TAMIL study

Treatment induced alterations in microenvironment in follicular lymphoma. A multi-center descriptive study

Primary investigator: Dr. D. de Jong[1]

Co-investigators: Dr. J.M. Kerst[2]
Dr. J.P. de Boer[2]
Dr. R.L.M. Haas[3]
Dr. B.P.M. Aleman[3]
Dr. J.W. Baars[2]
C. van den Bogaard[4]

EudractCT number: 2009-012303-26

[1]Department of Pathology, [2]Department of Medical Oncology, [3]Department of Radiotherapy, [4]Department of Clinical Chemistry; Netherlands Cancer Institute/ Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Dr. D. de Jong	Tel: 020-512 2752	Email: d.d.jong@nki.nl
Dr. J.P. de Boer	Tel: 020-512 2568	Email: j.d.boer@nki.nl
Dr. J.M. Kerst	Tel: 020-512 2568	Email: j.kerst@nki.nl

1. Introduction :

Follicular lymphoma (FL) is the second most frequent type of non-Hodgkin's lymphoma in adults. The disease is characterized by an indolent course with frequent relapses. Ultimately, resistance to chemotherapy or transformation to a more aggressive phase of the disease in the form of diffuse large B-cell lymphoma develops, and patients die as a result of their disease. Median survival is 8 to 10 years. The range is very wide, however, with patients surviving for more than 15 years and 10% to 15% of the patients who run a rapidly fatal course and die within 3 years after diagnosis. The translocation t(14;18) is the basic molecular defect in FL and results in protection from apoptosis by aberrant overexpression of bcl-2 protein. Accumulation of genomic alterations and clonal selection account for subsequent progression and transformation. Recently, the role of the immunologic microenvironment of FL in determining clinical behavior and prognosis has been substantiated. Combined genetic and immunologic data may now support a model for the development of FL as a disease of functional B cells in which specific molecular alterations infer intrinsic growth properties of the tumor cells as well as dictate a specific functional cross talk with the immunologic regulatory network resulting in extrinsic growth support.(1) The interaction of tumor cells with their microenvironment is essential for their growth and survival in vivo, as well as their localization and ability to disseminate. Several studies have recently emphasized the role played by numerous environmental factors on outcome FL, including immune response elements and stromal cells. Recent gene-expression data lend support to the crucial role of the immunologic context in the development and clinical behavior of FL, with a focus on the role of T cells and accessory cells.(2-6) From previous immunologic studies it is known that the microenvironment of malignant germinal centers encompasses a dynamic, functional molecular network of mutual developmental and maturating factors involving B cells, T cells, and follicular dendritic cells (FDCs).(7) The functional status of the FDC network, both in the reactive and malignant state, is marked by expression of complement receptors CD21 and CD35, immunoglobulin receptor CD23, and production of high levels of CXCL13.(8) Mature FDCs express all these markers and support a dense T-cell infiltrate. In the immature or a presumed functionally defective state, FDCs show loss of CD21 and CD23 with concomitant loss of T cells with spatial alterations of the T-cell infiltrate toward density at the follicular boundaries.(9;10)These features appear to be related to progression and prognosis.(10;11)
Gene-expression data show that FL with a poor response to anti-CD20 therapy (rituximab) and poor prognosis FL transforming to DLBCL within 3 years both have gene-expression signatures that are similar to normal activated lymphoid tissue (12;13) The overall gene signatures point to a relative activation of mediators of the cellular immune response, such as macrophages, follicular dendritic cells(14) and different classes of activated (CD69 positive) T cells.(15;16) Specifically, genes related to the functional status of FDCs are relatively overexpressed as compared to good-prognosis FL (eg, CCL19, CCL20, CCR1) and the T-cell repertoire seems to be skewed toward T-helper1 functions, producing IL-12, IFN-γ, and IL-2 as reflected by both upstream markers (signal transducer and activators of transcription

[STAT] -4) and down-stream effectors (IFN-induced proteins) and as supported by previous studies(17-20) In contrast, in the ‘‘good prognosis’’ group, terminal B-cell differentiation prevails in the context of a similarly dense, but a nonactivated T-cell population. Upon transformation, there is dramatic drop in absolute T-cell numbers that, however, retain their activated T-helper1 phenotype in the context of immature DC features (CCR1,CCL3, CCL5).(21-23). Although the results of gene-expression studies all point in a similar direction on the role of the immune microenvironment in FL, the results of immunohistochemical studies on specific T-cell subsets and accessory cells are very contradictory, however. Specific cell populations are claimed to significantly correlate with poor prognosis in some series, but with good prognosis or without any significant impact in others. In general, these studies are all well performed. Still some of the various results may be explained by technical scoring variations, but the explanations may be mostly directed by diversity of the clinical properties of the patients and variable treatments. Specifically, results thus far suggest a differential impact of very aggressive treatment as in the series reported by Farina and co-workers, versus the standard, more ”indolent” treatment in the UK and the Netherlands standard CHOP-like treatment as is common in many other countries. Moreover, due to its specific targeting of T-cells, fludarabine may have a different impact on the FL microenvironment. Therefore the prognostic value of different T-cell populations may be modulated as compared to other regimens.
This study aims to investigate the pre-treatment composition of the immune microenvironment in FL and to monitor changes as induced by different treatment modalities by serial fine needle aspirations of easily accessible FL localizations during treatment.

2. Objectives of the study:

The primary objective of this study is to assess the influence of specific treatment modalities on the composition of the immune microenvironment in FL. Using flowcytometry cell analysis (FACS) specific subsets of T-cells, follicular dendritic cells (FDC’s), macrophages and NK-cells will be investigated on cell suspensions obtained by fine needle aspiration of peripheral nodal lymphoma localizations. Second objectives are to correlate the observed differences in microenvironment to response.

3. Study design:

In patients with indolent FL pre-treatment and post-treatment (i.e. day 4) fine needle aspirations of one easily accessible FL localizations will be performed. Pre- and post-treatment changes in microenvironment will be assessed by FACS analysis and correlated with pretreatment histological biopsies, flowcytometry cell analysis in peripheral blood, advocated therapy, line of therapy and FLIPI score (Appendix A).

4. Flowcytometry cell analysis (FACS):

After standard fine-needle cytological aspiration, one smear is made. The remaining material is suspended into a FACS-tube containing RPMI 1640 fluid (see NKI intranet document CYT-AD-017 and related). This suspension should be stored on ice, i.e. 0-4°C, until futher analysis. Five-color flow cytometry will be performed on cell suspensions of cytological aspirates from lymphoma-positive lymph nodes and peripheral blood for markers to monitor tumor cells, total numbers of T-cells, T-cell subsets, including Thelper, Tcytotoxic/suppressor, Treg, NK-cells,

macrophages/monocyte classes, including M1 and M2 type DCs and follicular dendritic cells (table 1)

Table 1.

FITC	PE	ECD	PC5	PC7
CD4	CD8	CD45	CD25	CD3
CD20	CD16.56	CD45	CD19	CD3
CD35	CD21	CD45	CD14	CD23
CD68	CD36	CD45	CD25	CD14

5. Selection criteria:

Inclusion criteria:

- Histologically confirmed follicular lymphoma grade 1,2 and 3A according to the WHO criteria
- All lines of therapy (i.e. first line, second line etc.) are allowed. In case of radiotherapy fine needle aspiration of easily accessible FL localizations should be performed within the radiation field.
- Age $\geq$18 years
- Performance status $\leq$ 2 on the ECOG scale (see appendix B)
- Having previously signed written informed consent

Exclusion criteria:

-
- Any contraindication for fine needle aspiration (e.g. a history of severe bleeding)
- Any other co-existing medical or psychological condition that will preclude participation in the study or compromise ability to give informed consent

6. Study visits:

Pre-treatment Visit: (Visit 1)

At the first, pre-treatment visit, the following information is collected and recorded for entry into the study database:

- Date of histologically confirmed diagnosis of follicular lymphoma grades 1, 2 or 3A.
- Age
- Gender
- Ann Arbor staging
- Number of involved nodal sites
- Previous lines of treatment
- Hb, LDH

Peripheral blood immunophenotying is done according to protocol (see 4).A fine needle aspirate of a peripheral lymph node is performed under palpation by the pathologist or ultrasound-guided by the radiologist and material is collected in RPMI 1640 and should be stored on ice, i.e. 0-4°C, until FACS analysis (see 4). One smear is made for morphological assessment.

Day 4 of treatment:(Visit 2)
At day 4 of the chosen treatment modality the following information is collected and recorded:

- Treatment received
- Clinical assessment of local response according to standard criteria (Chesson)

Peripheral blood immunophenotyping is done according to protocol (see 4).
A fine needle aspirate of a peripheral lymph node is performed as previously under palpation by the pathologist or ultrasound-guided by the radiologist and material is collected in RPMI 1640 for FACS analysis (see 4). One smear is made for morphological assessment.

Post-treatment evaluation
At the first routine visit after completion of treatment, the following information is collected and recorded:

- Treatment received
- Date of completion of treatment
- Complete assessment of response according to standard criteria (Cheson criteria, Appendix D)

7. Statistical Considerations:

In this descriptive study it is hypothesized that significant differences in pre- and post-treatment fine needle aspiration and between all different treatment modalities could be assessed if at least 10 patients are included in every treatment modality and regimen. It is estimated that it will take approximately two years to achieve the desired amount of patients. A two-sided log rank test will be used for testing the differences in cell populations in the microenvironment in pre- and post treatment samples and between the different treatment modalities and regimens.

8. Ethical Considerations:

This study will be conducted in full conformance with the principles of the current revised version (2000) of the declaration of Helsinki. This study will be performed in accordance with the international Good Clinical Practice Standards and according to all local laws and regulations concerning clinical studies.
It is the responsibility of the investigator(s) to obtain informed consent from each subject participating in the study, after explanation of the aims, methods, benefits and potential hazards of the study. The consent must be obtained before any study-specific procedures are performed. It must be made completely and unambiguously clear to each subject that they are free to refuse to participate in the study, or that they can withdraw their consent at any time and for any reason, without incurring any penalty or withholding of treatment an part of the investigator. Signed informed consent must be kept on file by the investigator(s) and documented in the case report form and the subject's medical records.
The investigator(s) must ensure that the subject's anonymity will be maintained.

It is the responsibility of the investigator(s) to publish the clinical study results as soon as possible after completion of the study.

9. Insurance:

In accordance with Dutch law and WMO, insurance coverage for all participating patients from all centers, has to be arranged. Every participating hospital will arange the insurance coverage for all patients participating at their own center.

10. Reference List

(1) de Jong D. Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. J Clin Oncol 2005; 23(26):6358-6363.

(2) Herreros B, Sanchez-Aguilera A, Piris MA. Lymphoma microenvironment: culprit or innocent? Leukemia 2008; 22(1):49-58.

(3) Bohen SP, Troyanskaya OG, Alter O et al. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A 2003; 100(4):1926-1930.

(4) Dave SS, Wright G, Tan B et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med 2004; 351(21):2159-2169.

(5) Glas AM, Kersten MJ, Delahaye LJ et al. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 2005; 105(1):301-307.

(6) Kuppers R. Prognosis in follicular lymphoma--it's in the microenvironment. N Engl J Med 2004; 351(21):2152-2153.

(7) Park CS, Choi YS. How do follicular dendritic cells interact intimately with B cells in the germinal centre? Immunology 2005; 114(1):2-10.

(8) Chang KC, Huang X, Medeiros LJ, Jones D. Germinal centre-like versus undifferentiated stromal immunophenotypes in follicular lymphoma. J Pathol 2003; 201(3):404-412.

(9) Thomazy VA, Vega F, Medeiros LJ, Davies PJ, Jones D. Phenotypic modulation of the stromal reticular network in normal and neoplastic lymph nodes: tissue transglutaminase reveals coordinate regulation of multiple cell types. Am J Pathol 2003; 163(1):165-174.

(10) Shiozawa E, Yamochi-Onizuka T, Yamochi T et al. Disappearance of CD21-positive follicular dendritic cells preceding the transformation of follicular lymphoma: immunohistological study of the transformation using CD21, p53, Ki-67, and P-glycoprotein. Pathol Res Pract 2003; 199(5):293-302.

(11) Chang KC, Huang X, Medeiros LJ, Jones D. Germinal centre-like versus undifferentiated stromal immunophenotypes in follicular lymphoma. J Pathol 2003; 201(3):404-412.

(12) Glas AM, Knoops L, Delahaye L et al. Gene-expression and immunohistochemical study of specific T-cell subsets and accessory cell types

in the transformation and prognosis of follicular lymphoma. J Clin Oncol 2007; 25(4):390-398.

(13) Bohen SP, Troyanskaya OG, Alter O et al. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A 2003; 100(4):1926-1930.

(14) Dave SS, Wright G, Tan B et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med 2004; 351(21):2159-2169.

(15) Bohen SP, Troyanskaya OG, Alter O et al. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A 2003; 100(4):1926-1930.

(16) Glas AM, Kersten MJ, Delahaye LJ et al. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 2005; 105(1):301-307.

(17) Glas AM, Kersten MJ, Delahaye LJ et al. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 2005; 105(1):301-307.

(18) Bohen SP, Troyanskaya OG, Alter O et al. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A 2003; 100(4):1926-1930.

(19) Vyth-Dreese FA, Boot H, Dellemijn TA et al. Localization in situ of costimulatory molecules and cytokines in B-cell non-Hodgkin's lymphoma. Immunology 1998; 94(4):580-586.

(20) Airoldi I, Guglielmino R, Ghiotto F et al. Cytokine gene expression in neoplastic B cells from human mantle cell, follicular, and marginal zone lymphomas and in their postulated normal counterparts. Cancer Res 2001; 61(4):1285-1290.

(21) Glas AM, Kersten MJ, Delahaye LJ et al. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 2005; 105(1):301-307.

(22) de Vos S, Hofmann WK, Grogan TM et al. Gene expression profile of serial samples of transformed B-cell lymphomas. Lab Invest 2003; 83(2):271-285.

(23) Monti S, Savage KJ, Kutok JL et al. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. Blood 2005; 105(5):1851-1861.

Farinha P, Masoudi H, Skinnider BF, et al: Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). Blood 106:2169-2174, 2005

Lee AM, Clear AJ, Calaminici M, et al: Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. J Clin Oncol 24:5052-5059, 2006

Alvaro T, Lejeune M, Salvado M-T, et al: Immunohistochemical patterns of reactive microenvironment are associated with clinicobiologic behavior in follicular lymphoma patients. J Clin Oncol 24:5350-5357, 2006

Carreras J, Lopez-Guillermo A, Fox BC, et al: High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. Blood 108:2957-2964, 2006

Taskinen M, Karjalainen-Lindsberg M-L, Nyman H, et al: A high tumor-associated macrophage content predicts favorable outcome in follicular lymphoma patients treated with rituximab and cyclophosphamide-doxorubicin-vincristine-prednisone. Clin Cancer Res 13:5784-5789, 2007

Kelley T, Beck R, Absi A, et al: Biologic predictors in follicular lymphoma: importance of markers of immune response. Leuk Lymphoma 48:2403-2411, 2007

Canioni D, Salles G, Mounier N, et al: High numbers of tumor-associated macrophages have an adverse prognostic value that can be circumvented by rituximab in patients with follicular lymphoma enrolled onto the GELA-GOELAMS FL-2000 trial. J Clin Oncol 26:440-446, 2008

Appendix A: FLIPI score

Follicular lymphoma international prognostic index (FLIPI):

•Age >60			
•Serum lactate dehydrogenase concentration above normal			
•Hemoglobin level <12.0 g/dL			
•Ann Arbor stage III or IV			
•Number of involved nodal areas >4			
One point is given for each of the above characteristics present in the patient with follicular lymphoma (FL), for a total score ranging from zero to five. When applied to an international study of long-term survival in 4, 167 patients with FL diagnosed between 1985 and 1992, the following three risk groups and their corresponding 5- and 10-year overall survivals (OS) were, as follows:			
Score	Risk Group	5-yr OS, percent	10-yr OS, percent
0 to 1	Low risk	91	71
2	Intermediate risk	78	51
3 or more	High risk	52	36

Adapted from Solal-Celigny, P, Roy, P, Colombat, P, et al. Follicular lymphoma international prognostic index. Blood 2004; 104:1258.

Appendix B: ECOG-scale

Eastern cooperative oncology group (ECOG, Zubrod) performance scale

Performance status	Definition
0	Fully active; no performance restrictions
1	Strenuous physical activity restricted; fully ambulatory and able to carry out light work
2	Capable of all selfcare but unable to carry out any work activities. Up and about >50 percent of waking hours
3	Capable of only limited selfcare; confined to bed or chair >50 percent of waking hours
4	Completely disabled; cannot carry out any selfcare; totally confined to bed or chair

Excerpted from Oken, MM, et al. Am J Clin Oncol 1982; 5:649.

Treatment induced Alterations in Microenvironment in follicular Lymphoma (TAMIL-study)

Appendix C : Ann Arbor staging classification for non-Hodgkin lymphomas

Stage I	Involvement of a single lymph node region (I) or of a single extralymphatic organ or site (IE)*
Stage II	Involvement of two or more lymph node regions or lymphatic structures on the same side of the diaphragm alone (II) or with involvement of limited, contiguous extralymphatic organ or tissue (IIE)
Stage III	Involvement of lymph node regions on both sides of the diaphragm (III) which may include the spleen (IIIS) or limited, contiguous extralymphatic organ or site (IIIE) or both (IIIES)
Stage IV	Diffuse or disseminated foci of involvement of one or more extralymphatic organs or tissues, with or without associated lymphatic involvement

All cases are subclassified to indicate the absence (A) or presence (B) of the **systemic ("B") symptoms** of significant unexplained fever, night sweats, or unexplained weight loss exceeding 10 percent of body weight during the six months prior to diagnosis.
Clinical stage refers to the extent of disease determined by diagnostic tests following a single diagnostic biopsy. If a second biopsy of any kind is obtained, even if negative, the term pathologic stage is used.
* The designation "E" generally refers to **extranodal contiguous extension** (ie, proximal or contiguous extranodal disease) that can be encompassed within an irradiation field appropriate for nodal disease of the same anatomic extent. A single extralymphatic site as the **only site of disease** should be classified as IE, rather than stage IV.
Adapted from Carbone, PP, et al, Cancer Res 1971; 31:1860 and Lister, TA, et al, J Clin Oncol 1989; 7:1630.

Appendix D: Response Definitions for Clinical Trials for non-Hodgkin Lymphomas

Table 2. Response Definitions for Clinical Trials

Response	Definition	Nodal Masses	Spleen, Liver	Bone Marrow
CR	Disappearance of all evidence of disease	(a) FDG-avid or PET positive prior to therapy; mass of any size permitted if PET negative (b) Variably FDG-avid or PET negative; regression to normal size on CT	Not palpable, nodules disappeared	Infiltrate cleared on repeat biopsy; if indeterminate by morphology, immunohistochemistry should be negative
PR	Regression of measuable disease and no new sites	≥ 50% decrease in SPD of up to 6 largest dominant masses; no increase in size of other nodes (a) FDG-avid or PET positive prior to therapy; one or more PET positive at previously involved site (b) Variably FDG-avid or PET negative; regression on CT	≥ 50% decrease in SPD of nodules (for single nodule in greatest transverse diameter); no increase in size of liver or spleen	Irrelevant if positive prior to therapy; cell type should be specified
SD	Failure to attain CR/PR or PD	(a) FDG-avid or PET positive prior to therapy; PET positive at prior sites of disease and no new sites on CT or PET (b) Variably FDG-avid or PET negative; no change in size of previous lesions on CT		
Relapsed disease or PD	Any new lesion or increase by ≥ 50% of previously involved sites from nadir	Appearance of a new lesion(s) > 1.5 cm in any axis, ≥ 50% increase in SPD of more than one node, or ≥ 50% increase in longest diameter of a previously identifed node > 1 cm in short axis Lesions PET positive if FDG-avid lymphoma or PET positive prior to therapy	> 50% increase from nadir in the SPD of any previous lesions	New or recurrent involvement

Abbreviations: CR, complete remission; FDG, [^{18}F]fluorodeoxyglucose; PET, positron emission tomography; CT, computed tomography; PR, partial remission; SPD, sum of the product of the diameters; SD, stable disease; PD, progressive disease.

Adapted from Cheson, B.D. et al,J Clin Ocol,2007, 25(5): 579-586