The long-term impact of tumour infiltrating lymphocytes (TILs) in a premenopausal cohort with focus on primary luminal breast cancer

Christine Lundgren

Department of Oncology, Jönköping, Region Jönköping County, and Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden; Department of Clinical Sciences, Lund, Division of Oncology and Pathology, Lund University, Lund, Sweden

Project plan for study analysing the prognostic value of tumour infiltrating lymphocytes as a biomarker in a cohort of premenopausal women with long term follow-up

Background

Despite the increased effects of screening and more efficient adjuvant treatment, endocrine sensitive (luminal) breast cancers might cause recurrence beyond 5-20 years after diagnosis (1). The risk of recurrence is strongly correlated to tumour size, nodal status and tumor aggressiveness (1). For premenopausal women tamoxifen is still the most commonly recommended oral drug for adjuvant endocrine therapy. Oestrogen receptor status (ER) is the only routinely used predictive biomarker for selection of adjuvant hormonal therapy and is in clinical practice evaluated by immunohistochemistry (IHC) (2).

The role of inflammation in tumours, and especially tumour infiltrating lymphocytes (TILs) has become an important research area for detecting new protentional biomarkers for prognosis and prediction of treatment effects. TILs have previously been evaluated in large randomized controlled studies and have shown to be of different predictive and prognostic importance in the various intrinsic subtypes of breast cancer (3). TILs have shown to predict chemo-sensitivity (4) and is regarded as a promising biomarker for pathological complete responses (pCR) in the neoadjuvant setting (5). Likewise, for those patients with residual disease after neoadjuvant therapy, high TILs seem to indicate better prognosis in luminal breast cancer subtypes (6). In a recent study, a high fraction of TILs was associated with reduced risk of ipsilateral breast cancer recurrence and patients with tumours with low fraction of TILs, seem to better benefit from radiotherapy (7).

There are in general two different types of TILs; stromal TILs (sTILs) defined as the proportion of the stromal area containing infiltration of lymphocytes, with no direct contact with invasive tumour cells, and intratumoual TILs that are intraepithelial mononuclear cells within the nests of the tumours or in direct contact with tumours cells. Stromal TILs are most

frequently found in breast cancer and the amount is assessed by IHC by the pathologist. For standardization of the scoring, international guidelines have been implemented, as postulated by the Immuno-Oncology International TILs Working Group (3). The different TILs in breast cancer are mainly T cells; especially cytotoxic CD8 lymphocytes and a minority are CD4 T helper cells, T regulatory cells (Treg), macrophages, mast cells and plasma-cells (8). Even though TILs are of different amounts in different subtypes, the general composition is relatively conserved as stated by: Myian et al. (9). In the metastatic setting, previous studies have found that different metastatic sites, incorporates different amount of TILs, with high levels especially in lung and brain metastatic tissues (10). The tendency of breast cancer to preferentially metastasize to a specific organ, known as organotropism, is generally known for the different subtypes, and the immune cells are also dependent factors for organ-specific metastasis formation (11).

Compared to other tumor types, breast cancer shows a relatively low mutational burden; highest in HER2-enriched and basal-like molecular subtypes, followed by the luminal subtype. The phenotypes of the so-called lymphocyte predominant breast cancer (LPBC), is defined by 50% or 60% of stromal or intraepithelial TILs. In luminal subtypes, the LPBC phenotype (50% cut-off) ranges between 2.9-15 % (4). Although the relation to prognosis and responses of TILs has been reported for luminal subtypes, no association has been found between baseline TILs and overall survival. However, they might be an indicator of endocrine resistance and suggest a more aggressive luminal subtype (12) .

TILs are certainly an emerging biomarker in the clinical setting, but further studies are needed to analyse the clinical relevance of them in different breast cancer subtypes and populations, both in post- and premenopausal women with long-term (>10 years) follow up. The SBII:2

trial was a randomized controlled trial, initiated in the late 1980´s, were premenopausal women were allocated to receiving tamoxifen adjuvant or not. A follow-up study of this cohort, confirmed that two years of tamoxifen significant lead to a reduction in cumulative breast cancer-related mortality in patients with ER+ tumours (13, 14). Long-term follow up data along with tumour tissues are available from most of these patients. Therefore, this trial is a suitable basis for studies of prognostic biomarkers from tumours of premenopausal women.

Material and methods

Aims

The specific aims of this study are to define the prognostic impact of TILs in breast cancer in a premenopausal cohort with 30 years of follow-up and stratify this in relation to given adjuvant treatment of tamoxifen. Moreover, the aim is to explore the impact of TILs on the first metastatic site.

Primary end-point: BCFi (Breast cancer free interval; first event of local, regional or distant recurrence, contralateral breast cancer, breast cancer-related death).

Secondary endpoints: D-RFi (Distant Recurrence Free interval), overall survival (OS), first metastatic site.

Study population

The study population consist of patients included in the SBII:2pre study (13). During 1986-1991, 564 premenopausal women with stage II invasive breast cancer, irrespective of hormone status, were randomized between to 2 years of adjuvant Tamoxifen or not. This was a multicentre study in Sweden, were two centres were included; the South East Region (n =

137) where patients received 40 mg of Tamoxifen and the South Region (n= 427) were patients received 20 mg tamoxifen daily. The patients received radical surgery; modified radical mastectomy or breast conserving surgery with axillary lymph node dissection. Seven patients received additional adjuvant chemotherapy (cyclophosphamide, methotrexate, and fluorouracil). Patients were excluded for following reasons: metastatic disease, bilateral breast cancer, or history of other malignancies.

Follow-up

Follow-up data was first reported in 2005 after median 13.9 years (13). Long-term follow up data was presented 2016 and 2019 (30 years of follow-up) (14, 15). Recurrence, contralateral breast cancer, distant recurrence, first metastatic site and death have been documented based on data from the regional oncologic centres, medical reviews and by the Swedish Cause of Death Register.

Tumour samples characteristic

The following variables will be available from previous studies for each patient: histopathological classification, ER, PR, HER2, Ki67, histological grade, tumour size, age, nodal status, adjuvant therapy (tamoxifen or no adjuvant therapy), adjuvant radiotherapy, type of surgery. Surrogate subtypes (to differentiate ER+/HER2- tumours as Luminal A-like or Luminal B-like) can be constructed based on the aforementioned biomarkers and histological grade. Blocks of formalin fixed tissue from the SPII:2pre trial are stored at the pathological departments in Linköping, Norrköping, Jönköping, Kalmar, Växjö, Karlskrona, Halmstad, Helsingborg, Malmö, Lund and Kristianstad.

IHC analyses has previously been reported (13, 14). ER and PR were initially determined based on the cytosol-based method. Additional IHC analyses (on tissue microarrays) were done in 2003 on paraffin-embedded tumour samples (n = 500). Histological grade has been re-evaluated in 491 patients. The majority of patients (n = 362) were ER+ (regardless of PR-status), 68 patients were HER2-postivie (HER2+), and in total 99 patients had triple-negative breast cancer (TNBC).

After Biobank approval, formalin-fixed paraffin-embedded (FFPE) tumour tissues will be collected. Hematoxylin and eosin staining of new sections will be done. Microscopic assessment of TILs will be performed according to the Immuno-Oncology International TILs Working Group (3) by a pathologist (Ute Kruger). Stromal TILs (sTILs) will be assessed as a semicontinuous variable (deciles; <1%, 1-9%, 10-49%, ≥50%) and LPBC (lymphocyte predominant breast cancer) will be defined as ≥ 50% sTILs. A cut-off <10% will be defined as low-TILs, 10-49% as intermediate and ≥ 50% as high TILs.

Statistical analysis

A descriptive analyse will present the cohort tumour and patient characteristics in addition to surrogate subtype (Luminal A-like, Luminal B-like, HER2+, TNBC), first metastatic site and TILs (presented in the predefined categories of low, intermediate and high TILs as well as LPBC).

Univariable (and for significant factors, multivariable) analyses by Cox regression with 30 years follow-up regarding BCFi and the secondary aims; D-RFi, first metastatic site and OS, will be performed adjusting for clinicopathologic factors including treatment assignment, subtypes and levels of TILs (cut-off 10%) and LPBC. Hazard ratios (HRs) will be calculated

(proportional hazard regression, 95% confidence intervals (CIs)). Cumulative incidence curves will illustrate BCFi and D-RFi and survival curves will be generated by the Kaplan Meier-method, stratified by amount of TILs (cut-off 10%) and treatment allocation and log rank test to test for significant difference. Overall events will be presented separately for the different subtypes. All calculations will be done by IBM® SPSS® Statistics Version 25 and STATA®. The statistic test will all be two-sided, significance level set to α=0.05.

Ethics

The SBII:2pre study has previously been approved by both the ethical committees in Lund and Linköping, Sweden. Randomization was performed by the Regional Oncological Centres and oral informed consent was registered for all the patients. Additional approvals (Dnr LU 240-01 and Dnr Linköping 01-134) has been approved in 2003 and ethics approval for long-term follow-up, (Dnr number LU 2015/350) has been approved 2015-06-09. For the purpose of this study, an additional complementary approval (Dnr LU 2017/97) has been approved 2017-02-13. Approval from the Biobank Sweden has been achieved.

Expected results and implications

As breast cancer is the most common female cancer and a leading cause of mortality of younger women, there is a need to find trustable biomarkers that will help to better predict the outcomes and effect of adjuvant therapy. So far clinicopathological factors are used in combination to classify patients as high or low risk of relapse and ER is the only biomarker to predict effect of endocrine treatment. In the era of immune-oncology and the benefits of biomarkers such as TILs seen in other malignant tumours, it is of importance to also study this marker in breast cancer and especially luminal subtypes are of interest. This subtype does

however need long-term follow-up studies and this project will bring knowledge in this field. In addition this study will explore the impact of TILs of the metastatic pattern of breast cancer.

In conclusion, we believe that this study will increase the knowledge about TILs in luminal breast cancer and to decipher its long-term prognostic value and correlation to endocrine treatment in premenopausal women, a patient category less studied.

Finances and budget

The costs for this project will consist of labour costs to perform new tissue sections and H&E staining, TILs assessment of the primary tumour tissues, arranging the database with the cohort variables and statistical analyses. Later on, labour costs for writing the manuscript and travel costs will be added. External statistic help for data analyses will be needed. After manuscript has been finished, language review will be done. Costs for Biobank approval has been financed in another study by our research team.

The project will be funded by subsidies from the Faulty of Medicine, Lund University, Futurum-the Academy for Health and Care, Region Jönköping County and funds from FORSS, Linköping, The estimated costs are presented in Table 1.

Factors	**Estimated costs SEK**
Laboratory work (including evaluation by a pathologist)	110 000
Statistical analyses	50 000
Salary for the PhD student	180 000
Language review	10 000
Open Access	20 000
Travel costs for the PhD student	30 000
Sum costs	400 000
Overhead costs (40%)	160 000

Total costs	**560 000**

Table 1. Estimated costs for the project.

Schedules

This project is a part of a doctor´s thesis of Christine Lundgren.

Preliminary schedule:

- Biobank approval: 2018
- Collect tumour tissue and H&E staining: 2019
- Assessments of TILs: 2019
- Data base construction and statistical analyses: 2019-2020.
- Completed: 2020

Once finished the results will be presented in a scientific journal.

References

1. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, et al. 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. New England Journal of Medicine. 2017;377(19):1836-46.
2. Curigliano G, Burstein HJ, P. Winer E, Gnant M, Dubsky P, Loibl S, et al. De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. Annals of Oncology. 2017;28(8):1700-12.
3. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. (1569-8041 (Electronic)).
4. Loi S, Sirtaine N Fau - Piette F, Piette F Fau - Salgado R, Salgado R Fau - Viale G, Viale G Fau - Van Eenoo F, Van Eenoo F Fau - Rouas G, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. (1527-7755 (Electronic)).
5. Carbognin L, Pilotto S, Nortilli R, Brunelli M, Nottegar A, Sperduti I, et al. Predictive and Prognostic Role of Tumor-Infiltrating Lymphocytes for Early Breast Cancer According to Disease Subtypes: Sensitivity Analysis of Randomized Trials in Adjuvant and Neoadjuvant Setting. (1549-490X (Electronic)).
6. Denkert C, Loibl S Fau - Noske A, Noske A Fau - Roller M, Roller M Fau - Muller BM, Muller Bm Fau - Komor M, Komor M Fau - Budczies J, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. (1527-7755 (Electronic)).
7. Kovács A, Stenmark Tullberg A, Werner Rönnerman E, Holmberg E, Hartman L, Sjöström M, et al. 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. Journal of Clinical Oncology. 2019:JCO.18.02157.
8. Bense RD, Sotiriou C, Piccart-Gebhart MJ, Haanen J, van Vugt M, de Vries EGE, et al. Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer. LID - 10.1093/jnci/djw192 [doi]. (1460-2105 (Electronic)).
9. Miyan M, Schmidt-Mende J, Kiessling R, Poschke I, de Boniface J. Differential tumor infiltration by T-cells characterizes intrinsic molecular subtypes in breast cancer. Journal of translational medicine. 2016;14(1):227-.
10. Dieci MV, Tsvetkova V, Orvieto E, Piacentini F, Ficarra G, Griguolo G, et al. Immune characterization of breast cancer metastases: prognostic implications. Breast cancer research : BCR. 2018;20(1):62-.
11. Chen W, Hoffmann AD, Liu H, Liu X. Organotropism: new insights into molecular mechanisms of breast cancer metastasis. NPJ precision oncology. 2018;2(1):4-.
12. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. (1474-5488 (Electronic)).
13. Rydén L, Jönsson P-E, Chebil G, Dufmats M, Fernö M, Jirström K, et al. Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. European Journal of Cancer. 2005;41(2):256-64.
14. Ekholm M, Bendahl PO, Ferno M, Nordenskjold B, Stal O, Ryden L. Two Years of Adjuvant Tamoxifen Provides a Survival Benefit Compared With No Systemic Treatment in Premenopausal Patients With Primary Breast Cancer: Long-Term Follow-Up (> 25 years) of the Phase III SBII:2pre Trial. (1527-7755 (Electronic)).

15. Ekholm M, Bendahl PO, Fernö M, Nordenskjöld B, Stål O, Rydén L. Effects of adjuvant tamoxifen over three decades on breast cancer–free and distant recurrence–free interval among premenopausal women with oestrogen receptor–positive breast cancer randomised in the Swedish SBII:2pre trial. European Journal of Cancer. 2019;110:53-61.