LUNENBURG LYMPHOMA BIOMARKER CONSORTIUM
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Participants: Daphne de Jong, Ton Hagenbeek, Andreas Rosenwald, Eva Kimby, Birgitta Sander, Franck Morschhauser, Thierry Molina, Maria Calamanici, John Gribben, Marie José Kersten, Edie Weller, Randy Gascoyne, John Raemaekers, Laurie Sehn, , Andrew Jack, Wendy Stevens, Ranjana Advani, Yaso Natkunam, Michael Pfreundschuh

Not attending: Martin Dreyling, Elias Campo, Christoph Torns, Wolfram Klapper, Lorenz Trümper, CrhistianBuske, Philippe Gaulard, Gilles Salles, Wolfgang Hiddemann

Welcome (Andrew Jack)

FOLLICULAR LYMPHOMA PROJECTS

Summary of achievements so far (Wendy Stevens)

End of spectrum project: 3 biological study questions, with availability of samples (top priority question 1):

1. long remission (>5 years) versus early failure (R-chemo)
   early failure: 186 patients (86 biopsy material)
   long remission 196 patients (82 biopsy material)
2. Wait and see versus patients who need immediate treatment
   - W&S stage III/IV > 5 years: 171 patients Stage III/IV immediate treatment: 488 patients
3. stage I vs stage III/IV
   - stage I nodal disease: 225 patients
   - stage IV 1027 patients

For study question 2 and 3 we anticipate that for about 50-60% of the patients sufficient biopsy material will be available.

Q1: long remission vs early failure after R-chemo

The clinical database was completed by Edie (58 items).

Availability of clinical data/samples: (See attached presentation Wendy)

To reach a reasonable statistically sound cohort, more samples are needed; possible sources:

- LYSA: (PRIMA/FL2000): will be discussed by Thierry Molina/Franck with Gilles Salles/Luc Xerri during the Scientific Council meeting. TMAs for PRIMA have been built (n=400); data will be presented at the ICML meeting. For FL2000 320 (160 had rituximab) TMAs are available.

- Hochster study E4096: no R-chemo induction (CVP followed by rituximab maintenance); might influence biology even though the outcome is similar --> decided not to use these cases.

- StiL trial: no central pathology was performed, difficult to get the samples.

- Bart's: TMAs have been constructed from 180 cases; John will check how many patients fit the criteria

  We aim to increase the sample size to 120-150 cases per group.

Q2: Wait and see versus immediate treatment

- for the numbers see attached presentation Wendy

- approximately 20 extra cases can be obtained from Stanford

- Wait and see: Ardeshna trial: 170 blocks available from the patients in the wait and see arm; still unclear whether they can be used (Andrew will check)

Q3: Stage I vs stage IV

- for the numbers (see attached presentation Wendy)

For all research questions: clinical data are necessary for all the patients. Depending on the magnitude of the difference that is expected in expression of the markers, the numbers of controls vs cases can be established by Edie. Availability of blocks needs to be known before matching can be performed by either a case-control or case-cohort approach.

Technical IHC validation study (Birgitta)

Manuscript is almost ready to be submitted.

Design of the validation study:

- 25 cases IHC + flow data available (CD3, CD4, CD8)
- TMA 2 cores 1mm
- Stained in a single lab for CD3, CD4, CD8, FoxP3, CD68, MIB1, CD10, CD21, CD34.
- Different markers were studied, as representative of various staining patterns of non-malignant cell populations (membranous, cytoplasmic, nuclear) and to cover a variation of infiltration densities.

- Scored by 7 pathologists and 2 independent image analysis systems (Ariol, Cambridge and Ariol, Madrid (only for CD3,4,8)
- % of positive cells was scored as well as distribution patterns

Highlights/conclusions/recommendations:

- 2 cores are required and sufficient: for most markers, results were remarkably similar. Only for MVD (CD34) there was an essential difference between the two cores.
- Manual scoring:
    - Agreement in manual scoring between pathologists: excellent for CD10 but low to moderate for other markers, especially T cell markers. Rare distribution patterns were however well recognized (e.g. perifollicular FOXP3)
- Good correlation between the two machines and moderate to high concordance.
- Manual vs image: in general at the low end manual < image; at the high end manual > image, so the range (variability) is extended by manual scoring.
- Image vs flow: reasonable correlation but image in general higher than flow.
- For manual scoring obviously a reduction in variation can be reached with fewer categories
- With machine scoring (calibration, training), the process for evaluation with new markers is easier to implement
- Advantages of image analysis
    - vs flow: no fresh tissue required, new markers can be added
    - vs manual: more reproducible than manual, continuous scoring possible
- Vancouver results with 1.5 mm cores showed a better correlation to flow information than 0.6 or 1.0mm cores. Larger core diameters or whole sections were not tested. Since 1mm cores are used by almost all studies and used as a standard procedure in existing protocols, it was decided to stick to 2 cores of 1mm diameter.

FL: markers to be studied:

Immunohistochemistry: list of IHC markers to be used:

- Definite markers: CD3,CD4, CD8, FOXP3, PD1, CD68, CD163, and CD20 (as a quality control marker) P53 and EZH2

- New markers to be added:

    - EPHA7 (to be tested first by Randy and John/Jude; not necessary to do a formal validation study)

    - MYC will not be included

    - T cell markers (based on a study by John Gribben on purified T cells from FL samples --> lists of differentially expressed genes: candidate markers which can be analyzed by IHC: CD200, ETV1, PMCH, NAMPT

- TMAs will preferably be made in one lab --> agreed to do this in Wurzburg.

- IHC staining will be done in one lab:→Wurzburg, Amsterdam or London

- Scoring will be done by image analysis --> agreed to do this in Bart's by Wendy and Andrew Clear under the supervision of Maria and Daphne.

Genetic markers

For molecular analyses: use either scrolls or 1 mm cores, separately stored at -20°C (or -70°C).

Sequencing (MiSeq): a marker panel will be prepared by Randy/ Daphne to include at least relevant markers such as,TNFRSF14, EZH2, CREBBP, EP300, MLL2, MEF2B,TNFAIP3/A20.

FISH BCL2/BCL6: more relevant for the stage I vs stage IV study question; not so much for the long vs short remission.

Collection and storage of material for FL:

Total amount of material needed: 6 cores or 2 cores and 4 scrolls (build 1 TMA block with duplicate cores and store 4 cores or 4 scrolls for molecular analyses in 2 separate Eppendorf tubes). A protocol will be written with optimal and minimal amount of material and prioritization. In principle priority will be: TMA > DNA > RNA.

Action list FL

1. Thierry/Franck: discuss availability of FL2000/PRIMA TMAs at upcoming LySA meeting
2. John: check database Bart's for patients who fit the criteria for the 3 study questions
3. Ranjana/Yaso: check availability of Stanford cases
4. Wendy/Edie: will complete the database, help the groups with the final proposal for collecting the clinical data and send instructions for the collection of the clinical data
5. Randy; John/Jude: test EPHA7 IHC
6. John G/Daphne: list of IHC markers for second round selection
7. Wendy/Daphne: SOP for collection and storage of cores/scrolls
8. Wendy/Andreas: construction of TMAs (two-tiered approach: cases collected thus far, additional cases). US cases: depending on legal issues whether TMAs will be built in US or also in Wurzburg
9. Wendy/Andrew Clear/Maria: will score the IHC markers on the TMA in London
10. Immediate treatment vs W&S and stage I vs stage IV: cases will be identified by all groups
11. Wendy/Daphne: a budget (for molecular studies) will be submitted to the board

DIFFUSE LARGE B-CELL LYMPHOMA PROJECTS

DLBCL FISH validation study (Andreas)

A validation study was done on 11 test TMAs to possibly obviate the need for redoing and rescoring the FISH for BCL2/BCL6/MYC. 5 labs did the FISH and IHC with their own reagents and scored it.
- BCL2: 2 out of 4 labs each had one discordant result (different cases).
- BCL6: 1 lab had 1 discordant result
- MYC: 3 labs had 1 discordant result in the same case.
- MYC IHC: 4 discordant results with a cutoff of 40% for positive versus negative.

NB For MYC IHC it is very important to use freshly cut slides, since slides stored at room temperature for >6 weeks were shown to have lost all immunogenicity.
Conclusion: good concordance for FISH and likely for IHC. These data will be submitted to Edie to evaluate reproducibility to decide if indeed existing data can be used.

DLBCL2000FISH study

It was decided that there is sufficient rationale to perform a large validation study on the impact of MYC/BCL2/BCL6 FISH based on existing data within our group. It will be most valuable to add MYC and BCL2 protein expression, but BCL6 protein expression will not be included.
It remains debatable if we can work with existing stains for BCL2 and MYC or that all stains need to be redone according to LLBC set protocols. Although the latter option is certainly optimal, it will imply a very large amount of work and may not be feasible if unstained slides are not available for all studies. Re-scoring of existing slides by LLBC pathologists is the second best option. It needs to be decided if a single cut-point will suffice or rather scoring should be done according to our previous scoring classes. A training and validation set may be used to establish a set cut point. Also it may be considered to perform automated scoring (Ariol), although this is not validated in our hands.
To allow a founded decision, more information needs to be retrieved:

- Formal evaluation of MYC-IHC reproducibility on the validation series described above
- Re-visiting LLBC validation study information on scoring reproducibility of BCL2 stained in different labs and in one lab (round 1/round 2 data)
- Retrieve information on availability of stained and unstained slides from the selected trials/series
- Retrieve information on the antibodies used for BCL2 and MYC in the selected trials/series.

<u>Cohorts of patients</u>:

- RICOVER (200 RCHOP), MegaCHOEP (100) (done)
- LNH03: 375 RCHOP, 240 R-ABCVP
- HOVON63, HOVON84, HOVON46 (needs to be done)
- Leeds registry 900 (500 also have GEP based on paraffin)
- Lancet series UK (done) 700
- BCCA: DLBCL (220 ready; approx 300 will be added --> 500). GEP data available on 300-400 patients (fresh frozen material). In

addition, RNA sequencing and WGS data will be available for a subset of these patients (project focusing on refractory disease).
- Stanford: 200 cases

Additional markers that were discussed:
- EBV (EBER)? In German trials/Vancouver: prevalence only 1-2%
- p53
- Top 5 candidates recurrent mutations from sequencing data? Should we already store DNA/RNA? At this point wait for more data
- Of the cohorts discussed: how many blocks will be available to be able to collect scrolls/cores for molecular analyses (PCR, nanostring)?

Organizational part: this project will be organized by Andreas (+ fellow) in collaboration with Laurie and Edie, who have already done a lot of work for this project; Yaso and Ranjani will join this working party. Laurie and Edie have already done an inventory on the series of patients and trials and will circulate a proposal to study clinical factors in this large series. A teleconference will be organized end of July.

Action list DLBCL:
1. Laurie/Edie: circulate proposal for DLBCL project
2. All: confirm numbers of patients with:
- available FISH data
- TMAs available for IHC MYC/BCL2
- blocks available to collect cores/scrolls for molecular analyses

FUTURE DIRECTIONS FOR LLBC:

Possible diseases to expand to:
- Hodgkin (focus on relapsed/refractory disease; paired biopsies)
- (T cell lymphoma)
- (MCL (European MCL network))
- Paired samples FL (primary/relapsed/transformed)
- Primary mediastinal

For the next meeting, Andreas Engert (HD) will be invited.

Date for the next meeting (London): May 14th-16th, 2014