Association between tumor immune response and risk of recurrence in breast cancer patients treated with radiotherapy

Background:

Breast cancer (BC) is the most common type of cancer among Danish women with a yearly incidence of 4.700. All Danish BC are registered in the DBCG (Danish Breast Cancer Group) database.

Benefit from radiotherapy:

Standard treatment for BC is surgery, and approximately 75% of all Danish BC patients are today treated with breast-conserving surgery (BCT) followed by radiotherapy (RT). RT is administered to eradicate remaining tumor stem cells and improve local tumor control, with the ultimate aim of improving patient survival. RT is offered to all patients treated with BCT, and to patients treated with mastectomy in whom clinico-pathological variables suggest a high risk of loco-regional recurrence (LRR)[1]. Preventing LRR is of high priority, in part to save the patient from distress, but also because LRR may act as nidus for subsequent distant metastasis (DM) and thereby compromise survival. The proportion of patients eligible for RT is increasing, and was offered to >75% of newly diagnosed BCs in 2019 *(data from the DBCG)*. However, the effects of RT have been found to be variable, not all BC patients benefitting from the treatment. Thus, some patients suffer the risk of RT-associated side-effects (including ischemic heart disease[2,3] and secondary cancer[4]) with limited survival advantage, and some develop recurrences in spite of RT. At the same time, the 5-year risk of LRR has decreased substantially from 7% in the period 1980-90[5] to approximately 3% at present[6,7]. Given these factors, it is of great importance to improve the identification of patients who are at high vs. low risk of developing LRR, and who are more or less likely to benefit from RT.

At present, no validated biomarkers can reliably predict benefit from RT or identify those BC patients who are at high or low risk of developing a recurrence after RT.

Intrinsic subtypes in breast cancer

Numerous tumor gene expression profiles in BC have been developed with the aim of classifying patients more precisely and individualizing systemic treatment. The most important has been the identification of prognostically relevant gene-expression based intrinsic subtypes (IS; Luminal A, Luminal B, HER2-enriched, Basal-like)[8,9]. A commercially available product based on IS (Prosigna Breast Cancer Prognostic Gene Signature Assay) has been implemented in the DBCG guidelines to assist treatment decisions with regard to chemotherapy and endocrine therapy[10,11]. For practical and economical reasons, immunohistochemical (IHC) surrogates for the IS-profiles have been introduced, that use a limited number of antibody markers, e.g., estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2) and Proliferation marker Ki-67 (Ki67)[12,13], but these do not provide identical prognostic information compared with the gene expression based subtypes. The value of IS for predicting RT-response or LRR risk has also been examined with Basal-like and HER2-like tumors being associated with

higher, and Luminal A tumors with lower LRR rates[14–16]. However, these findings are not robust enough to guide RT-treatment decisions.

Radiotherapy and the immune system

A limited number of gene expression profiles have been published that are either prognostic for LRR, or predictive of radiosensitivity or radio-resistance[17–20]. Among these, a DBCG-RT gene profile showing both prognostic impact in terms of LRR and benefit from RT has been identified and validated by Tramm *et al*[19], and has been found to be independent of the IS[21]. The profile was developed from pre-treatment tumor-tissues from high-risk BC patients included in the nationwide DBCG82bc cohort. Patients were diagnosed in 1983-89 and randomized to +/- RT after mastectomy[22,23]. Among patients not treated with post-mastectomy RT (PMRT), the DBCG-RT profile identified two groups with significantly different LRR risks ($p<0.0001$). PMRT reduced the risk of LRR in the "High LRR risk" patients ($p<0.0001$), whereas the "Low LRR risk" patients experienced no additional benefit from RT ($p=0.93$). The genes in the DBCG-RT profile were found to be related to the immune system, *e.g.*, T-cell activation and antigen presentation, suggesting that the beneficial effect of RT could be associated with the presence of immune cells in the tumor before treatment. Preclinical data also suggests that part of the efficacy of RT is due to an immunogenic cell death mediated through activation of STING/cGAS pathway triggering activation of tumor-specific, cytotoxic T-lymphocytes[24]. A high level of tumor infiltrating lymphocytes (TILs) is only found in approximately 10% of BCs. This is seen more frequently among the aggressive Basal-like- and HER2-enriched subtypes than among the luminal subtypes, although BCs of all subtypes may show low, intermediate and high TILs levels[25] (Figure 1). Recently, studies of the same DBCG82bc patients have indeed shown a correlation between the DBCG-RT profile and TILs (unpublished data), and high TILs have been found to be associated with significantly increased benefit from RT in terms of overall survival (OS), with a trend in terms of lower DM risk, but without significant effect on local control[26] (Figure 2). However, TILs comprise various cell types (e.g., lymphocytes and plasma cells), and it is likely that the association is dependent on specific immune cells (e.g., cytotoxic or regulatory T-lymphocytes or B-lymphocytes). The mechanism behind the observed association between TILs, RT and clinical outcome is unclear.

It would be of great clinical value in BC if evaluation of immune cells in primary tumors could provide prognostic information and identify patients at high *vs*. low risk of recurrence after RT.

Purposes:

1. To study the composition of immune cells in different subtypes of BC
2. To study the differences in subtype and immune response in paired primary tumors and corresponding local and/or distant recurrences in BC
3. To study the association between the immune response in pretreatment primary tumors of various subtypes and risk of local and/or distant recurrence and breast cancer specific mortality in BC patients treated with various RT-regimes

Material and methods:

Study population and data

BC patients from three national cohorts exploring varying RT-regimes will be included.

- **DBCG-HYPO**: low risk patients > 40 years of age (y/a), diagnosed 2014-2019, treated with BCT and randomized to RT at a dose of either 50 Gray (Gy)/25 fractions (fr) or 40 Gy/15 fr (hypofractionation). In 1538 patients, 44 LRR and 38 DM are recorded with a clinical follow-up time of approximately 7 years.
- **DBCG-PBI:** low risk BC patients ≥ 60 y/a, diagnosed 2014-2019, treated with BCT and randomized to either whole or partial breast RT (40Gy/25 fr). In 880 patients, 15 LRR and 7 DM are recorded with a clinical follow-up time of approximately 7 years.
- **DBCG:IMN2**: high risk BC patients with lymph node metastasis or tumorsize > 5 cm, diagnosed 2008-2014, treated with mast/BCT and axillary dissection and RT including irradiation of the internal mammary nodes (IMN) in right-sided BC. Approximately, 17% of the 5627 patients (≈ 966 patients) are expected to originate from the Central Region of Denmark (RM). Based on results from the IMN1 study, we expect to find approximately 12 LRR and 120 DM in the RM cohort. Median clinical follow-up time will be approximately 10 years.

The study set up will be as a **case-control study** encompassing 72 LRR and 165 DM (= 237 cases) from the three cohorts; each case matched with three controls who have not developed a recurrence (=711 controls). From all 948 cases/controls, formalin-fixed, paraffin-embedded tissue (FFPE) from primary tumors and recurrences will be collected. We expect to be able to retrieve FFPE from approximately 80% of recurrences, since DM in particular may not be histologically verified or biopsy material may be too sparse for further analysis. Patients will be identified through the DBCG database; information on surgical procedure, RT-regime, histopathological information and clinical outcome data will be applied for from the DBCG.

Subproject 1

Hypothesis

- *The composition of the immune cells present in the BC tumor tissue before treatment varies according to the tumor´s intrinsic subtype*

Research-plan

To investigate, whether not only level but also the composition of TILs vary according to IS, the presence of specific immune cells will be examined in primary tumors from the total cohort of patients, ensuring representation of BCs of all subtypes. Immunohistochemical surrogates of IS will be constructed using ER, HER2 and Ki-67 indexes (data available from the DBCG). IHC information will be supplemented if missing. Stromal TILs will be examined in whole slide Hematoxylin & Eosin (H&E)-stained sections using lightmicroscopy according to DBCG guidelines[27,28]. Multiplex IHC, allowing evaluation of up to five markers on the same tissue slide, will be used to determine the composition of the immune cells, e.g., as cytotoxic and regulatory T-cells, and B-cells including tertiary structures (using CD4, CD8, FOXP3, CD20, etc.). The stains will include

markers of tumor epithelium (CK7/19) and myoepithelial cells (p63) in order to differentiate between invasive carcinoma, in situ lesions and tumorrelated stroma. Furthermore, markers of the STING-pathway may be included (e.g., STING, cGAS, IFI16). The stained slides will subsequently be digitally scanned, to allow for automated digital image analysis to aid in the generation of quantitative estimates of marker expression, together with information on co-expression of markers and the localization of the immune cells (intraepithelial, stromal etc.)(Figure 3). In non-biopsy material with adequate FFPE-tissue, tissue micro arrays (TMAs) may be used to minimize running costs for IHC stains, after first ensuring that the use of TMA cores is validated and provides equivalent results to those based on whole slide sections (data available, but not yet analyzed).

Subproject 2

Hypothesis

- *The immune response decreases in magnitude and differs in composition comparing primary tumors with subsequent, corresponding recurrence*
- *The intrinsic subtype is retained from primary tumor to corresponding local/distant recurrence or converts to a more aggressive subtype*

Research plan:

To investigate differences in the presence of immune cells comparing primary tumors and subsequent recurrences, corresponding pairs of primary tumor and available recurrences among the group of 237 cases will be examined. In general, levels of TILs is lower in metastases[29]; however it is unclear if (and eventually how), the specific composition differs. IS is largely maintained from primary tumor to metastasis; in particular, Basal-like tumors are found to be very stable, whereas > 50% of Luminal A tumors convert to more aggressive subtypes such as Luminal B and HER2-enriched[30]. In the paired samples, changes in the immune response analyzed as described in Subproject 1, will as such be correlated to IS in order to also evaluate possible associations related to a change in subtype. In a subgroup of patients with availability of tissues from both local and distant recurrences, gene-expression analysis (nCounter Breast Cancer360™ Panel) including evaluation of IS and immunogenic pathways, will be performed to expand the characterization of the immune response and allow for a possible correlation between protein- and mRNA gene expression of the immunogenic markers. This part of the project will be carried out in collaboration with Therese Sørlie, Oslo University Hospital.

Subproject 3

- *The immune response in pretreatment, primary breast cancers of various subtypes is associated with risk of local and/or distant recurrence and breast cancer specific mortality in BC patients treated with various RT-regimes*

Research plan

To examine if the immune response in the primary tumor tissue (before systemic treatment and RT) is associated with risk of recurrence in BC patients treated with RT, results from the histopathological characterization of the immune response in the various subtypes will be correlated to clinical outcome data for all 948 patients included in the case-control study. Clinical outcome data will include information on allocation to RT-

regime (+/- hypofractionation, partial *vs*. whole breast RT, +/- nodal irradiation), systemic treatment given and data on survival (breast cancer specific mortality and OS) etc.

Statistics:

All subprojects are planned to include a sufficient number of patients to secure conclusive results with a power of >80%. Simple agreement measures and Spearman correlation will be used to evaluate correlations between TILs and IS. Univariate survival analysis and multivariate proportional hazard evaluations will be used for survival analysis using BC specific mortality and OS as endpoints. Cumulative incidence proportions will be used for LRR and DM taking competing risks into account.

Perspectives:

If the immune response in the primary tumor (before treatment) can predict clinical outcome in BC patients, it may lead to more individualized RT treatment and modification of DBCG RT-treatment guidelines. The applied methods are readily applicable in a daily pathological routine and can be performed on pre-operative biopsies allowing for an early treatment planning.

Ethics:

The project relates to already treated patients and does not raise any problematic ethical issues. The study will be registered at RM (RMs Interne Fortegnelser over Forskningsprojekter) and approval will be applied for from the local Ethical Committee. For patients diagnosed after 2004, the Danish Registry for Use of Tissue (Vævsanvendelsesregister) will be checked for matches.

The applicant's role in the project (for timeframe, please see Figure 4):

1) Contributing to fund application and coordinating the project
2) Collecting FFPE materials from the Danish pathology departments
3) Updating missing IHC data on Ki-67, ER and HER2 status
4) Estimating TILs on H&E-sections from all primary tumors and recurrences
5) Identifying tumor areas on H&E-sections to be used for eventual TMAs
6) Participating in digital image analysis of multiplex IHC and gene expression analysis
7) Analyzing and evaluating data

The applicant has been invited to visit Professor Therese Sørlie´s laboratory at Oslo University Hospital for a stay of 3-4 months. Sørlie´s group has substantial, multifaceted expertise in the molecular biological methods and bioinformatics needed to support the proposed project. The project is related to another planned PhD project ("The DBCG IMN2 study") and collaboration with mutual benefit for the involved PhD students is expected.

Supervisors:

Aarhus University Hospital:

- Trine Tramm, Consultant, Associate Professor, PhD, Department of Pathology (Main supervisor)
- Birgitte Vrou Offersen, Consultant, Professor, PhD, Department of Oncology
- Lise Bech Jellesmark Thorsen, MD, PhD, Department of Oncology

Oslo University Hospital, Norway:

- Therese Sørlie, Group leader, Professor, PhD, Department of Cancer Genetics

Figure 1-4

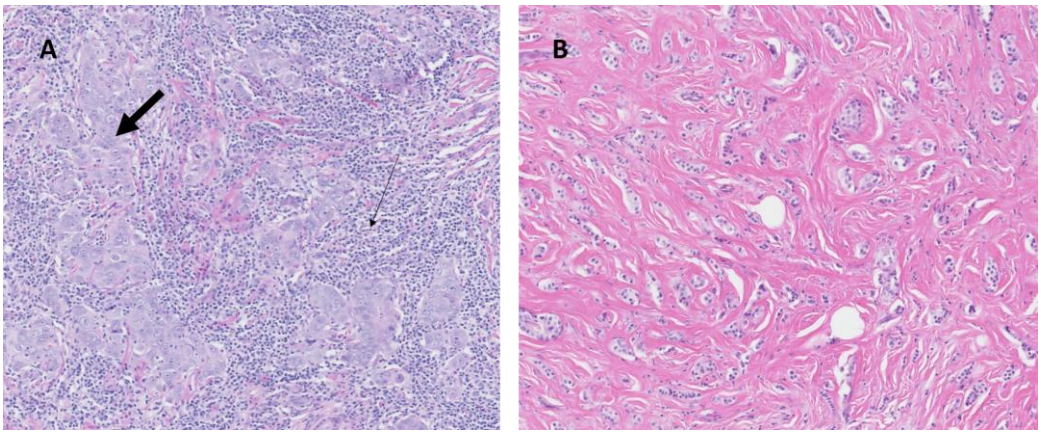

Figure 1 Hematoxylin-eosin staining of breast cancer with (**A**) high level of tumorinfiltrating lymphocytes (TILs) (thin arrow) in the stroma between tumorcells (fat arrow) and low levels of TILs (**B**). TILs encompass a varity of mononuclear celltypes (lymphocytes, plasmacells etc.)

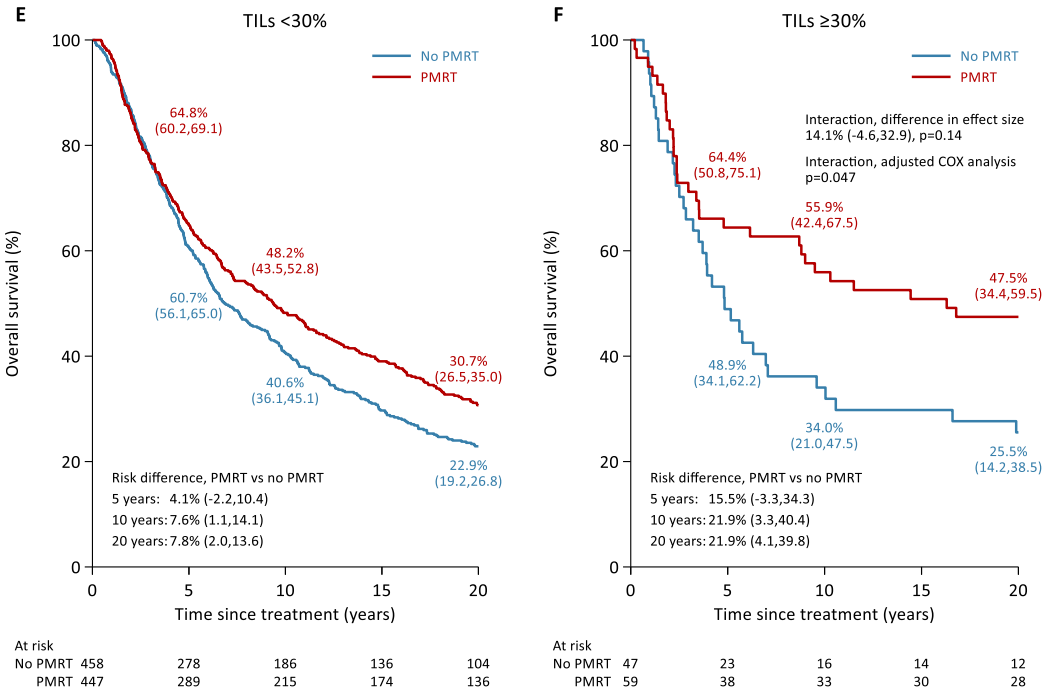

Figure 2 TILs estimated on HE-sections in 1011 patients from the DBCG82bc cohorte showing a significantly increased overall survival benefit in patients treated with postmastectomy radiotherapy (PMRT, red line) with high level of TILs (F) as compared to patients with low TILs (cut off 30%) (interaction test =0.047)(E)[26]

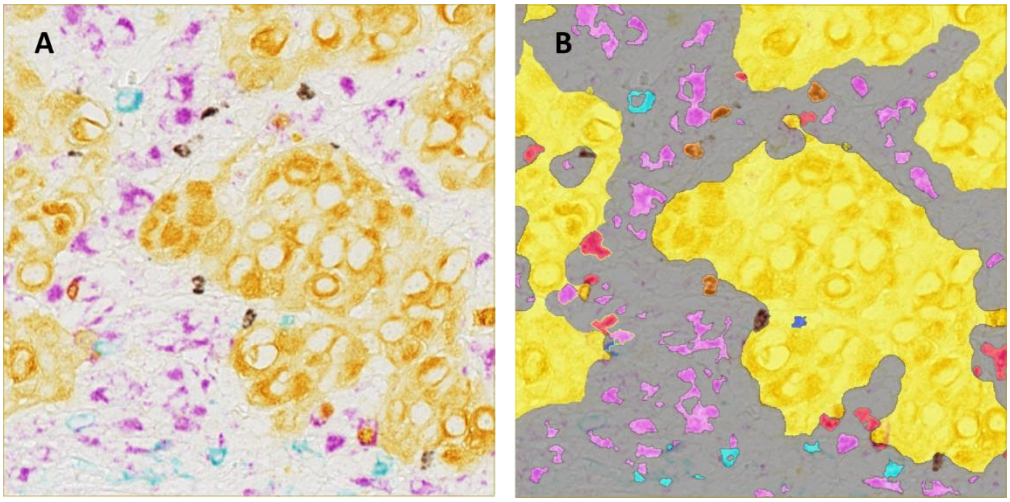

Figure 3A Tumor tissue from a breast cancer patient showing multiplex immunohistochemical staining allowing discrimination of specific TILs (tumor epithelium = yellow, CD8 (cytotoxic T-lymphocytes) = brown, FOXP3 (regulatory T-lymphocytes) = turquoise, CD4 (helper T-lymphocytes) = pink). The multiplex technique also enables evaluation of the specific localization of the inflammatory cells (e.g., in the stroma or tumor epithelium) and co-expression of various markers in the same cells **B** The different staining´s are annotated for subsequent digital image analysis offering the possibility of detailed quantitative analysis and also of a spatial evaluation (e.g., distance of inflammatory cells to tumor epithelium etc.)

Study Time Frame	**2021**			**2022**				**2023**				**2024**		
	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3
Setup and approvals	x	x												
Funding applications	x	x	x	x										
Collection of FFPE material and clinical data			x	x	x									
Generation of multiplex IHC and gene expression data					x	x	x	x	x	x				
Research study visit, Oslo								x						
Writing of papers						x	x		x	x		x	x	x
Writing PhD thesis												x	x	
Return of FFPE material and reporting														x

Figure 4 Time frame for the planned PhD study planned to start 1/6-2021 (Q3) and anticipated to finish 31/5-2024 (Q2)

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