expanding on the notion that an enhanced patient stratification for RT benefit is achieved by integrating markers of tumor aggressiveness and immunological activation, we used the clinically available biomarkers to characterize the state of the immune infiltrate and the aggressiveness of the tumor. Again, we observed that this had the potential to improve RT individualization. We refer to the unpublished manuscript for additional details.

Immunological biomarkers from the primary tumor provide independent information on the risk of local recurrences, which can be used to stratify patients according to the need for postoperative radiotherapy. An immune infiltrate's implications depend on tumor-intrinsic characteristics, and successful implementation of immunological biomarkers in clinical practice, therefore, requires a co-analysis of such factors. Tumors with an activated immune response may have a low risk of a local recurrence and constitute a group where de-escalation of RT may be feasible.

Keywords: Tumor-infiltrating lymphocytes, CD8 T cells, FOXP3 T regulatory cells, radiotherapy, local recurrence, tumor-intrinsic characteristics

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SAMMANFATTNING PÅ SVENSKA

Bröstcancer behandlas i dagsläget generellt av en kombination av kirurgi, läkemedel, och strålbehandling. Genom strålbehandling kan man generellt sett undvika lokala återfall trots en mindre extensiv operationsmetod, så kallad bröstbevarande kirurgi. Strålbehandling utgör i dagsläget därför en central komponent av bröstcancerbehandlingen och gör stor nytta på populationsnivå. På individnivå varierar dock nyttan av strålbehandling mycket. De flesta patienterna botas utan strålbehandling medan en minoritet får ett lokalrecidiv trots strålbehandling. Det finns därför behov av att utveckla nya metoder som möjliggör en bättre behandlingsindividualisering där patienter som inte behöver strålbehandling kan besparas behandlingen medan de som får återfall trots behandling kan ges en utökad behandling. Syftet med denna avhandling var att undersöka om en karakterisering av patientens egna immunsvar mot tumören tillför information som kan användas för att förstå behovet av strålbehandling på individnivå.

I första delstudien undersökte vi hur mått på lymfocyter i tumören (så kallade tumör-infiltrerande lymfocyter) påverkar risken för lokalrecidiv och nyttan av strålbehandling. Lymfocyter är namnet på gruppen av immunceller som tros vara viktigast för immunsystemets förmåga att bekämpa cancerceller. Vi fann att höga nivåer av tumör-infiltrerande lymfocyter innebar en förbättrad prognos och tecken till ett minskat behov av strålbehandling.

I delstudie två undersökte vi om en djupare karakterisering av tumörinfiltrerande lymfocyter kunde tillföra ytterligare information. Vi studerade hur balansen mellan CD8+ T-lymfocyter, som tros hämma tumörväxten, och FOXP3+ T-regulatoriska lymfocyter, som tros gynna tumörväxten, påverkade risken för återfall. Vi fann att patienter med höga nivåer av CD8+ T-lymfocyter och låga nivåer av FOXP3+ T-regulatoriska lymfocyter hade bäst prognos och minst nytta av strålbehandling. Ytterligare analyser av genuttrycket i tumören gav en liknande bild om att tumörer med ett effektivt immunsvar har bättre prognos och mindre nytta av strålbehandling.

I den tredje delstudien inkluderade vi analyser av tumörens genuttryck i försök att bättre kunna förutsäga i vilka tumörer det fanns bra förutsättningarna för att ett gynnsamt immunsvar skulle kunna etableras.

Alla patienter med tumör-infiltrerande lymfocyter har inte en förbättrad prognos och vi ville undersöka om dessa gick att identifiera. Immunceller reagerar mot förändrade proteiner som uttrycks av tumörceller och det är sannolikt därför främst i tumörer med ett brett uttryck av abnorma proteiner som immunsvaret gör störst nytta. Vi fann att tumörer med aggressiva karakteristika hade störst nytta av en immunologisk aktivering. Dessa hade en god prognos och ett sänkt behov av strålbehandling. Vidare var det tumörer med aggressiva karakteristika där ett aktiverat immuninfiltrat saknades som hade sämst prognos.

I den fjärde och sista delstudien undersökte vi om vi genom vad vi hade lärt oss från studier I-III kunde åstadkomma en förbättrad behandlingsindividualisering genom att använda immunologiska och tumör-relaterade variabler som redan används i kliniken. Vi biomarkörer som ses vid ett aktiverat immunsvar för att karakterisera immunsvaret. Vidare använde vi histologisk grad, som är en välkänd aggressivitetsmarkör, för att karakterisera tumörens egenskaper. Vi fann att ett aktiverat immuninfiltrat var starkt prognostiskt bland högrisktumörer (d.v.s. tumörer av hög histologisk grad) och att detta innebar en lika stor riskminskning för lokalrecidiv som strålbehandling. Däremot sågs detta inte bland lågrisktumörer. Vidare såg högrisktumörer med immunaktivering till och med ut att ha en bättre prognos än lågrisktumörer.

Sammanfattningsvis antyder den här avhandlingen att immunologiska biomarkörer från tumören ger oberoende information om risken för ett lokalt återfall, vilket kan användas för att stratifiera patienter efter behov av postoperativ strålbehandling. Tumörer med ett aktiverat immunsvar ser ut att ha en låg risk för lokala återfall och utgöra en grupp där nedtrappning av strålbehandling kan vara möjlig. Tumörer utan ett aktiverat immunsvar utgör sannolikt gruppen där utökad strålbehandling gör störst nytta.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kovacs, A., Stenmark Tullberg, A., Werner Rönnerman, E., Holmberg, E., Hartman, L., Sjöström, M., Lundstedt, D., Malmström, P., M. Fernö, and Karlsson, P., Effect of Radiotherapy After Breast-Conserving Surgery Depending on the Presence of Tumor-Infiltrating Lymphocytes: A Long-Term Follow-Up of the SweBCG91RT Randomized Trial.

J Clin Oncol, 2019. 37(14): p. 1179-1187.

II. Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., Holmberg, E., Chang, S.L., Feng, F.Y., Speers, C., Pierce, L.J., Lundstedt, D., Killander, F., Niméus, E., Kovács, A., and Karlsson, P., Immune Infiltrate in the Primary Tumor Predicts Effect of Adjuvant Radiotherapy in Breast Cancer; Results from the Randomized SweBCG91RT Trial.

Clin Cancer Res, 2021. 27(3): p. 749-758.

III. Stenmark Tullberg, A., Sjöström, M., Niméus, E., Killander, F., Chang, S.L., Feng, F.Y., Speers, C., Pierce, L.J., Kovács, A., Lundstedt, D., Holmberg, E., and Karlsson, P., Integrating tumor-intrinsic and immunological factors to identify immunogenic breast cancers from a low-risk cohort-results from the randomized SweBCG91RT trial

Manuscript

IV. Stenmark Tullberg, A., Sjöström, M., Tran, L., Niméus, E., Killander, F., Kovács, A., Lundstedt, D., Holmberg, E., and Karlsson, P., Risk of local recurrence and benefit from radiotherapy based on integrated assessments of histological grade, TILs, PD-1, and PD-L1- results from the randomized SweBCG91RT trial

Manuscript

Papers not included in the dissertation

- Schiza, A., Thurfjell, V., Stenmark Tullberg, A., Olofsson, H., Lindberg, A., Holmberg, E., Bremer, T., Micke, P., Karlsson, P., Wärnberg, F., and Strell, C.. Tumour-infiltrating lymphocytes add prognostic information for patients with low-risk DCIS: findings from the SweDCIS randomised radiotherapy trial
  European Journal of Cancer, 2022. 168: p. 128-137.
- Strell, C., Stenmark Tullberg A., Jetne Edelmann, R., Akslen, L.A., Malmström, P., Fernö, M., Holmberg, E., Östman, A., and Karlsson, P., Prognostic and predictive impact of stroma cells defined by PDGFRb expression in early breast cancer: results from the randomized SweBCG91RT trial
  Breast Cancer Res Treat, 2021. 187(1): p. 45-55.

CONTENT

ABBREVIATIONS

BCR	B cell receptor
BCS	Breast-conserving surgery
BCT	Breast-conserving therapy
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin-embedded
FF	Fresh-frozen
HR	Hazard ratio
HER2	Human epidermal growth factor receptor-2
IHC	Immunohistochemistry
MRI	Magnetic resonance imaging
PgR	Progesterone receptor
RT	Radiotherapy
SweBCG91RT	Swedish Breast Cancer Group 91 Radiotherapy Trial
TCR	T cell receptor
TMA	Tissue microarray
TILs	Tumor-infiltrating lymphocytes

1 INTRODUCTION

1.1 BREAST CANCER EPIDEMIOLOGY & TREATMENT

1.1.1 OVERVIEW

Epidemiology

Breast cancer is the most common form of cancer and the second most common cause of cancer death in women globally[1]. It accounts for around one in four diagnosed cancers in women[2]. The incidence has increased in recent years while the 10-year survival rate has improved in Europe and North America, but not in the rest of the world[3]. The incidence of breast cancer is strongly correlated with age, with around 25% occurring before age 50[2]. However, the increased incidence is not restricted to older women, indicating that an increased life expectancy is unlikely to be the sole explanation[1]. The improved 10-year survival in developed countries may be due to earlier detection and more effective treatment[3]. Risk factors include estrogen exposure (exogenous or endogenous via low parity, early menarche, late menopause, obesity, et. c.), genetic predisposition, ionizing radiation, certain types of benign proliferative breast disease, and increased breast density[2]. In addition, a Western lifestyle, including diet and alcohol consumption, also contributes to an increased risk[2].

Screening

The implementation of mammography screening is estimated to have reduced breast cancer mortality by around 20%[2]. Screening is most beneficial among women aged 50-69, and there is limited evidence for a benefit among younger or older women[2]. Ultrasound may be used as a complementary screening method, but there is currently no consensus regarding how to use this. For women with a strong genetic predisposition, annual magnetic resonance imaging (MRI) combined with mammography increases the chances of diagnosing cancer earlier, although it is unclear if it provides a mortality benefit[2].

Classification

Breast cancer is a heterogeneous disease that can be divided into subtypes based on gene expression, specific receptors, or histology. Classifying breast cancer as subtypes can guide treatment decisions as different subtypes confer varying prognoses and benefit from specific treatments. With gene expression information, breast cancer can be classified as the molecular subtypes Luminal A and B, characterized by ER signaling; HER2 positive, characterized by HER2 signaling; and the Basal subtype [4, 5]. HER2-positive tumors can further be subgrouped

as HER2 luminal (ER signaling present) or HER2 non-luminal (no or low ER signaling). Commercially available kits that incorporate measurements of genes associated with molecular subtypes are today used in clinical practice to assist decision-making regarding systemic treatment[6, 7]. In the absence of gene expression data, breast cancer is classified into subtypes based on the expression of the estrogen receptor (ER), the progesterone receptor (PgR), and the human epidermal growth factor receptor-2 (HER2). ER-negative and HER2-positive subtypes tend to have a higher proliferation rate, which indicates a less favorable prognosis[8]. If no ER, PgR, or HER2 expression is observed, the tumor is classified as triple-negative, corresponding to the Basal molecular subtype. ER-positive subtypes are divided into Luminal A-like and Luminal B-like based on Ki67 and PgR expression. Histologically, breast cancer is divided into ductal, lobular, mixed, or less common types such as mucinous, tubular, medullary, and papillary[9]. Finally, tumor stage, another essential prognostic factor, is determined by tumor size, lymph node involvement, and the presence or absence of distant metastasis[10]. In addition to subtype and tumor stage, patient age, histological grade, and lymphovascular invasion are important prognostic factors for early non-metastatic breast cancer[11-13].

Treatment

Breast cancer treatment has evolved dramatically over the last decades and generally consists of surgery, systemic therapy, and radiotherapy (RT), with the vast majority of patients with early breast cancer being successfully cured. RT and systemic treatment can be given before (neoadjuvant) or after (adjuvant) surgery, and the decision is generally based on tumor size and aggressivity. Although most breast cancers are treated with curative intent, many new therapies are also being developed in the palliative setting, prolonging life in these patients.

Surgical treatment

Surgical treatment is provided as modified mastectomy or breast-conserving surgery (BCS)[14]. BCS with subsequent RT is referred to as breast-conserving therapy (BCT) and is preferred in most cases as it produces similar outcomes to mastectomy despite less invasive surgery, resulting in a shorter recovery time[15]. In addition, BCT may be associated with improved overall survival compared to mastectomy, although the reason for this is unclear[16, 17]. Mastectomy is generally preferred for inflammatory breast cancer, when RT is contraindicated

(e.g., previous RT against the same breast or a current pregnancy), or if the tumor is difficult to operate on (e.g., large size, multifocality, or persistently positive surgical margins). Axillary lymph node dissection is generally performed if palpable pathological lymph nodes are present or if more than two positive sentinel nodes were found in the sentinel node biopsy, a technique that allows the first lymph nodes to drain the area of the tumor to be examined.

Systemic treatment

The systemic treatment of breast cancer has revolutionized over the past decades[14], and treatment recommendations are based mainly on tumor biology. In general, high-risk and/or tumors that are difficult to operate on may be recommended neoadjuvant systemic therapy as this can downstage the tumor stage and increase the chances of successful subsequent surgery[18]. Chemotherapy is generally given to all but Luminal A tumors with a low risk of recurrence. Antihormonal treatment is given if the tumor is ER-positive; the exact choice of treatment is based on menopausal status[19]. Anti-HER2 treatment is given if the tumor overexpresses HER2. Neoadjuvant immunotherapy is generally recommended for triple-negative and HER2-positive breast cancer of stage T2 or with lymph node metastases[20]. Systemic therapy can also be given for metastatic breast cancer, e.g., chemotherapy or targeted therapies, such as cdk4/6 inhibitors or PARP inhibitors[21-23], et. c.

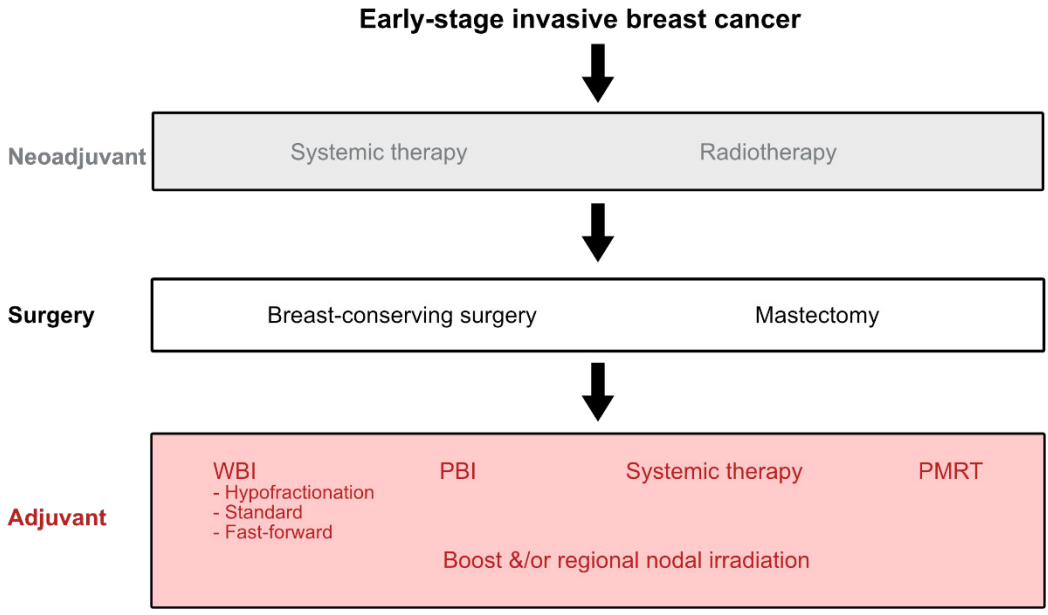
Radiotherapy

RT has not undergone the same development as medical treatment regarding the use of treatment-predictive biomarkers based on tumor biology. It is generally provided to eliminate potential residual tumor foci among patients who have undergone BCS[14]. RT is regarded as a local therapy that primarily prevents local recurrences. A local recurrence is associated with an increased risk of subsequent distant metastasis and death[24, 25]. RT is most often provided as adjuvant whole-breast RT in the form of hypofractionation (total dose 40-42.5 Gy), but can also be given as standard fractionation (total dose of around 50 Gy) or partial breast irradiation[26]. In addition, recent studies support the use of ultrashort whole-breast RT, FAST Forward, given as five fractions to a total dose of 26-28.5 Gy[26]. For low-risk patients aged 65 or older, omission of RT can be considered if antihormonal therapy is given[26]. For high-risk patients with inoperable tumors,

neoadjuvant RT may be recommended. If the patient is considered to be at high risk of recurrence, intensified treatment with RT boost may be recommended[27]. The boost can be simultaneously integrated during hypofractionation or given as additional fractions following standard RT treatment[28]. In addition, regional lymph node irradiation (RNI) may be recommended if a high axillary tumor burden is suspected[29], e.g., among patients with palpable pathological axillary lymph nodes or with more than two positive sentinel nodes after sentinel node biopsy. RNI generally includes axillary, supraclavicular, and upper internal mammary lymph nodes. RT can also be given after mastectomy in high-risk patients[26]. Henceforth, any mention of RT in this thesis will refer to adjuvant whole-breast irradiation unless otherwise stated, as this is the type of RT studied in this thesis and most commonly used.

Despite the proven benefits, RT is not without drawbacks. Both acute and chronic side effects may develop. The most severe long-term side effects are an increased risk of heart disease and secondary malignancy[30, 31]. Acute side effects occur early and cause local symptoms in the form of, for example, arm edema, skin fibrosis, pneumonitis, neuropathy, et. c[32]. The risk is higher if an RT boost is given[27]. These risks are reduced with improved techniques that reduce radiation doses to internal organs.

Figure 1. Overview of treatment for early-stage breast cancer



WBI= Whole-breast irradiation. PBI= Partial breast irradiation. PMRT= Post-mastectomy radiotherapy.

Early-stage (I-IIIA) breast cancer is usually treated with systemic therapy, surgery, and radiotherapy. High-risk- or tumors difficult to operate on may be recommended neoadjuvant therapy. Breast-conserving surgery combined with adjuvant whole-breast irradiation and systemic therapy is usually preferred. If the patient has a high risk of a local recurrence, the addition of an RT boost may be recommended. Furthermore, if there is nodal involvement, regional nodal irradiation may be recommended.

1.1.2 PRECISION MEDICINE IN RADIOTHERAPY

Estimating the RT benefit

Radiotherapy is based on the assumption that there are tumor microfoci after surgery that adjuvant RT eliminates. Most patients do not develop a local recurrence even though they do not receive RT and can, therefore, in theory, be spared the risk of side effects from RT[33]. Research on RT in breast cancer has identified clinical variables associated with an increased risk of a local recurrence despite treatment with RT. Young age is perhaps the strongest risk factor[33]. Other risk factors can be summarized as aggressive tumor characteristics and non-radical surgery[33, 34]. In these cases, an RT boost is sometimes recommended. However, although these clinical variables are prognostic, they do not seem predictive and are, therefore, insufficient to identify patients with differing relative benefits from RT[33]. Currently, no prospectively validated RT-predictive biomarkers exist[14].

Most prospective research on RT individualization in recent years has focused on identifying patients with favorable prognostic factors resulting in a sufficiently low risk of local recurrences that do not need RT. Along with earlier detection and more effective systemic treatment, the risk of a local recurrence has also decreased. The low absolute risk of a local recurrence among low-risk patients, therefore, reduces the absolute benefit from RT[35]. However, the relative benefit from RT remains unchanged, indicating that more research is needed to accomplish true treatment individualization, similar to systemic therapy. Finally, a minority of patients develop a local recurrence despite receiving RT and would likely benefit from an RT boost[24, 25]. In summary, there is a need to develop new methods to improve the treatment individualization of RT by accurately identifying patients who can safely be omitted from RT and those who benefit from intensified RT[36, 37]. So far, clinical variables alone have not convincingly achieved this, although ongoing studies indicate that identifying low-risk groups, as defined by clinical variables, that can be omitted from RT may be possible[38].

RT and the tumor biology

Analysis of the tumor's gene expression enables a deeper insight into the specific underlying biology. In recent years, several research groups

have used genomic methods that allow for a better characterization of tumor biology to create tools predictive of the relative RT benefit. One way this has been attempted is by identifying genes associated with resistance to RT, thereby attempting to quantify tumor-intrinsic radioresistance. Some groups have focused on pan-cancer molecular signatures developed from cell lines based on the assumption of common biological pathways across cancer forms associated with radioresistance[39]. Other groups have focused on a specific type of cell line, e.g., breast cancer cells[40]. Examples of biological processes that are enriched in these radioresistance signatures are proliferation[41], hypoxia[42], and DNA damage response[40]. However, most molecular signatures developed from cell lines have failed external validation[43]. A possible cause of difficulties in translating in vitro findings to the clinical setting is that cell lines are clonal populations not representative of the complex tumor heterogeneity seen in vivo[43]. Furthermore, cross-talk with the tumor microenvironment, where different types of stromal cells influence tumor progression, is challenging to consider in the in vitro setting[43].

Another approach has been to identify molecular signatures associated with radioresistance through retrospective analyses of clinical cohorts. The Danish Breast Cancer Cooperative Group developed a signature stratifying patients who had undergone mastectomy into two groups with differing risks of a local recurrence[44]. The signature aimed to identify patients who do not benefit from postmastectomy RT. Finally, our group has developed the ARTIC classifier using a cohort treated with BCS and RT[45]. The signature was shown to be strongly prognostic of locoregional recurrence and predictive of benefit from RT in a retrospective analysis in a randomized independent RT validation cohort.

Before molecular signatures can be implemented in the clinic for RT prediction, they must undergo external validation in prospective trials. Common to many classifiers identifying radioresistance is that they tend to identify aggressive, highly proliferating tumors, as it seems that tumor-intrinsic radioresistance and tumor aggressiveness often go hand in hand. However, there is reason to believe that there may be another side to the coin and that it is also possible to identify such aggressive tumors that have a favorable prognosis. This is supported, for example, by a recent study that showed an excellent prognosis in young women with triple-negative breast cancer with high levels of tumor-infiltrating lymphocytes[46]. Finally, one can argue that existing RT predictive

tools primarily evaluate tumor-intrinsic factors and that their performance can be improved by including other perspectives related to the risk of local recurrence. Examples of information that could enhance these decision-making tools are those that provide independent information on the degree of residual tumor burden after surgery.

Only a minority of operations with negative margins can be assumed to have a high enough residual tumor burden to cause a local recurrence unless additional treatment is given[47]. This is supported by large meta-analyses estimating that most patients are cured by surgery and endocrine therapy alone[33]. Presumably, it is among the remaining minority of patients with residual tumor foci that prediction of radioresistance is useful. Independent information informing the likelihood of residual postoperative disease, for example, related to the surgery or stromal factors, would affect the pretest prevalence for developing a local recurrence despite RT. This information could be used to further select patients where radioresistance-predictive tools provide the most utility. Furthermore, the exclusion of cured patients in external validations of radioresistance classifiers would reduce unexplained variance and improve the prospects for successfully validating tumor-intrinsic radioresistance signatures. In addition, a better biological understanding of how cancer-stroma crosstalk influences RT benefit may improve the prospects of applying concepts related to RT benefit to heterogeneous patient populations and enable new treatment options with targeted therapy. An example of such a therapy is TGFβ inhibition which has the potential to activate the immune response and induce radiosensitivity simultaneously[48, 49]. In light of the above, it is relevant to investigate whether characterizations of the immunological activity in the primary tumor can contribute independent information that can improve the individualization of postoperative RT in breast cancer. An activated immune response may be hypothesized to decrease the risk of tumor micro-invasion. Furthermore, an immune response is generally mounted in secondary lymphoid organs[50] and, therefore, not restricted to the primary tumor, suggesting that its effect may also remain postoperatively. Consequently, immune activation in the removed primary tumor could inform the likelihood of tumor regrowth if there is residual tumor burden.

1.2 THE IMMUNE SYSTEM

Overview

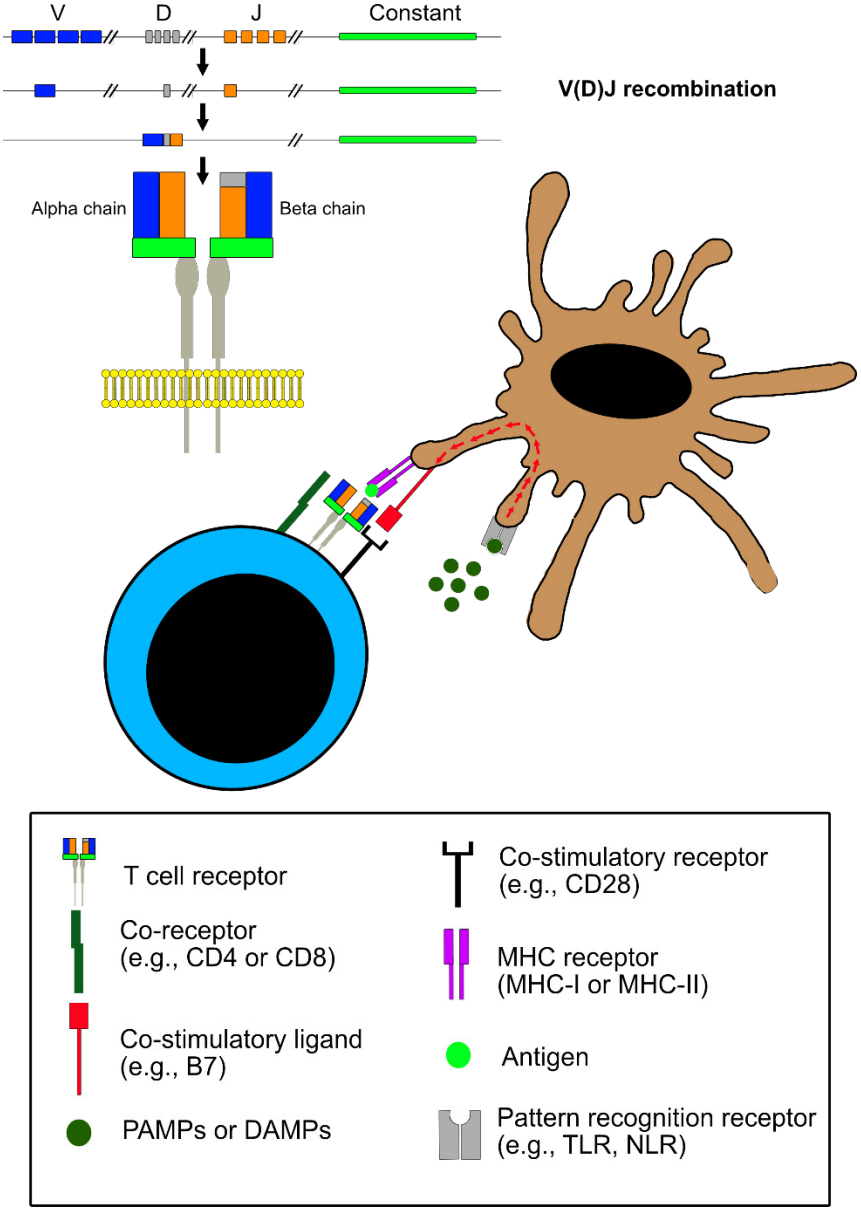
The immune system aims to protect us against pathogens and cancer cells and is involved in tissue repair[51]. It is activated primarily via tissue damage and foreign substances and can be divided into two components; innate and adaptive. The innate immune system is nonspecific and constitutes the first-line defense, while the adaptive part takes time to activate but generates a potent and specific immune response. The innate part includes barrier protection and cells primarily from the myeloid lineage, and the adaptive part includes lymphocytes and plasma cells derived from the lymphoid lineage[51]. An effective immune response against manifest cancer generally requires activating the adaptive part of the immune system, capable of distinguishing normal from transformed cells[52].

The adaptive immune system

The specificity of the adaptive immune system stems from its extreme diversity. Foreign proteins (or in some cases also carbohydrates or lipids) are recognized by the two lymphocyte types, T cells, and B-cells, via the T-cell receptor (TCR) and B-cell receptor (BCR), respectively. These receptors consist of a variable and a constant part[51]. As evident from the name, the variable part is variable. It consists of two components of two and three parts[51, 53]. These are encoded by the Variable (V) and Joining (J) segments (light chain in B cells and alpha chain in T cells), and Variable (V), Diversity (D), and Joining (J) segments (heavy chain in B cells, beta chain in T cells), respectively. There are many different variants of the different gene segments V, D, and J, which means numerous combinations are possible. During T- and B cell maturation, one of the many variants from each segment for each of the two chain types of the respective cell is combined in a random process resulting in a tremendous diversity[51, 53]. Diversity is why this part of the immune system is called adaptive- no matter what unknown foreign substance is encountered, there is probably at least one lymphocyte whose unique receptor has an affinity for the substance, resulting in activation. An activated lymphocyte then undergoes clonal expansion, meaning an army of clones generated from the specific lymphocytes being activated can be formed. Adaptability, which thus occurs by mobilizing the most capable lymphocytes when exposed to a foreign

substance, is also the reason why this part of the immune system is activated with some latency at the time of the first exposure[51].

Figure 2. Schematic overview of V(D)J recombination and the process of T cell activation



PAMP= Pathogen-Associated Molecular Pattern. DAMP= Damage-Associated Molecular Pattern. T cell receptors undergo V(D)J recombination, resulting in a tremendous repertoire of unique receptors that can bind to various antigens. Additional so-called co-stimulatory signals from antigen-presenting cells are needed for T cell activation, transmitted via a co-stimulatory receptor. Furthermore, T cells can be subdivided based on the additional co-receptor termed Cluster of Differentiation (CD).

Preventing autoreactivity

With the potency of the adaptive immune system, several layers of safety barriers are required to prevent self-harm. Early in the maturation of T and B cells, the negative selection process in primary lymphoid organs (thymus and bone marrow) eliminates those with the ability to bind self-antigens[51]. However, this process is not without error which necessitates additional layers of protection, mainly related to T cell regulation, as this is the cell type that coordinates many aspects of the adaptive immune response[51].

The next step of protection involves limiting lymphocytes to secondary lymphoid organs to prevent them from accidentally encountering self-antigen found in peripheral tissue but not in lymphoid organs (for which they have, therefore, not undergone negative selection)[51]. If, after all, a lymphocyte were to encounter a self-antigen for which it has a high affinity, peripheral tolerance prevents the immune cell from being activated. Antigen-presenting cells upregulate costimulatory receptors if they pick up an antigen in conjunction with specific signals (e.g., tissue damage or conserved molecular patterns associated with pathogens), which indicate that the antigen comes from an actual threat. Presentation of the antigen together with costimulatory signals then activates antigen-specific T cells. Lack of costimulation instead induces anergia, which prevents the T cell from functioning. The same applies to B cells, where binding to an antigen without simultaneous T-cell stimulation induces anergia[51].

Finally, some mechanisms inhibit and kill lymphocytes in the event of a prolonged immune response. This includes, for example, the immunosuppressive FOXP3-expressing T-regulatory cell, which can be induced in the peripheral tissue by cytokine signaling and which, via immunosuppressive cytokines, inhibits several components of the immune response[51]. Another example is activation-induced cell death and T cell exhaustion, which can be likened to a built-in shutdown mechanism where a continuous stimulation of a T lymphocyte eventually leads to cell death or decreased function through the upregulation of inhibitory receptors, such as PD-1[51]. A healthy immune response to an invading pathogen is generally transient. On the other hand, continuous stimulation suggests that the antigen cannot be easily eradicated, which may indicate that lymphocytes are reacting to a self-antigen. This may explain the evolutionary benefit of such built-in off-switches. However, the physiological mechanisms that prevent autoimmunity are highly

relevant in the cancer context as these are critical mechanisms by which cancer cells escape detection and/or elimination.

The role of lymphocytes in cancer

In recent years, the interest in the immune system in cancer research has skyrocketed. Most research has focused on the adaptive immune response due to its ability to recognize cancer cells[54]. Since cancer is a genetic disease with DNA abnormalities, cancer cells will exhibit RNA changes that are translated into abnormal proteins, so-called neoantigens, which subsequently can be detected by neoantigen-specific lymphocytes[55].

One measure of the antitumoral immune response often used is immunohistochemical quantification of tumor-infiltrating lymphocytes (TILs)[56]. The concept of TILs includes T cells, B cells, and NK cells (the latter of which is part of the innate immune response). Increased levels of TILs correlate with improved prognosis and treatment effects of immunotherapy and possibly systemic therapy in different cancers[57-60].

The natural outcome of the fight between an activated immune response and cancer seems to be that the cancer cells eventually win by, for example, hijacking the physiological mechanisms that have evolved to prevent extensive inflammation[51]. Without intervention, these physiological defenses against prolonged inflammation generally mean that the initial immune response is eventually blunted and extinguished. In addition, establishing an immunological selection pressure leads to immune evasion through the preferential survival of cancer clones with the ability to resist the attack of the immune system[61]. An example of a mechanism of immune evasion is the upregulation of inhibitory immune checkpoint molecules that shut down immune cells, with PD-L1 perhaps being the most well-known. This explains why blocking the PD-L1/PD-1 signaling pathway can re-activate an ongoing but inhibited immune response. An effective antitumoral immune response can be re-established by blocking inhibitory signaling in lymphocytes, resulting in tumor regression and sometimes cure[62].

Role in breast cancer

The immune system's role in breast cancer is not as clear-cut as in other cancers, and there is conflicting information about the prognostic effect. TILs, and other measures of an immune response in the primary tumor, confer an improved prognosis, primarily in the more aggressive subtypes[63]. It is also among these subtypes that immunotherapy appears to be most beneficial[20]. The relevance of the immune system within more aggressive subtypes may have to do with a broader neoantigen repertoire and better costimulatory conditions. Within the more aggressive breast cancers, a treatment predictive effect is also seen from TILs regarding chemotherapy and anti-HER2 therapy [57, 58]. In the case of less aggressive, ER-positive tumors, an immune infiltrate tends to either be non-prognostic or confer an unfavorable prognosis[64-66]. However, it is not clear why low-risk breast tumors do not derive a benefit from an immune infiltrate[67]. Furthermore, it is unknown if a subgroup of these tumors can be identified where immunotherapy benefit is seen. A better understanding of the immune system's role in breast cancer and the influence of tumor-intrinsic factors on its implications for cancer progression may enable improved prognostic stratification and identification of patients benefiting from immunotherapy. This, in turn, would translate to improved treatment individualization.

The immune system and RT

RT eliminates cancer cells by damaging their DNA. This results in cell death, inflammation, and exposure of neoantigens and cancer DNA to the tumor microenvironment. In mechanistic studies, RT-induced cancer death has been shown to act as a stimulus for the immune response[68], which creates a double anti-cancer effect – direct and indirect cancer-killing through DNA damage and immune activation, respectively. The beneficial effect of an RT-induced immune activation is not restricted to the radiation field, and the generation of an immune response resulting in cancer destruction outside of the radiation field is termed the abscopal effect[68]. The abscopal effect is believed to occur from RT acting as a vaccine through the local release of neoantigens and activation of antigen-presenting cells, which leads to a systemic immune response with regression of tumor lesions at distant sites[68].

The ability of RT to activate the immune response forms the basis for ongoing clinical trials combining RT with immunotherapy in the hope of an improved local tumor-killing effect and a possible abscopal effect[69]. However, there is a lack of clinical studies on the interaction

between RT and the immune system. Since RT in breast cancer is mainly given postoperatively, it is not obvious that preclinical findings, where a preexisting solid tumor is irradiated, can be translated to the clinical setting. Furthermore, RT has inherent immunosuppressive effects, which theoretically means that it could inhibit an activated local immune response[70]. Therefore, research on the interaction between RT and the immune response in the clinical setting is needed. The fact that immunological biomarkers are prognostic, treatment-predictive, and therapeutic illustrates the potential of harnessing assessments of the immune infiltrate to tailor RT and improve patient outcomes.

2 AIM

2.1 IMPROVED INDIVIDUALIZATION OF RADIOTHERAPY THROUGH IMMUNOLOGICAL BIOMARKERS

The thesis aimed to investigate whether immunological biomarkers can contribute to an improved treatment individualization of RT in breast cancer.

The specific sub-projects included the following aims:

- Study I: To study how tumor-infiltrating lymphocytes affect the risk of local recurrence and the benefit from RT
- Study II: Based on the findings from study I, we wanted to refine further the characterization of the immune infiltrate by analyzing how the CD8:FOXP3 balance, as well as specific immune cells measured via gene expression, affect the benefit from RT and the risk of local recurrences.
- Study III: To further understand the underlying biology, we wanted to investigate whether the integration of tumor-intrinsic and immunological factors improves the prognostication of immunological biomarkers. Furthermore, we studied if this improved characterization of the immune infiltrate could provide additional information regarding RT benefit.
- Study IV: Based on the findings from study III, we used the clinical variables TILs, PD-1, PD-L1, and histological grade to test the feasibility of using clinical variables to accurately determine the influence of the immune response on the risk of a local recurrence and the need for RT among high-risk tumors.

3 PATIENTS AND METHODS

3.1 PATIENT POPULATION

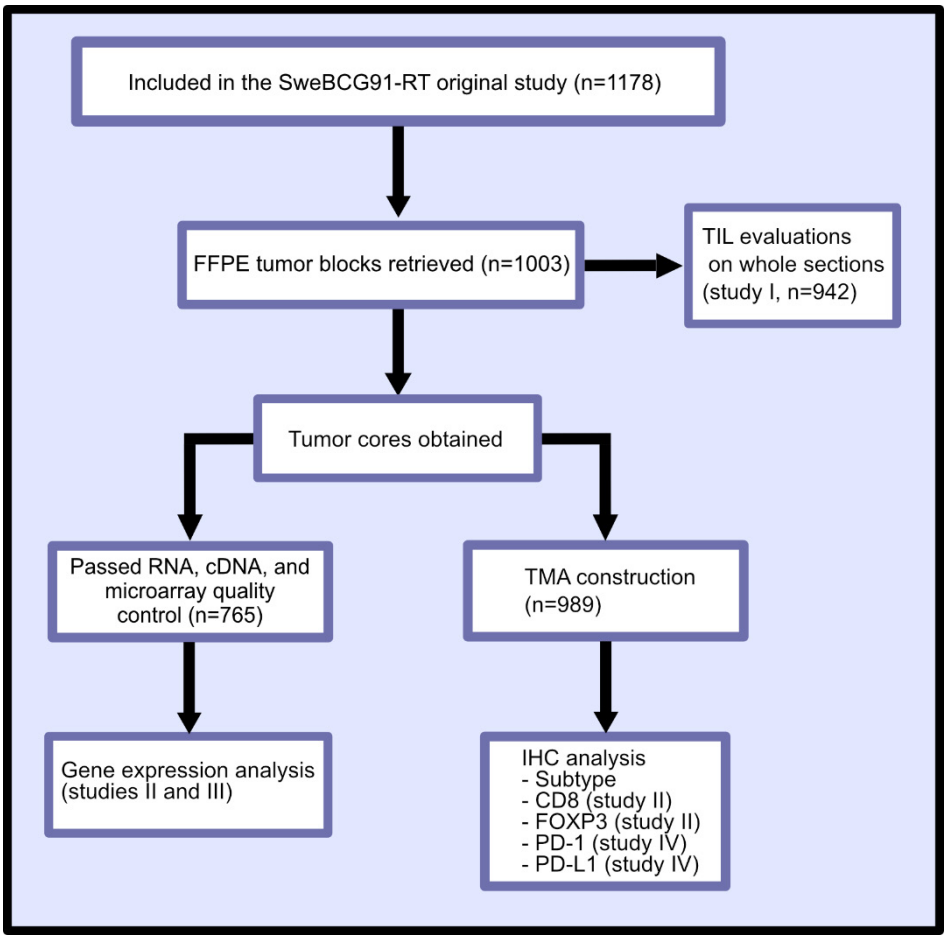
In all four studies included in this thesis, retrospective analyses of the SweBCG91RT study, a randomized RT trial, were performed[71, 72]. This study was started in 1991 to assess the feasibility of omitting postoperative RT after breast-conserving surgery (BCS). Although several randomized trials had demonstrated a beneficial effect from RT, the similar but smaller Uppsala-Örebro multicentre trial had found no significant difference in local recurrences with or without RT with a median follow-up time of three years[72]. In addition, the introduction of mammographic screening at this time in Sweden raised the question of whether screening-detected tumors should be treated differently.

The SweBCG91RT study included 1178 patients with stage I-II lymph node-negative breast cancer who, between 1991-1997, were allocated to BCS with or without postoperative RT. A microscopic examination of at least five lymph nodes was recommended. Patients were younger than 76 years, and the breast tumors were detected clinically or via screening. Treatment with RT was given as a total dose of 48-54 Gy in 24-27 fractions against the remaining breast without a boost. A standardized sector resection and axillary lymph node dissection of levels one and two were performed. Negative margins were mandatory for inclusion. Systemic therapy was given per the current regional guidelines and was not recommended for lymph-node negative stage I tumors. It was only recommended for patients with tumors larger than 2 cm. This meant that a minority of patients received hormone therapy (6%), chemotherapy (1%), or both hormone therapy and chemotherapy (1%). Patients were followed yearly with physical examinations and mammography during the original trial. Long-term follow-up information was later retrieved from the medical records, The Swedish Cause of Death Registry, and the Swedish population registry. The median follow-up time for patients without an event was 15.2 years, 15.2 years, 20.0 years, and 21.2 years for the endpoints local recurrence, any recurrence, breast cancer death, and death from any cause, respectively. Follow-up information from the different sources and endpoints was collected at different times, explaining the differing median follow-up times.

Formalin-fixed paraffin-embedded (FFPE) tumor blocks from the primary tumors were collected, and tissue microarrays (TMAs) were constructed, Figure 3. In total, 1003 tumor blocks were collected, of which 989 could be used to construct TMAs. Tumors of patients included in the follow-up studies had similar characteristics to those

excluded, except for tumor size and histological grade. Excluded patients generally had smaller tumors that were of a lower histological grade.

Figure 3. Consort diagram over methods for studies I-IV



Tumor blocks were collected from patients included in the original SweBCG91RT trial. TMAs were constructed and gene expression information was obtained.

In studies II and III, the Servant[73], Sjöström[74], and Van der Vijver[75] cohorts were used as these include irradiated patients treated with BCS with long-term follow-up and information about local recurrences, which allows for some degree of external validation of findings from the SweBCG91RT cohort. It should be noted that public breast cancer cohorts tend to be more aggressive than the general breast cancer population and thus may not be fully representative[76]. This is consistent with our observations, where a higher recurrence rate was generally observed in public cohorts compared to the SweBCG91RT cohort. In Study II, we used the METABRIC[77], Mainz[78], and Van der Vijver[75] cohorts for the gene expression analyses. Study III used 21 cohorts that were downloaded using the MetaGxBreast package[79].

3.2 TISSUE MICROARRAYS

We used two cores of 1 mm per patient for TMA construction for tumors of the SweBCG91RT cohort. The use of TMAs is convenient because it allows for maximum utilization of the tissue[80]. For example, a tumor block can usually be cut into sections 3-5 micrometers thick, allowing for around 50-100 stainings. However, several hundred TMAs from 1 mm cores can be created from a single tumor block, and each TMA can, in turn, undergo thousands of stainings, allowing for hundreds of thousands of assays[81]. In addition, TMAs allow for the original block to be preserved to a greater degree than whole sections. Furthermore, since TMAs allow different tumor samples to be stained simultaneously, batch effects are effectively prevented[80]. Finally, using TMAs is efficient as an entire cohort can be stained simultaneously.

However, there are several disadvantages to the use of TMAs. Since each staining includes only a small portion of each tumor, there is a risk of the sample not being representative of the whole tumor due to tumor heterogeneity. Heterogeneity is a problem that can exist between different tumor cores and different depths of a given core[80, 81]. Despite examination of an H&E-slide by a pathologist, it is difficult to know that deeper levels of a core contain tumor cells. In evaluating ER status as positive or negative, a good concordance is observed between whole sections and TMAs (96% of assessments are concordant)[80]. However, when evaluating continuous immunological variables, it is likely better to use whole sections. Previous studies show that up to three TMA evaluations are needed to classify a tumor as having high or low TILs reliably but that an accurate estimate of the absolute number of TILs requires many more cores[82].

3.3 SUBTYPE CLASSIFICATION

The tumors of the SweBCG91RT cohort were classified into biological subtypes by two board-certified pathologists, in accordance with the St Gallen International Breast Cancer Conference 2013[83]. This was done on TMAs by analysis of ER, PgR, Ki67, and HER2 amplification by silver in situ hybridization[84]. Assessments were performed until consensus was reached. Ki67 categorizations were made according to the existing guidelines of the Swedish Quality and Standardization Committee (KVAST), which meant that a third of tumors were classified as having high Ki67 values[84]. This was adjusted against a matched regional cohort as the SweBCG91RT cohort was deemed to represent a low-risk cohort. The resulting Ki67 cut-off at 10% resulted in 27% of tumors being classified as having high Ki67. HER2 positivity was defined as 3+ staining or amplification[84]. The criteria for subtype classification used were as follows:

- Luminal A-like: ER ≥1%, PgR ≥20%, Ki-67 low, HER2-negative
- Luminal B-like: ER ≥1%, PgR <20% or Ki67 high, HER2-negative
- HER2-positive: HER2-positive, any Ki-67, ER, and PgR status.
- Triple-negative: ER <1%, PgR <20%, HER2-negative, any Ki67.

The distribution of clinical variables is seen in the table below. In summary, most tumors were ER-positive (68.6%), the median age was 60 years, the median tumor size was 12 mm, and most tumors were classified as histological grade II (59.8%).

Table 1. Distribution of clinical variables among available tumors from the original SweBCG91RT cohort.

Variables	No RT	RT
HER2-positive	29 (4.8%)	35 (6.1%)
Luminal A-like	286 (47.1%)	269 (47.1%)
Luminal B-like	133 (21.9%)	126 (22.1%)
Triple-negative	49 (8.1%)	32 (5.6%)
Missing	110 (18.1%)	109 (19.1%)
ER-negative	76 (12.5%)	76 (13.3%)
ER-positive	348 (57.3%)	324 (56.7%)
Missing	183 (30.1%)	171 (29.9%)
PgR negative	124 (20.4%)	127 (22.2%)
PgR positive	299 (49.3%)	273 (47.8%)
Missing	184 (30.3%)	171 (29.9%)
Grade I	70 (11.5%)	78 (13.7%)
Grade II	288 (47.4%)	285 (49.9%)
Grade III	136 (22.4%)	101 (17.7%)
Missing	113 (18.6%)	107 (18.7%)
No hormone therapy	557 (91.8%)	535 (93.7%)
Hormone therapy	50 (8.2%)	36 (6.3%)
Missing	0 (0%)	0 (0%)
No chemotherapy	593 (97.7%)	563 (98.6%)
Chemotherapy	14 (2.3%)	8 (1.4%)
Missing	0 (0%)	0 (0%)
Age, median years (IQR)	60 (52-67)	59 (51-66)
Missing	0 (0%)	0 (0%)
Tumor size, median mm (IQR)	12 (9-16)	12 (9-15)
Missing	6	3

HER2= Human epidermal growth factor-2 receptor. ER= Estrogen receptor. PgR= Progesterone receptor. IQR= Interquartile range. The ER and PgR variables represent the distribution of tumors with positive and negative expression as determined in the original SweBCG91RT trial. Subtype was later determined using immunohistochemistry methods. Most tumors from the SweBCG91RT cohort were estrogen receptor-positive, and most were classified as Luminal A-like. Few patients were treated with systemic therapy. All patients had negative lymph nodes.

In public gene expression datasets, we used PAM50 and the genefu package to characterize different subtypes based on gene expression[85]. This information was used in study III to characterize an active immune infiltrate in the training cohort (additional details in the section on immunological evaluations).

3.4 BIOINFORMATICS

3.4.1 GENE EXPRESSION ANALYSIS IN THE SWEBCG91RT COHORT

In conjunction with the recollection of FFPE blocks and TMA preparation, cores were obtained for RNA extraction, Figure 3. Tissue was available from 922 tumors. A board-certified breast pathologist confirmed cancer content, and a representative tumor area was selected from which a 1.5 mm punch biopsy was taken. cDNA was amplified and hybridized onto GeneChip Human Exon 1.0 ST Arrays[45]. Single Channel Array Normalization (SCAN) was used to normalize the data[86].

Studies I and IV used immunohistochemistry (IHC) methods to analyze patients from the SweBCG91RT cohort, while studies II and III used both IHC and gene expression. In addition, gene expression from publicly treated breast cancer cohorts was also used for studies II and III to increase external validity.

Formalin-fixed paraffin-embedded versus fresh-frozen tissue

The gene expression data from SweBCG91RT was derived from FFPE tissue. RNA extracted from FFPE is generally of poorer quality than that extracted from fresh-frozen (FF) tissue. Fresh tissue immediately begins to degrade after it is removed from the host. The purpose of fixation is to slow and prevent this process. There are several factors influencing the quality of the RNA from FFPE tissue[87-89]. Longer time to fixation increases endogenous RNA degradation by endogenous RNases. A larger sample of FFPE tissue also increases RNA degradation because formalin takes longer to penetrate the tissue. Formalin fixation in itself also has direct unwanted effects on RNA integrity[90]. Although these were FFPE tumor blocks from the 1990s, it was possible to obtain gene expression data of good quality from the SweBCG91RT cohort. However, correlations between genes involved in similar biological pathways (e.g., immunological genes) tended to be weaker in the SweBCG91RT cohort (FFPE tissue) compared to publicly available cohorts (FF tissue), which, as would be expected, may indicate somewhat lower RNA quality in the SweBCG91RT cohort.

Gene expression microarray considerations

We used microarrays to profile the transcriptome. RNA is converted to cDNA, which is then hybridized against predefined arrays complementary to known transcripts. The degree of binding to a particular array is then quantified by a fluorescence-based method, which generates an arbitrary measure of the abundance of the specific complementary transcript. In recent years, an alternative method, RNA sequencing, has gained popularity due to its ability to fully sequence the entire transcriptome instead of being limited by predefined transcripts, in contrast to microarrays[91]. This enables investigation of splice variants and non-coding RNAs and more. However, an advantage of microarrays is that they can reliably profile the transcriptome despite a reduced RNA quality, which was particularly advantageous in our case involving FFPE tissue[92].

In an optimal scenario, all measured gene expression differences between samples are due to biological variance. Unfortunately, in reality, that is not the case. Various factors related to the method or samples introduce non-biological variance, which explains parts of the measured differences[93]. Examples of such factors are different RNA quantities between samples or systemic bias due to the samples being analyzed in different batches. Gene expression data is generally pre-processed to reduce the impact of such methodological artifacts. A central component of pre-processing is normalization. Using various assumptions, one can adjust measured gene expression to minimize non-biological causes of variance[93]. An example of such an assumption is that the proportion of expressed genes is relatively constant between samples. In our case, the SCAN method was used, a single-sample technique aiming to maximize the signal-to-noise ratio within individual samples[86]. Furthermore, normalization at the single-sample level, rather than batch level, makes this technique suitable for precision medicine as the normalization does not have to be repeated every time a new sample is added. The SCAN method has shown favorable results on the Human Exon ST array 1.0, the microarray platform used to profile the transcriptome of the SweBCG91RT cohort[86].

The problem of potential batch effects exists for all the studies of this thesis but is especially relevant for study III, where a large number of genes in 21 different cohorts were analyzed to train two models. This meant the use of different batches and analyses across different microarray platforms. We used a rank-based method when analyzing

gene expression to reduce the risk of batch effects in this study[94]. Furthermore, we calculated enrichment scores for different gene sets to avoid the problem of examined genes not being present in all cohorts[95]. The methodology for study III is described in greater detail in the methods section of immunological evaluations pertaining to study III.

3.4.2 DERIVING BIOLOGICAL MEANING FROM GENOMEWIDE EXPRESSION ANALYSIS

The introduction of genomic analyses in cancer research dramatically increased the available information to researchers. However, this development resulted in problems extracting biological meaning from statistically significant findings. Individual genes can be expressed by numerous cell types and be part of multiple signaling pathways, sometimes making it difficult to make statements about the underlying biology. Furthermore, most randomly generated gene signatures are prognostic in breast cancer[96], further justifying the importance of study designs that anchor investigated variables and interpretations in underlying biology. A prognostic association does not mean that a gene is biologically relevant. Instead, random signatures being prognostic likely has to do with correlations with other relevant biological processes such as proliferation[96]. For every hypothesized causal relationship between a variable and an observed outcome, many more potential non-causal associations with no direct biological meaning could explain that relationship. However, for predictive purposes, one can argue that understanding the underlying biology is not necessary and that the ability of a method to make accurate predictions is of sole importance. Nevertheless, cancer is a heterogeneous disease, and disease progression is affected by many variables, all of which are unlikely to be accounted for in a training set. Understanding the underlying biology may make accounting for heterogeneity in a new population easier and improve generalizability.

Gene Set Enrichment Analysis (GSEA) was developed to facilitate the interpretation and increase the generalizability of findings from gene expression studies[97]. GSEA works by evaluating predefined gene sets linked to specific biological processes rather than individual genes. The method compares two populations and calculates the enrichment for a list of genes, with corresponding values of differential expression, by ranking these genes in relation to all other available genes. The method then seeks to answer whether the list of genes is overrepresented at the extremes of the ranked gene list, after which statistical significance is estimated, and multiple hypothesis testing corrections are performed. This process is performed for different biological pathways, represented by unique gene lists. An estimate is then provided regarding up- and down-regulated biological signaling pathways in the two compared populations. This type of approach

provides replicable results across different studies and gene expression platforms in contrast to what may be obtained from analyses at the gene level[97]. Rank-based methods have also been shown to be more resistant to batch effects and cross-platform variation[94], further justifying the use of methods such as GSEA to understand the underlying biology of an outcome across cohorts.

3.4.3 IMMUNE CELL QUANTIFICATION USING RNA

In study II, we used the gene-signature-based tool xCell to quantify different types of immune cells in the primary tumor[98]. As with GSEA, xCell uses a rank-based gene set enrichment approach to quantify the enrichment of gene sets specific to different immune cells. This provides robustness across different microarray and RNA-seq platforms despite batch effects and different normalization methods[98]. To discriminate between neighboring cell types that cause similar gene expression profiles, xCell also uses a correction method, the so-called spillover correction adjustment. We considered this beneficial as neighboring cell types can have different effects (e.g., different CD4 T helper cell subsets). The tool generates an arbitrary measure of the absolute abundance of a given immune cell compared to the other examined samples. xCell has been validated against flow cytometry[98] and performs well compared to other tools designed to estimate immune cell infiltration from bulk RNA data[99].

3.4.4 SINGLE-SAMPLE GENE SET ENRICHMENT ANALYSIS

Barbie et al. extended the concept of GSEA by introducing a method calculating the enrichment of gene sets at the sample level instead of the group level, termed single-sample Gene Set Enrichment Analysis (ssGSEA)[100]. Gene expression values of constituent genes in a given gene set and sample are ranked based on the absolute levels, and an enrichment score is then calculated through empirical cumulative distribution functions of constituent genes and remaining genes. This method has all the advantages of GSEA, including the extraction of biologically relevant information and robustness to cross-platform analyses and batch effects. Cross-platform robustness is also benefited by the fact that all genes do not need to be present in every cohort being examined as the method calculates enrichment scores based on the available genes. In addition, sample-level data allows for analysis at the individual level rather than the group level, which may be more suitable for precision medicine as this does not necessitate the categorization of patients and allows for treatment-predictive modeling. Apart from the above advantages of ssGSEA, we hypothesize that biological processes, rather than specific genes, ultimately drive prognosis and treatment prediction. When it comes to therapeutic applications, however, it becomes relevant to target specific targets, exemplified by the successful therapeutic blockade of targets such as ER, HER2, and immune checkpoint receptors. The ssGSEA method was used in study III, where we integrated tumor-intrinsic and immunological factors to improve predictions of the prognosis associated with an immune infiltrate and benefit from RT.

3.4.5 REGULARIZATION

A standard linear model uses least squares fitting, which minimizes the residual sum of squares, to produce coefficients[101]. However, alternative fitting procedures can reduce overfitting and model complexity, thereby increasing accuracy and interpretability[101]. The methods we chose to use in study III aimed to achieve this through the established principles of subset selection, shrinkage, and dimension reduction.

Study III evaluated a large number of gene sets through Cox regression analyses in a training dataset containing 21 different cohorts. A subsequent meta-analysis was then performed, and the top 50 gene sets were selected. This reduced the initially evaluated thousands of gene sets to the top 50 ranked gene sets. Following this, we further eliminated highly correlated gene sets by calculating the mean correlations between each possible pair of the 50 top-ranked gene sets. Including several highly correlated features would mean duplicated information, thereby risking bias and increased variance. In addition, it would result in unnecessary model complexity.

One wants to avoid overfitting the model to the training data to maximize prediction accuracy in independent data sets. This was done in study III through the penalized regression elastic net, a machine learning method that can shrink the coefficients and perform variable selection (by shrinking coefficients to zero)[101]. Shrinkage of coefficients reduces variance and excluding irrelevant variables that do not add information increases interpretability because it removes unnecessary complexity from the model[101].

Finally, dimension reduction is another method of increasing model performance by transforming the original information into fewer integrated measures and thus reducing the number of dimensions[101]. This was accomplished by calculating the enrichment of a given biological process using the ssGSEA method for genes of a gene set. This meant that values from a handful to thousands of genes (depending on how large the given gene set was) were reduced to a single value of enrichment of the biological process represented by the gene set.

To choose the best elastic net model, we used five-fold cross-validation, which means that the training data is randomly divided

into k parts (five in our case) and that the model is trained on k-1 of the parts and tested on one of the parts[101]. For each iteration, the mean squared error (MSE) is calculated. This is repeated k times- each of the k parts is left out once[101]. The cross-validation value is obtained by averaging the MSE for the k cross-validations[101]. This process is repeated with various tuning parameters (lambdas), which, in turn, determine the degree of shrinkage of the coefficients. The tuning parameter that results in the lowest MSE during cross-validation is then selected. Finally, the model is re-fitted using all observations and the selected value of the tuning parameter[101].

Using the above methods, we created an immunological model and a tumor-intrinsic model designed to capture tumor-intrinsic qualities predicting the prognostic implications of an immune infiltrate. These two models were subsequently integrated to create a one-dimensional measurement considering the interplay between the local immune infiltrate and immunomodulatory tumor-intrinsic qualities. This was done by training an elastic net model using the expression: *endpoint~(Immunescore + Proliferative Index)^2*. We hypothesized that transforming the biological information achieved by integrating immunological and tumor-intrinsic qualities into a one-dimensional measurement would allow us to best test the clinical utility of our findings and that such a measure would be more useful clinically. In addition, it would allow us to avoid the subjective steps of determining arbitrary cut-offs for different combinations of Immunescore and Proliferative Index.

Refer to Table S4 of Study III for the complete Integrated model. By studying the resulting coefficients of the Integrated model, one can conclude that the highest predicted risk is observed among tumors with high values of Proliferative Index and low values of Immunescore. Furthermore, a high Immunescore among tumors with a high Proliferative Index downgrades the predicted risk and produces a risk estimate similar to tumors with a lower Proliferative Index, where Immunescore also has a more negligible impact on the predicted risk. Therefore, the Integrated model can combine patients with different values of Immunescore and Proliferative Index but with similar predicted risk- a simplification that may be necessary for the clinical implementation of methods that integrate tumor-intrinsic and immunological factors.

3.4.6 STATISTICAL METHODS

In general, we limited the follow-up of our analyses to 10 years in all studies except in public cohorts with overall survival as the endpoint. In the latter case, we assumed that the time from a distant metastasis to death is approximately 5 years (i.e., death within 15 years corresponds to distant metastasis within 10 years). Age was included as a covariate in these instances due to its strong influence on overall survival.

Local recurrence as the first event within 10 years was used as the primary endpoint for analyses of the SweBCG91RT cohort in all studies except for study II, where we used any recurrence based on the non-significant trend toward a reduced RT benefit with high TILs observed in study I. We, therefore, attempted to increase the power in study II by using any recurrence as the endpoint. The Sjöström and Servant cohorts also used local recurrence as the endpoint. Any recurrence, distant metastasis, or overall survival was used as the endpoint for the remaining public cohorts.

The following adjustments for covariates were made for analyses of the SweBCG91RT cohort:

- Study I: RT, histological grade, age, and subtype.
- Study II: RT, histological grade, age, subtype, and systemic therapy.
- Study III: RT, histological grade, age, ER status, and tumor size.
- Study IV: RT and age (histological grade was used to create the examined tumor-intrinsic variable).

In study III, the meta-analysis and training of the model were performed in public cohorts. We prioritized the endpoints according to the following order as we deemed this to be the order most likely to effectively isolate the favorable prognostic signal of an activated immune response: distant metastasis-free survival (DMFS) > any recurrence > overall survival. The aim was to find gene sets that capture the biological pathways associated with immune activation. For this reason, it was not crucial that the patients had similar treatment and characteristics to the validation cohorts (SweBCG91RT, Sjöström, Servant) as we hypothesized that immune activation has an independent effect on tumor progression

and does not depend on treatment (although it is also treatment predictive). This hypothesis was supported by findings of a prognostic effect from antitumoral immune cells in study II despite differing endpoints and treatments (METABRIC[77], Mainz[78], and Van der Vijver[75] cohorts).

In all studies, we used cause-specific Cox regression to calculate hazard ratios for tables. For figures, we used proportional hazards models based on a cumulative incidence function, according to the method described by Fine and Gray, to calculate subdistribution hazards. A fundamental difference is how they handle competing events, which in turn means both have advantages and disadvantages.

Cause-specific Cox regression right-censors subjects when a competing event occurs or at the last follow-up date (i.e., the patient survived without an event to the time of censoring, but it is not known how much longer). The patient is, therefore, removed from the risk set (i.e., patients at risk) at censoring. This results in estimates representing the biological effect, and the method does not consider competing events. A weakness of this method is the risk of informative censoring, as this would introduce bias into the model[102]. Informative censoring refers to censoring associated with the outcome, e.g., a randomized study where patients drop out due to becoming too sick. Unfortunately, there is no way to test whether censoring is non-informative or informative[103]. One way to deal with this problem is to include risk factors for the causes of informative censoring in the model[103]. The subdistribution hazard method differs in that it does not assume whether censoring is informative or not.

Fine and Gray described a method for studying outcomes in the presence of competing events[104]. They developed a model based on the cumulative incidence function, which produces the cumulative probability of the outcome. Similar to Cox regression, the individual is removed from the risk set if an event or true censoring (i.e., the patient reaches the end of the follow-up time) occurs, but not when competing events occur. The authors acknowledge that the latter is unnatural, but it is necessary for the method to work for modeling the effect of covariates on cumulative incidence functions[104]. Unlike a Kaplan-Meier survival function analysis, which describes the distribution of events in a hypothetical

situation without competing risks, the subdistribution hazards method provides estimates in the presence of competing risks. By including covariate information, predictions at the individual level can be made, making it particularly suitable for prediction. However, one objection to the method is its inherent inability to estimate the causal effects of covariates for different events. A covariate that increases the cause-specific risk of a competing event will also appear to reduce the subdistribution hazard for other events. This is due to the fact that the individual will remain in the risk set despite not being able to experience other events.

Since there are advantages and disadvantages to both methods described above, we have used both variants in the studies. In tables, we used cause-specific Cox regression to account for the biological effect, while in figures, we used the subdistribution hazard method to describe the cumulative incidences in the presence of competing risks. In general, both methods produced similar estimates throughout the work included in this thesis.

In the SweBCG91RT cohort, patients were primarily followed until a first recurrence. Past 15 years, information was collected from the medical registry and via linkage to the Swedish Cause of Death Registry and the Swedish population registry[105]. Other recurrences and death were considered competing events for local recurrence, any death a competing event for any recurrence, and any death not from breast cancer a competing event for breast cancer death. In analyses of public datasets using Cox regression, there was no distinction between competing events and censoring. However, because we used cause-specific Cox regressions, the lack of information on competing events had no significance on the estimates.

For interaction tests, likelihood ratio tests were conducted where regression models with and without interaction terms were compared. A p-value <0.05 has been considered statistically significant.

The proportional hazards assumption underlies the Cox proportional hazard model and follows that the method does not assume the functional form of baseline hazard functions. The hazard function (or hazard rate) describes the risk of an event in non-censored individuals at a given time. Cox proportional hazard regression

creates a baseline hazard which is multiplied by a factor (representing a relative risk) determined by an individual's feature vector compared to if these features had been missing. The proportional hazards assumption refers to the fact that the model assumes that the relative hazard remains constant at different covariate values and over time[106, 107]. If the proportional hazards assumption is violated, biased effect estimates may result.

In several of our studies, violations of the proportional hazards assumption are seen. This is seen for histological grade and RT throughout and can be explained by the fact that a high histological grade and omission of RT preferentially causes a local recurrence in the first five years. Because less aggressive subtypes (i.e., tumors of a lower histological grade) tend to produce later recurrences, the increased risk of recurrence associated with a higher histological grade is attenuated and later reversed when comparing non-censored patients at a late date. The same goes for RT, which causes the most significant risk reduction for local recurrences in the first five years. Part of the risk reduction from RT may even be due to delaying the recurrence. Therefore, when comparing non-censored patients at a late time, there is no apparent risk reduction associated with RT. Therefore, the relationship between the risk of local recurrences between non-censored patients with different values of these covariates changes over time, resulting in the proportional hazards assumption being violated.

There are different ways to deal with violations of the proportional hazards assumption. One way is to stratify the non-proportional covariate[108]. Other strategies include introducing time-dependent coefficients[109], estimating the average hazard ratios[110], or using restricted mean survival time methods[111]. However, non-proportional hazards do not automatically mean that estimates are biased, and it is common to report estimates as averages over the follow-up time[107]. If the censoring occurs independently of the covariates causing the violation of the proportional hazards criteria, calculated hazard ratios can be interpreted as the mean over time[107]. Studies I and II reported hazard ratios as averages over time due to violations of the proportional hazards assumption. In study III, we included time-dependent effects for covariates not meeting the proportional hazards assumption, and this only marginally affected the estimates. In study IV, we added models for 0-5 years, where the proportional hazards assumption was met, in

the supplement, due to non-proportionality in the 10-year analysis. The estimates were very similar to those for 10 years, indicating that considering models with 10-year follow-up estimates is valid.

For study III, we used flexible parametric survival analysis according to the method described by Royston and Parmar[112]. We chose this method instead of the Cox proportional hazard model because it allows for the direct estimations of absolute measures and their uncertainties at any given time[113]. We deemed it important to include confidence intervals in our illustrations due to the complexity of the analysis. We hypothesized that tumor-intrinsic factors (as measured by Proliferative Index) determine the biological implications of an immune infiltrate (as measured by Immunescore) and that the interplay between these two, in turn, determines the benefit derived from RT. Instead of using a three-way interaction test between Proliferative Index, Immunescore, and RT, we stratified patients depending on RT and created two separate models with an interaction term between Proliferative Index and Immunescore. We then overlaid these models to estimate the benefit of RT along the two axes of the Proliferative Index and Immunescore. To illustrate the uncertainty of the models, we included 95% confidence intervals made possible by the flexible parametric survival analysis.

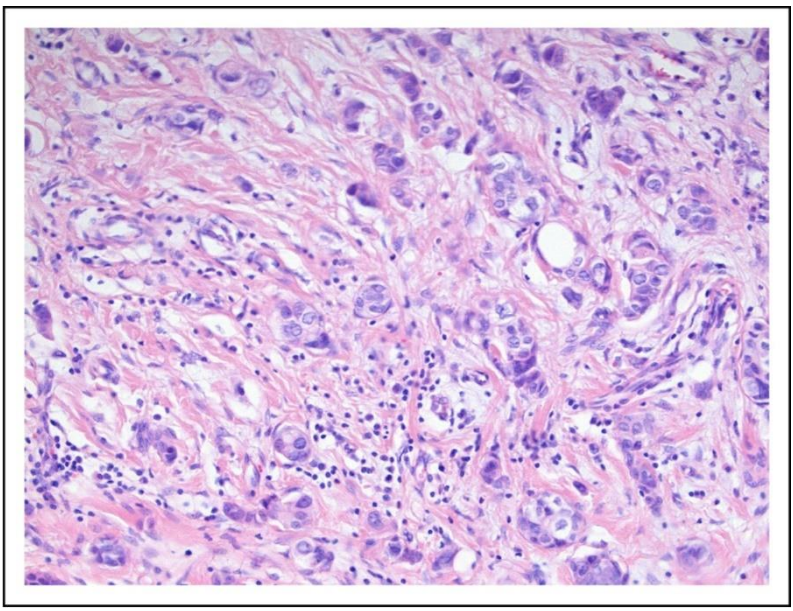
The ethical review board approved all included studies. The studies were carried out following the Helsinki Declaration. Reference numbers for approved ethics applications are 2010/127 and 2015/548.

3.5 IMMUNOLOGICAL EVALUATIONS

3.5.1 STUDY I

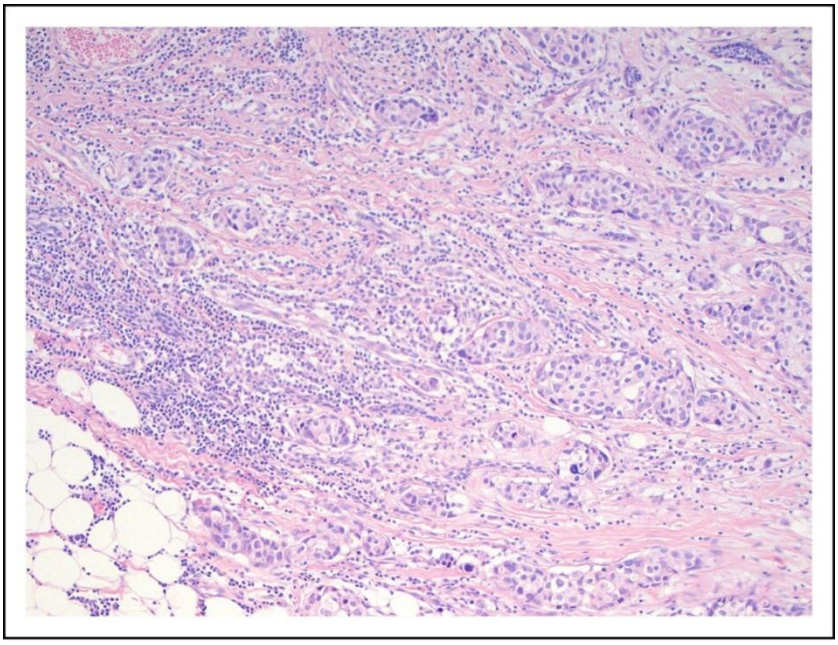
TILs were evaluated on hematoxylin & eosin (H&E) stained whole sections and included as a component in the measures for studies II and IV to increase the robustness of the TMA evaluations. Evaluation of TILs was done by two board-certified pathologists, following the recommendations of the International Immuno-Oncology Biomarker Working Groups[114], as semicontinuous values; <1%, 1-9%, 10-49%, 50-74%, ≥75%. TILs were evaluated in the stroma as the proportion of stromal area occupied by lymphocytes. A predetermined cut-off of 10% was used to define high and low levels, respectively. Images of TILs can be seen below.

Figure 4. Images of stromal tumor-infiltrating lymphocytes in the SweBCG91RT cohort



Stromal tumor-infiltrating lymphocytes 1% to 9%.

First published in Kovács, A., Stenmark Tullberg, A., Werner-Rönnerman, E., et al. Journal of Clinical Oncology (2019) 37:14. Reprinted with permission.



Stromal tumor-infiltrating lymphocytes 10% to 49%.

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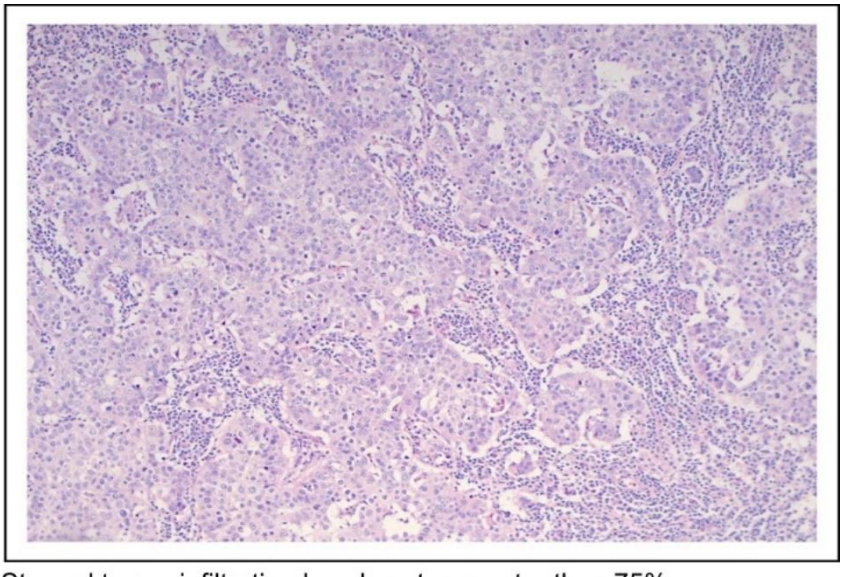

Stromal tumor-infiltrating lymphocytes greater than 75%.

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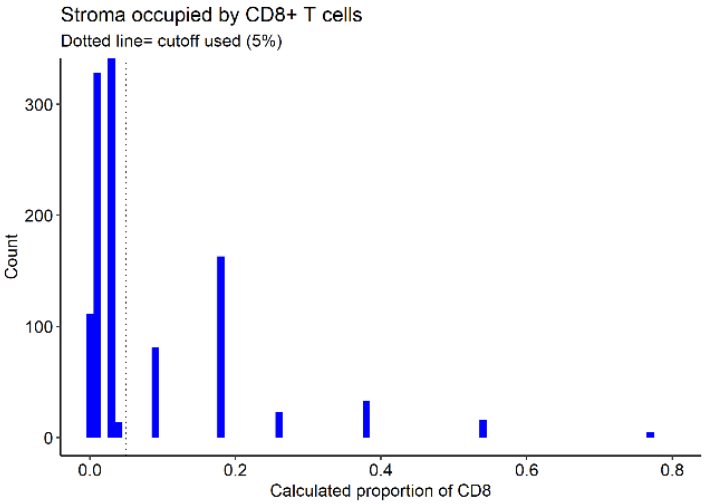
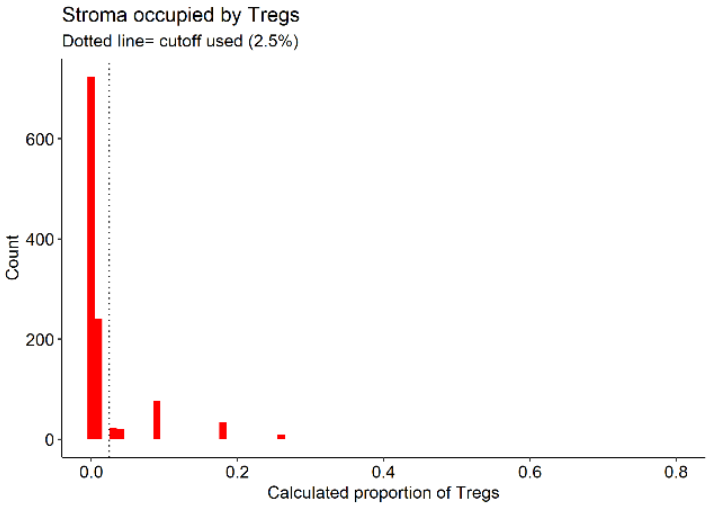
3.5.2 STUDY II

In study II, we examined the CD8:FOXP3 balance. CD8+ T lymphocytes are considered the primary effector cell of the antitumoral immune response[115, 116], while FOXP3+ T regulatory cells are instead believed to promote tumor growth[117]. We wanted to further refine our analysis of TILs by assessing the balance between these two lymphocyte subsets. Therefore, evaluations of CD8- and FOXP3-positive lymphocytes on TMAs were performed by estimating the proportion of lymphocytes that expressed CD8 or FOXP3, respectively. Two TMAs were evaluated per cell type, and the highest value was selected. Since our underlying hypothesis was that the absolute number of cells (i.e., the amount of CD8- or FOXP3-positive lymphocytes) is biologically more important than relative measures (i.e., the proportion of lymphocytes positive for CD8 or FOXP3), we created a measure to quantify the balance between the absolute number of CD8 and FOXP3 cells. To do this, we used the median value of the range of stromal TILs from whole sections (see above) and then multiplied it by the median value of the range of the proportion of lymphocytes with CD8 and FOXP3 positivity, respectively, from TMA evaluations. Cut-offs were then determined based on the distribution. We created the following groups; $CD8^{Low}FOXP3^{Low}$, $CD8^{High}FOXP3^{High}$, and $CD8^{High}FOXP3^{Low}$ (no group had the $CD8^{Low}FOXP3^{High}$ combination).

Figure 5. Distributions of CD8+ and FOXP3+ T cells in the SweBCG91RT cohort



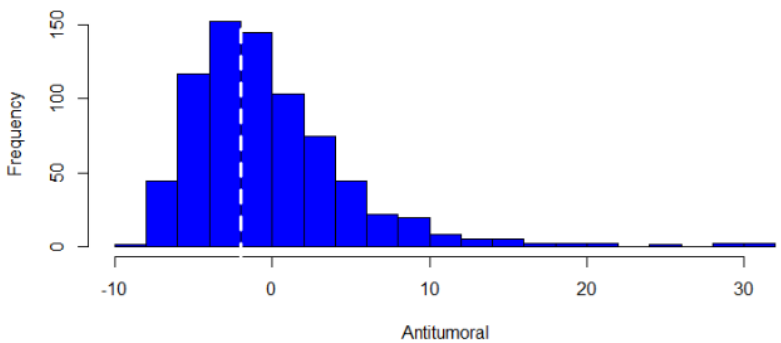
The majority of tumors were classified as having low levels of CD8+ T cells. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.



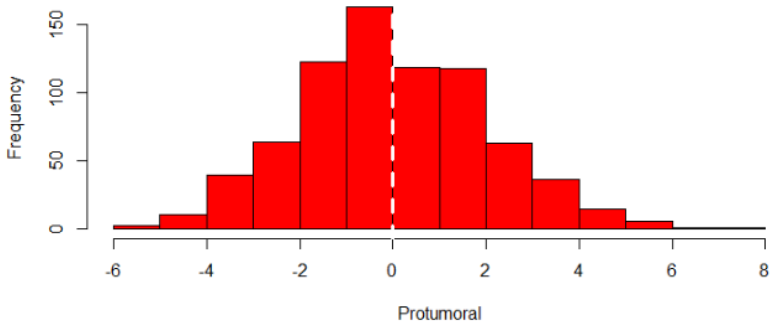
The majority of tumors were classified as having low levels of FOXP3+ T cells. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.

We also wanted to perform a similar analysis at the gene expression level but with all available immune cells grouped as antitumoral or protumoral. We chose the deconvolutional tool xCell[118], which can quantify various types of immune cells based on bulk RNA data. We first performed analyses in the public METABRIC[77], Mainz[78], and Van der Vijver[75] cohorts and found that the lymphocyte subsets included in TILs, except for Th1 and Tregs, as well as plasma cells, were consistently prognostically favorable. Conversely, tumor-associated macrophages, Tregs, Th1, and NKT cells were associated with unfavorable prognoses. Each cell type was scaled, and the values of the anti- and protumoral immune cells were then summed to create a protumoral and an antitumoral variable. Cut-offs were determined based on the distribution and ability to generate prognostically distinct groups in public cohorts. The selected cut-offs were -2 and 0 for the antitumoral and protumoral immune cell variables. The distributions of immune cells quantified by gene expression were not as positively skewed as for the IHC evaluations of CD8+ and FOXP3+ T cells, permitting the creation of more balanced groups.

Figure 6. Distributions of the antitumoral- and protumoral variables in the SweBCG91RT cohort.



The antitumoral group of cells was created using gene expression analysis and consisted mainly of cell types included in the notion of TILs. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.



The protumoral group of cells was created using gene expression analysis and consisted T regulatory cells, the Th1 subset of CD4+ T cells, and NKT cells. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.

3.5.3 STUDY III

In the third study, we set out to explain the discrepancy between our findings of immunological biomarkers, such as TILs, being prognostically favorable in a low-risk population, and other studies where the opposite tendency has been observed[64-66]. We hypothesized that there are tumor-intrinsic factors that effectively predict the biological implications of an immune infiltrate and that a better understanding of these could improve the treatment individualization of patients. We wanted to test the hypothesis by, in public cohorts, training two models that quantify these two dimensions; immune activation and tumor-intrinsic factors that predict the significance of the immune activation. We used gene sets from msigdb as features to evaluate[119]. The gene sets contain genes linked to a specific biological process identified through previous studies. We hypothesized that using gene sets, rather than individual genes, would make the features more biologically relevant as genes can be expressed by various cell types and included in different pathways. To quantify different biological processes, we chose the ssGSEA method, which calculates the enrichment of a gene set by ranking the constituent genes to the expression of all the remaining genes[95]. We deemed this an advantage as rank-based methods have produced more robust results in cross-platform analyses[94]. See Figure S1 in the supplement of Study III for an overview of the methods used to create the immunological, tumor-intrinsic, and integrated models.

We started by developing the immunological model, where we hypothesized that we could quantify an activated local immune response by selecting the immunological gene sets most strongly linked to prognosis. The premise of our analyses was that an activated immune response would be consistently prognostically favorable in aggressive tumors, following previous literature[120]. The gene sets were therefore evaluated in HER2+ and Basal tumors of the training cohort after performing Pam50 subtype analysis using the genefu R package[85].

Immunological gene sets were selected from msigdb (category C7), and the enrichment of each gene set was calculated using ssGSEA. The calculated enrichment values for each gene set were subsequently tested in separate Cox regression analyses in each cohort. A meta-analysis was then conducted for all cohorts,

weighted by the root of the cohort size according to the method proposed by Stouffer et al. [121]. The top 50 ranked gene sets were then selected. Since an immune response consists of many parallel immunological processes (i.e., many interacting immune cells belonging to both innate and adaptive immune responses), we hypothesized that a large part of the selected processes would be highly correlated. We, therefore, wanted to eliminate processes that were too strongly correlated and thus meant duplicated information. To accomplish this, we calculated correlations between each possible gene set pair in each cohort, after which we calculated the mean of each correlation across all cohorts and eliminated the gene set with the lowest ranking for each pair with mean a Spearman correlation > 0.7 or < -0.7, as this indicates a strong positive or negative correlation[122]. In total, 22 gene sets remained after the collinearity reduction. An elastic net model was then trained among HER2+ and Basal tumors. Since the immunological model was not trained against direct measurements of the immune infiltrate, we compared its correlation with TILs, as measured in study I, to other methods designed to measure immune infiltration based on bulk RNA data. We used the global measurements of immune infiltration produced by xCell[118] and ESTIMATE[123], which have both been validated in independent datasets. See Figure S2 in the appendix of Study III for a comparison of the correlation between TILs, the developed immunological model, xCell, and ESTIMATE. In summary, TILs showed the strongest correlation with the immunological model, indicating that the assumptions that were used to create it were valid.

A tumor-intrinsic model was created by performing a similar meta-analysis using the interaction values between Immunescore and each gene set from the H, C2, and C6 categories. We hypothesized that these categories primarily represent tumor-intrinsic biological processes. By testing these hypothesized tumor-intrinsic biological processes in interactions with the immunological model, information is obtained on whether the prognostic effect of the immunological model in the training set varies depending on a tested tumor-intrinsic biological process. Highly significant interactions mean that the prognostic effect of the immunological model is better understood by a co-analysis of the given tumor-intrinsic biological process than by analyzing the immunological model in isolation. In order to be able to use immunological biomarkers for precision medicine fully, an understanding of these interaction effects is

therefore required. In total, eight proposed tumor-intrinsic gene sets remained after collinearity filtering. An elastic net model was then trained against prognosis and was based on the same presumption used to develop the immunological model- that biological processes predicting the prognostic implications of an immune infiltrate are associated with tumor aggressiveness. By training the model against prognosis, the proposed prognostic and immunomodulatory signals could be simultaneously isolated. Furthermore, the model was trained among tumors with a score for the immunological model in the lowest tertile (n=2312). This allowed for the exclusion of aggressive tumors where an immune infiltrate was hypothesized to attenuate the prognostic signal and thereby add noise to the signal we were trying to isolate. After elastic net modeling, only three gene sets were included in the final model. The model correlated strongly with proliferation and tumor aggressivity. The interaction between the immunological and tumor-intrinsic models was then validated in the Servant, Sjöström, and SweBCG91RT cohorts.

The immunological and tumor-intrinsic models were subsequently integrated to create a one-dimensional measure of prognosis based on immunological activation combined with tumor-intrinsic characteristics, which allowed us to investigate the clinical applicability of our findings in a group of high-risk tumors, as defined by clinical variables as age <60 with any histological grade or age <70 with histological grade III.

3.5.4 STUDY IV

In the fourth and final study, we wanted to build on the findings from study III by integrating tumor-intrinsic and immunological variables using IHC variables, as this could bring our findings closer to the clinic. To define an activated immune response, we used TILs and added PD-1 and PD-L1. We hypothesized that PD-1 and/or PD-L1 would be seen in an activated immune infiltrate as these correlate with T-cell activation and inflammation and provide independent information in addition to TILs[124]. Therefore, we defined an activated immune infiltrate as high TILs combined with a high expression of at least one of PD-1 or PD-L1. The cut-off for PD-1 and PD-L1 was the same as in clinical practice for breast cancer (≥1%)[20]. Two TMAs per patient and marker were used. By including TILs from whole sections and four TMAs, we aimed to counteract the problem of heterogeneity associated with TMAs. As a tumor-intrinsic measure, we used histological grade since the tumor-intrinsic model correlated best with this variable and is a well-established measure of tumor aggressiveness[125, 126]. Furthermore, we chose histological grade instead of subtype because the latter is not one-dimensional and likely contains greater heterogeneity, as illustrated by the Luminal B-like subtype that can exhibit a wide range of proliferation[125, 126].

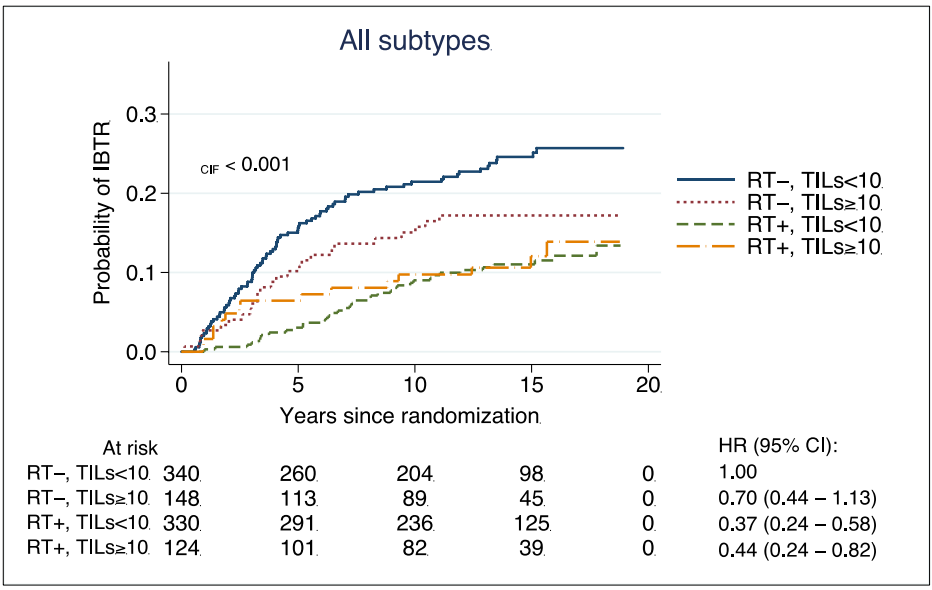
Tumors of histological grade III were classified as high-risk. Since histological grade II fails to provide clinically meaningful information, we also wanted to stratify these based on aggressivity, measured by the tumor-intrinsic model from study III. See Figure S1 of the supplement of study IV for a comparison of scores for the tumor-intrinsic model between different histological grades. In order to avoid diluting the aggressivity characteristics of the high-risk group, we used a high threshold to upgrade grade II tumors to high-risk; see Figure 2 of study IV for a flow chart of the categorization of high- and low-risk tumors.

4 RESULTS

4.1 STUDY I

Most patients had tumors with TILs <10% (71%). TILs correlated with clinical variables associated with a less favorable prognosis (higher histological grade, the ER-negative/HER2-positive subtypes, younger age). High TILs conferred a non-significant, but numerically reduced, benefit from RT (HR 0.63, CI 95% 0.31-1.27) compared to patients with low TILs (HR 0.37, CI 95% 0.25-0.57). High TILs were also associated with a reduced risk of a local recurrence (HR 0.61, CI 95% 0.39-0.96, p=0.033) in multivariable analysis. A significant interaction between TILs and RT was not observed (p=0.317).

Figure 7. Benefit from radiotherapy depending on TILs in the SweBCG91RT cohort



Having TILs ≥10% was associated with an improved prognosis in multivariable analysis when adjusting for histological grade, age, and RT. Although not significant, the benefit from RT also appeared reduced among tumors with high TILs, compared to tumors with TILs <10%. First published in Kovács, A., Stenmark Tullberg, A., Werner-Rönnerman, E., et al. Journal of Clinical Oncology (2019) 37:14. Reprinted with permission.

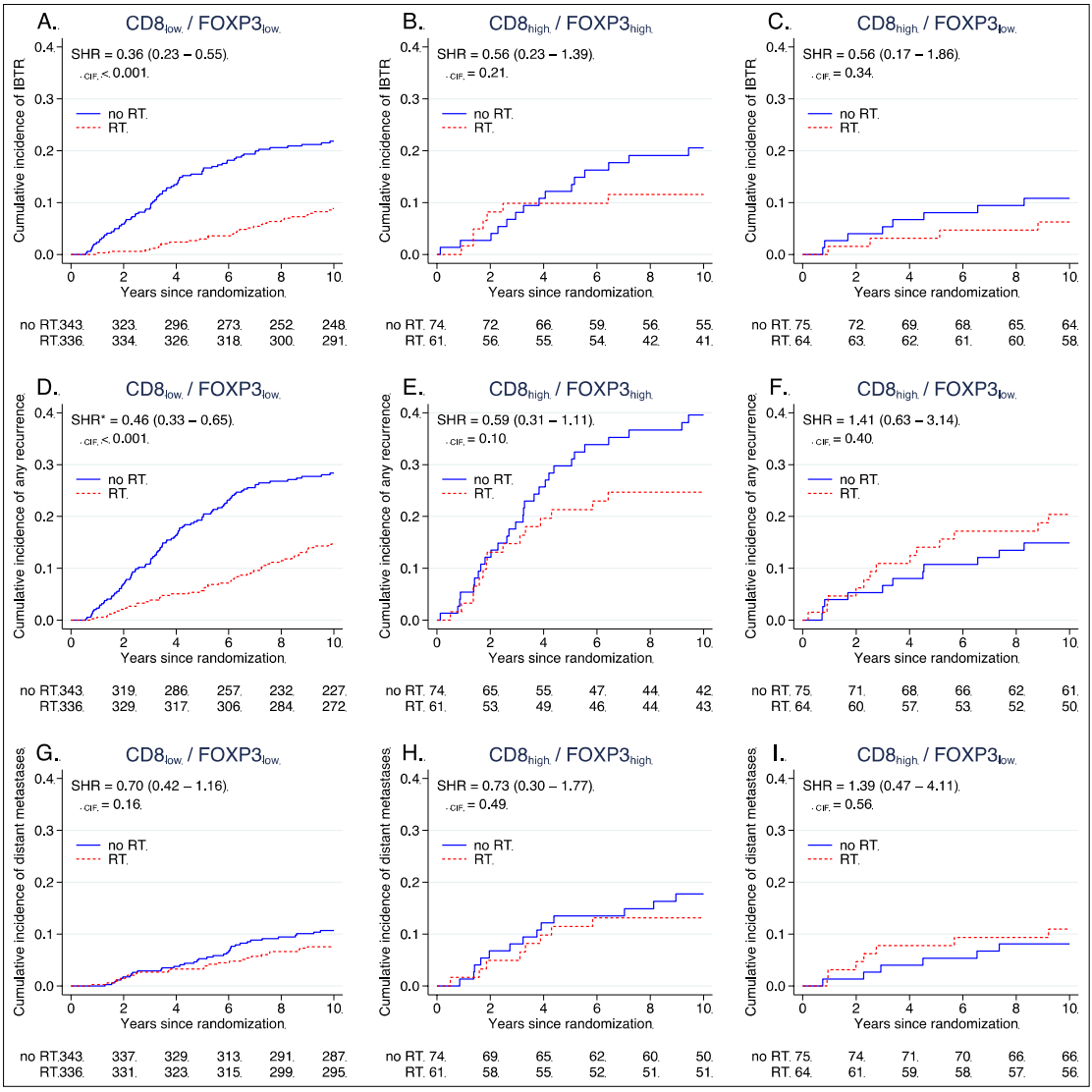
4.2 STUDY II

4.2.1 IHC

Having a high amount of FOXP3-positive lymphocytes correlated with unfavorable clinical variables such as non-luminal subtypes, higher histological grade, and younger age, similar to what we observed for TILs. However, CD8 positivity was not as strongly correlated with these variables in the absence of concurrent FOXP3 positivity.

As described in the statistics section, we used any recurrence as the primary endpoint in study II to increase power given the non-significant interaction test in study I, which we hypothesized was due to insufficient power. We found that the $CD8^{High}FOXP3^{High}$ group had the highest risk of any recurrence (HR 1.7, CI 95% 1.2-2.4, p=0.002) with the $CD8^{Low}FOXP3^{Low}$ group as the reference group (HR 1.0). In univariable analysis, we found no significant difference in prognosis between $CD8^{High}FOXP3^{Low}$ compared to $CD8^{Low}FOXP3^{Low}$ (HR 1.0, reference) regarding any recurrence (HR 0.79, CI 95% 0.51-1.2, p=0.28) or local recurrence (HR 0.55, CI 95% 0.30-1. 0, p=0.052). In order to investigate the prognostic effect independent of RT, we conducted a multivariable analysis of unirradiated patients. In this group, the $CD8^{High}FOXP3^{Low}$ had a reduced risk of any recurrence (HR 0.41, CI 95% 0.21-0.77, p=0.006) and IBTR (HR 0.41, CI 95% 0.19-0.86, p=0.018) compared to $CD8^{Low}FOXP3^{Low}$ (HR 1.0), Figure 8. The $CD8^{High}FOXP3^{Low}$ group, which had the best prognosis among unirradiated patients, numerically showed the least benefit from RT, Figure 8. The interaction test was significant for any recurrence (p=0.039) but not for IBTR (p=0.68).

Figure 8. Cumulative incidence IBTR, any recurrence and distant metastasis among immune groups created by immunohistochemical assessment

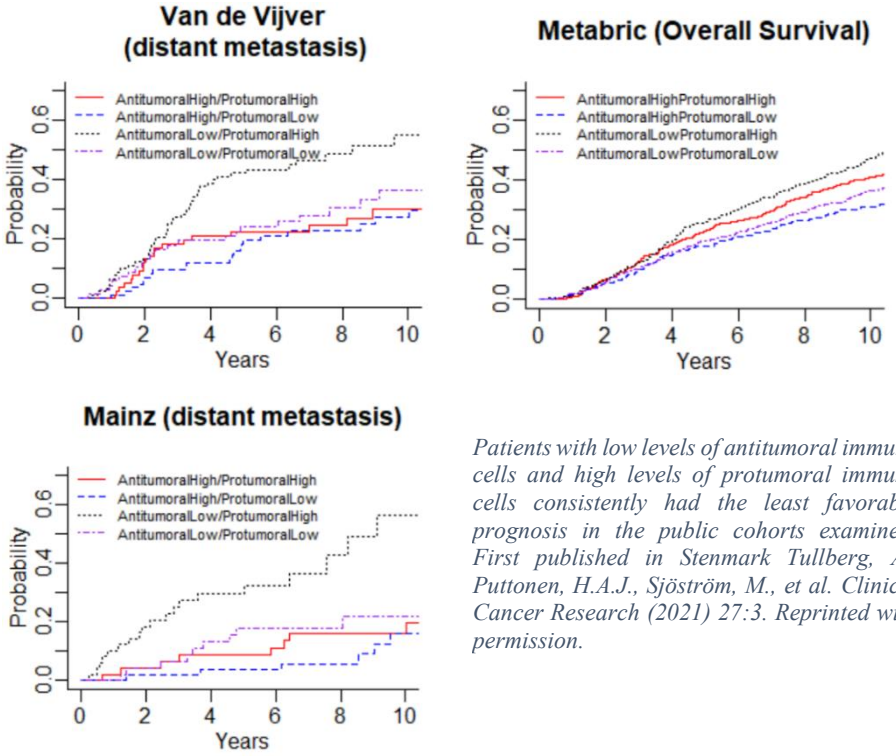


The group without an immune infiltrate ($CD8^{Low}/FOXP3^{Low}$) appeared to have the greatest benefit from RT, while patients with the immune balance hypothesized to be most favorable ($CD8^{High}/FOXP3^{Low}$) numerically derived the smallest benefit from RT. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.

4.2.2 GENE EXPRESSION

Using publicly available cohorts, we selected individual cell types that showed a consistent prognostic effect, as quantified by xCell[98]. They were then scaled and summed into an antitumoral- or a protumoral variable depending on if they conferred a favorable or an unfavorable prognostic effect. Below are figures of the prognostic impact of the balance between the antitumoral and protumoral variables in the public cohorts.

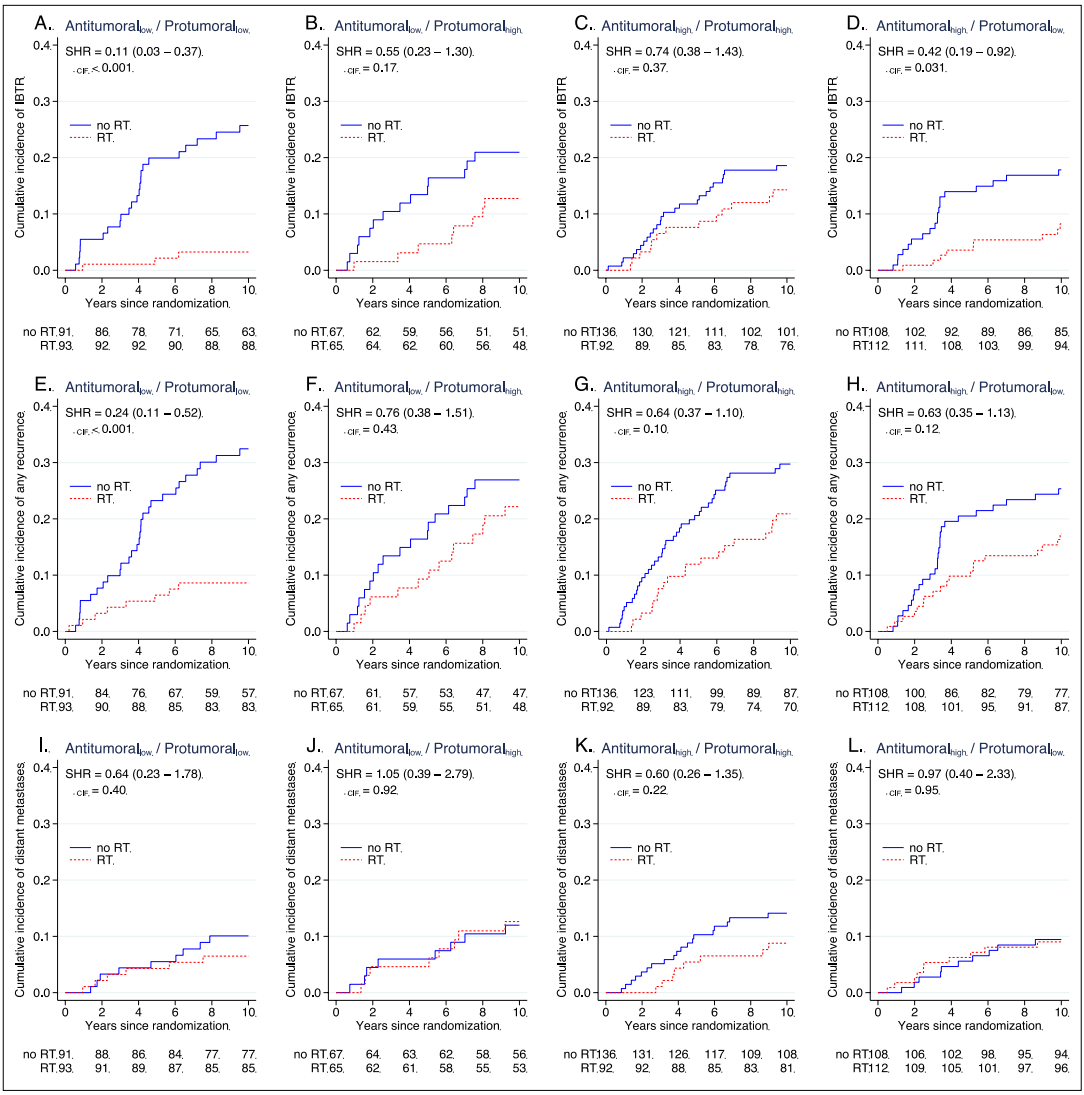
Figure 9. Prognosis associated with the balance between antitumoral- and protumoral immune cells.



Patients with low levels of antitumoral immune cells and high levels of protumoral immune cells consistently had the least favorable prognosis in the public cohorts examined. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.

In the SweBCG91RT cohort, none of the immune groups were significantly associated with the risk of any recurrence or local recurrence. The largest benefit from RT was seen in the immune-depleted group (any recurrence: SHR 0.22, CI 95% 0.10-0.49, p<0.001, local recurrence: SHR 0.11, CI 95% 0.03-0.37, p<0.001). The interaction analysis between the immune groups and RT was significant for any recurrence (p=0.035) and local recurrence (0.025).

Figure 10. Cumulative incidence of IBTR, any recurrence, and distant metastasis among immune groups created by gene expression



The most immune-depleted group (Antitumoral^{Low}/Protumoral^{Low}) appeared to derive the largest benefit from RT. First published in Stenmark Tullberg, A., Puttonen, H.A.J., Sjöström, M., et al. Clinical Cancer Research (2021) 27:3. Reprinted with permission.

4.3 STUDIES III & IV

Refer to the results sections of the unpublished manuscripts of studies III and IV for a description of the results from these studies. In summary, we found that integrating tumor-intrinsic and immunological factors provided superior predictions of the risk of local recurrence than either factor alone. This information could also be used to estimate the absolute benefit from RT in terms of reducing the risk of local recurrences.

5 DISCUSSION

RT has long been an essential component of breast cancer treatment, primarily aimed at preventing local recurrences[24, 25]. Systemic breast cancer treatment has undergone rapid development and is now tailored based on tumor biology to maximize the benefit to the patient[127]. Unfortunately, RT has not undergone the same development, and today's guidelines are based primarily on crude clinical prognostic measures rather than the biology of the tumor[36, 37]. Immunological biomarkers may predict the benefit from chemotherapy and anti-HER2 therapy, indicating a biological relevance for treatments other than immunotherapy[57-59]. Mechanistic studies indicate that RT may affect the local immune response in a favorable way[69], which is corroborated by studies of cancer types other than breast cancer [60, 128, 129]. However, it is unclear how this translates to the adjuvant clinical setting in breast cancer. This thesis shows that immunological biomarkers provide independent information in addition to standard clinical variables and can differentiate patients based on the risk of local recurrences. This information may be used to improve RT individualization.

An immune infiltrate is associated with an improved prognosis among aggressive breast cancer subtypes[124]. However, this is not seen in less aggressive subtypes[64-66], and a better understanding of tumor-intrinsic factors that modify the importance of the immune infiltrate is required. In this thesis, we studied how immunological activation, measured via IHC and gene expression, affects the local recurrence risk and benefit from RT in breast cancer, primarily by retrospective analyses of the randomized SweBCG91RT trial[71, 130]. We found that a favorable immune response translates to an improved prognosis and perhaps a possibility of RT de-escalation.

A favorable antitumoral immune response is based on the activation of T cells targeting tumor-specific proteins (neoantigens) encoded by DNA mutations[55]. However, T cells require the support of antigen-presenting cells for activation, which is why a favorable tumor microenvironment with adequate antigen presentation and costimulation also is essential[131, 132]. In this thesis, we consistently observed that tumor aggressiveness and proliferation predict a beneficial prognostic effect of immune infiltrates. This was already seen in study I for high TILs when stratifying for subtypes.

Non-luminal and Luminal B-like subtypes appeared to be favored by high TILs in contrast to Luminal A-like tumors. Study III confirmed that tumor aggressiveness is predictive of the implications of an immune infiltrate regarding local recurrences. Interestingly, most tumors of the high-risk group in study IV consisted of ER-positive tumors, a group not considered to be particularly immunogenic[67]. Therefore, our findings indicate that subtyping is insufficient to accurately predict an immune infiltrate's significance. This is particularly important for Luminal B tumors, which exhibit a broad spectrum of aggressiveness that ranges from slightly to significantly higher proliferation than Luminal A[133]. Here we see the potential for improved treatment individualization by, for example, adding additional proliferation thresholds for Luminal B tumors to identify those that benefit from an immune infiltrate. Although we have not explicitly tested the latter, examples of measurements that can be used are gene expression, histological grade, and probably Ki67. We hypothesize that proliferation contributes to properties in the tumor microenvironment that increase the likelihood of an activated antitumoral immune response[134]. These include an increased amount of neoantigens, increasing the possibilities for a diversified T-cell response[55, 135], or increased replication stress[136, 137], resulting in an improved costimulatory environment with activation of antigen presentation pathways[138].

Highly proliferating tumors with an activated immune response have an excellent prognosis, as observed in studies III and IV, despite a lack of systemic treatment and intensified RT (i.e., RT boost). The prognosis may even be more favorable than that of less aggressive tumors. This finding conforms with another recent study showing a favorable prognosis among high-risk patients with aggressive tumors and an immune infiltrate[139]. The incidence of local recurrences in the unirriadiated group of patients with a favorable immune infiltrate, identified by integrating tumor-intrinsic and immunological variables in study IV, was lower than in an unirradiated low-risk group of patients aged ≥65 years, with N0 luminal A-like tumors previously studied from the SweBCG91RT cohort[84]. Therefore, these findings suggest that de-escalation of RT treatment is possible in this patient group— either by omitting the RT boost or even omitting RT altogether.

The group consistently exhibiting the worst prognosis were those with aggressive tumors without an active immune infiltrate, as indicated by studies III and IV. Study III also showed that these tumors may be radioresistant. However, signs of radioresistance were not seen in study IV, possibly due to the reduced ability of the categorical variables histological grade, TILs, and PD-1/PD-L1, to identify tumors with the most extreme characteristics as opposed to the continuous gene expression models. Nevertheless, it is likely among patients with aggressive immune-depleted tumors that an RT boost is most beneficial. No patient in the SweBCG91RT cohort received an RT boost, and this patient group's benefit should be evaluated in future studies. Another exciting treatment possibility for these patients may be TGFb inhibition, which can induce radiosensitization and immune activation by inhibiting immunosuppressive pathways[48, 49].

The correlation between immune infiltration and proliferation makes the integration of immunological biomarkers and tumor-intrinsic factors, to some extent, occur by itself. This can explain why we saw a prognostically favorable effect of immune infiltration in study I and, to some extent, study II despite not stratifying our analyses by tumor-intrinsic factors. Tumors with TIL infiltration tend to have a higher proliferation rate but, due to an activated immune response, at the same time, a good prognosis. The tumor group without immune infiltration is more heterogeneous and consists of less aggressive tumors with a good prognosis and highly aggressive tumors with a poor prognosis. Not taking into account clinicopathological confounders may underestimate the influence of an immune infiltrate on the risk of local recurrences. The $CD8^{High}FOXP3^{High}$ group in study II most clearly exemplifies this. These patients had an increased risk of breast cancer recurrence, likely primarily due to the strong correlation between FOXP3 and unfavorable clinicopathological variables. This is a potential cause of the discrepancy in the literature regarding the prognostic impact of an immune infiltrate. Failure to adjust for confounders, or to stratify patients based on tumor-intrinsic factors associated with a benefit from an immune infiltrate, can make immunological biomarkers appear less favorable or even unfavorable. Public gene expression cohorts of breast cancer patients generally contain more aggressive tumors, where an immune infiltrate is favorable[76]. In contrast, population-based cohorts may include less aggressive

tumors, which explain the unfavorable effects of an immune infiltrate observed[64-66].

Although several tools predictive of radioresistance have been developed, most have failed external validation[43]. There are several reasons for this. Firstly, there is a lack of high-quality randomized RT cohorts. Secondly, in vitro studies on cell lines are likely to successfully identify predictors of tumor-intrinsic radioresistance but fail to account for tumor heterogeneity and stromal crosstalk seen in vivo[43]. In addition, RT is based on an assumption of residual tumor microfoci, and it is among patients where this assumption is valid that tools predicting radioresistance can be of benefit. In vitro studies mimic the neoadjuvant, rather than the adjuvant, setting and therefore fail to account for the problem of accurately predicting the residual tumor burden after surgery. The radioresistance of a primary tumor does not matter if the patient is completely cured by surgery. Despite negative margin status, about one-third of tumors have microfoci[47]. Although measures of tumor aggressiveness appear to provide information on this, as illustrated by the prognostic significance, it does not provide a complete picture. This is exemplified by the fact that unirradiated clinically defined low-risk groups in the SweBCG91RT cohort have almost as high a risk of a local recurrence as unirradiated patients with more aggressive tumors[140]. This thesis suggests that measures of the local immune response provide independent information on the risk and extent of tumor micro-invasion.

The application of radioresistance profiles to entire breast cancer cohorts, where most patients are likely already cured when postoperative RT is given, likely contributes unexplained variance that obfuscates the true biological signal of radioresistance and makes external validation difficult. This thesis indicates that the local immune system may predict the probability of residual tumor cells and the development of a subsequent local recurrence in unirradiated patients. Immunological biomarkers may, therefore, be used both for evaluating patients who may be cured by surgery alone and as a pretest prevalence tool for gene profiles to assess tumor-intrinsic radioresistance. A group with radioresistant tumors where the vast majority are cured by surgery alone should probably be assessed differently concerning RT individualization than a similar group where a significantly larger proportion is estimated to have residual tumor burden post-surgery. We hypothesize that the biology

of any residual tumor foci and the associated tumor microenvironment have characteristics similar to that of the resected primary tumor. An immune response is generally mounted in secondary lymphoid organs[50] and, therefore, likely not restricted to the primary tumor, suggesting that its effects also remain postoperatively. Apart from reducing the risk of micro-invasion preoperatively, a favorable immune activation may, therefore, also indicate that the patient is protected from tumor regrowth postoperatively. In other words, a beneficial immune activation in the removed primary tumor could raise the threshold for the residual tumor burden needed for tumor regrowth to occur.

Much of the prospective clinical research on RT individualization has focused on low-risk clinical groups by identifying elderly patients with ER-positive small tumors[141]. These have been hypothesized to have such a good prognosis that they do well on endocrine therapy alone. However, the relative benefit from RT regarding local recurrences appears to be unchanged compared to large meta-analyses on the entire breast cancer population[24, 141]. Nevertheless, the absolute benefit is reduced due to the favorable prognosis. Immune infiltration is not as frequent in low-risk tumors as in high-risk tumors, and our studies indicate that immune infiltration among low-risk tumors does not translate to an improved prognosis. Therefore, immune infiltrates among these tumors should probably not warrant RT omission. On the contrary, tendencies toward an unfavorable effect from an immune infiltrate were seen in studies III and IV, which conforms with previous studies[64-66]. Potential explanations for these observations are associations between immune infiltrates and unfavorable clinical variables[142] or that an immune response in these tumors is dysfunctional and favors tumor growth[143, 144]. Future studies should try to confirm whether immune infiltration in low-risk tumors predicts an increased risk of local recurrences, making RT omission unsuitable.

In vitro studies show synergistic effects between RT and immunotherapy, capable of generating an abscopal effect. An immune-stimulating effect of RT can explain this through the release of neoantigens and the creation of an immunogenic tumor microenvironment[68, 131, 145]. We saw no clear evidence that an activated immune response was associated with a more significant benefit from RT in studies I, II, or IV. In study III, when tumor-intrinsic factors were considered, tendencies toward a larger RT

benefit were seen in the high-risk group with an activated immune response. With a lower tumor burden, the postoperative setting may not allow for RT-induced immune activation.

5.1 WEAKNESSES

Several weaknesses should be addressed in our studies. First, the gene expression information from SweBCG91RT is based on FFPE tissue, suggesting lower-quality RNA with increased non-biological variance. We noted that correlations between immunological genes were significantly weaker in the SweBCG91RT cohort compared to public cohorts with fresh-frozen material. This may indicate lower gene expression quality in SweBCG91RT. The method of calculating enrichment scores for gene sets, rather than individual genes, in study III was an attempt to increase the robustness of our measurements due to the potentially reduced RNA quality.

For immunohistochemical analyses in studies II and IV, TMAs were used, which entails problems with potential tumor heterogeneity and risk of non-representative evaluations[80]. This problem was likely the largest for study II, where the classification of CD8 and FOXP3, respectively, relied on two TMAs each despite questionable concordance. It has been found that three TMAs are a lower limit of what is needed to estimate immune infiltration reliably[82]. In study IV, the evaluation was based on four TMAs in addition to the TILs evaluation, which we believe should provide a reliable measure of the immune infiltrate.

Our findings overall suggest that the use of immunological biomarkers requires stratification based on tumor-intrinsic qualities. Therefore, the SweBCG91RT cohort is heterogeneous in the context of how to interpret immunological biomarkers, as indicated by studies III and IV. Furthermore, subgroup analyses reduce the power, necessitating additional analyses in independent cohorts to confirm our results. In study III, we attempted to compensate for this problem by including analyses of the independent Sjöström[74] and Servant[73] cohorts.

RT has been shown in previous randomized trials to be so effective at reducing the risk of local recurrences that there are few randomized RT cohorts available where the benefit of RT can be studied retrospectively. Therefore, we have not been able to validate any biomarkers predictive of RT benefit in independent cohorts. Instead, we used public gene expression cohorts to test the generalizability of parts of our findings (i.e., the influence of immunological biomarkers among irradiated patients regarding

local recurrences). This means that our findings on the relative effects of RT in different groups are hypothesis-generating and should be confirmed in future studies.

A further objection to the generalizability is that patients of the SweBCG91RT cohort received different treatment than today's breast cancer patients. A small proportion of patients received systemic treatment. However, in today's situation, all patients with ER-positive tumors would receive endocrine therapy, all but luminal A tumors with a low recurrence risk chemotherapy, and all HER2-positive tumors anti-HER2 therapy. In addition, immunotherapy may have been given to high-risk tumors[20]. Furthermore, preclinical data suggest that anti-HER2 therapy is both RT-sensitizing and immune-stimulating[146], and anti-HER2 treatment may have directly influenced our results. No patients received an RT boost, which today would have been given to patients at high risk of a local recurrence. All in all, this suggests that the prognosis of the patients would have been better today and that our findings are not fully representative of the modern setting. However, despite the objection to generalizability, we believe that the absence of treatment with an RT boost and systemic therapy has allowed us to study which tumors can undergo safe de-intensification of treatment.

6 CONCLUSION

- Breast tumors can be classified according to the two axes of proliferation and immune infiltration, each containing independent information. An accurate interpretation of the prognostic implications of one requires a mutual co-analysis of the other.
- Highly proliferating tumors with an activated immune infiltrate may have a remarkably low risk of local recurrences. Patients with such tumors may be candidates for RT de-escalation.
- Highly proliferating tumors without an activated immune infiltrate have the highest risk of local recurrences and likely constitute the group needing an RT boost.
- A subset of high-risk ER-positive tumors may be immunogenic and benefit from an immune infiltrate. In addition, some of these may be candidates for therapies targeting the antitumoral immune response.
- An immune infiltrate cannot be used to guide RT de-escalation among supposedly low-risk tumors as it may indicate an increased local recurrence risk.

7 FUTURE PERSPECTIVES

Although most breast tumors with TILs may be ER-positive, most research has focused on more aggressive subtypes[67]. Immunological biomarkers are, therefore, incompletely utilized in breast cancer. Additional research is required to understand why breast cancer is generally non-immunogenic and whereby the ER-positive subtypes do not seem to benefit from an immune infiltrate. Understanding the tumor-intrinsic factors that predict immunogenicity and favorable immune activation may enable the identification of a subgroup of ER-positive tumors that benefit from immunotherapy. It could also contribute to improved treatment individualization in other respects by providing independent prognostic information in addition to the tumor-intrinsic factors evaluated in clinical practice.

Most research on the de-escalation of RT has so far focused on low-risk tumors[147]. As more studies show a strongly favorable prognostic effect of an antitumoral immune response in high-risk tumors[46], it can be hypothesized that these are also candidates for RT de-escalation, despite exhibiting aggressive characteristics. An effective immune response could, for example, justify omitting RT boost in borderline indication. Prospective studies should investigate whether reducing the amount of RT given to these patients is safe. One can hypothesize that these patients do well without intensive RT treatment when given immunotherapy and adequate additional systemic treatment. Furthermore, future attempts to create tools predictive of radioresistance may try to implement the independent information provided by a successful characterization of the immune infiltrate. One proposal is to stratify primary tumors with presumed radioresistance based on immunological activation. It is likely to be immune-depleted aggressive tumors, which thus have a high risk of residual local tumor burden, where information about tumor-intrinsic radioresistance is most likely to be translated to a benefit from RT intensification.

Clinical trials combining RT and immunotherapy are ongoing[148], and it will be interesting to see if this allows synergistic effects to be obtained in the clinical setting. The neoadjuvant setting may be most beneficial due to the higher tumor burden, and the greatest probability is likely observed for rapidly proliferating tumors with a wide range of neoantigen targets. The optimal fractionation scheme to produce favorable immunological effects remains to be elucidated, but

mechanistic studies suggest that higher doses and fewer fractions are the most beneficial[149]. If RT can be combined with immunotherapy to enhance the antitumoral immune response, RT may go from being considered a local to systemic therapy.

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