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Critically Appraised Topic

Laboratory diagnostics of leptomeningeal invasion by lymphoproliferative disorders

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Clinical Bottom Line

Leptomeningeal invasion by lymphoproliferative disorders is rather an uncommon, but a harmful complication which requires a thorough investigation and subsequently adjusted treatment.

The gold standard in laboratory diagnostics for leptomeningeal invasion by lymphoproliferative disorders remains conventional cytomorphological analysis of CSF. However, conventional microscopy is known for a good specificity, the sensitivity is rather low. A combination of cytological examination and flow cytometric immunophenotyping may increase the sensitivity and slightly augment specificity.

Although flow cytometry has proven useful, it is limited by the low cell count, limited sample volume, rapid cell decay, peripheral blood contamination, free immunoglobulins,… in CSF samples. (Pre-)analytical quality improvement might compensate these limitations.

To prevent rapid cell loss, the CSF sample should be analysed within 1 hour after collection or stabilised with a stabilisation medium f.e. Transfix or RPMI. Besides a proper choice of antibody panel, a two-step approach consisting of a screening and an extended immunophenotyping has been proposed for analytical improvement.

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Clinical/Diagnostic scenario

Leptomeningeal invasion in patients with leukemia or lymphoma is a relatively uncommon, but a devastating clinical complication which implies an adverse prognosis.[1,2] Non-Hodgkin's lymphoma (NHL) may affect both the peripheral nervous system and central nervous system (CNS). Metastasis in the subarachnoid space (leptomeningeal spread) is the most common pattern of metastatic involvement. Hematogenous spread of NHL to the CNS is less common, but nodular deposits within brain parenchyma can be formed by infiltration of the subarachnoid space through the Virchow-Robin spaces. Furthermore focal neurological complications can occur by development of tumour nodules along the central axis or the spread of tumour to lymph nodes may cause spinal cord compression.[3]

Incidence of CNS involvement may be increased by improvement in survival and remission rates of patients with lymphoproliferative disorders.[4] As many secondary CNS metastases are diagnosed early after initial diagnosis, during or shortly after treatment, the suggestion has been made that initial CNS involvement may be underdiagnosed.[5].

Although CNS prophylaxis seems to reduce CNS relapse, it is not recommended to give additional CNS treatment to all patients with aggressive NHL. Because the incidence of CNS involvement is not high enough and it augments systemic toxicity.[5,6]

At present, diagnosis of leptomeningeal invasion is based on standard diagnostic procedures, including clinical presentation and follow-up of clinical signs and symptoms, neuroimaging with magnetic resonance imaging (MRI), conventional cytology and flow cytometric immunophenotyping of cerebrospinal fluid (CSF). However each of these diagnostics have their own limitations leading to limited sensitivity or specificity.[4,7]

Clinical signs and symptoms in leukemic or lymphomatous meningitis may be difficult to distinguish from symptoms of the primary disease or neurological complications of treatment.[4,7] However patients with hematological leptomeningeal disease have a different clinical presentation than those with solid tumour invasion. Leptomeningeal metastases (LM) can present with a variety of symptoms, but patients with hematologic LM present more often with cranial nerve dysfunction while those with solid tumours mostly with spinal or radicular symptoms.[8,9]

According to the systematic review of Giglio *et al.*, the overall risk of CNS involvement (parenchymal and leptomeningeal metastases) after treatment of NHL was 4.2%. First, the risk of CNS involvement depends on the subtype of lymphoma with a frequency of 5% (i.e. diffuse large B-cell lymphoma (DLBCL)) to 30% for aggressive NHL (i.e., Burkitt lymphoma (BL) and B-cell lymphoblastic lymphoma (B-LL)).[3,5,10] Second, additional risk factors can be identified: features of advanced disease[2], elevated serum lactate dehydrogenase (LDH), hypoalbuminemia (< 35g/L), age less than 60 years, retroperitoneal lymph node involvement, involvement of more than one extra nodal site[3,11], CSF pleocytosis[11,12], elevated CSF protein content[1,12], performance status (Eastern Cooperative Oncology Group (ECOG) ≥ 2, neurological symptoms and elevated serum Beta 2- microglobulin.[11]

In CNS lymphoma, especially primary CNS lymphoma (PCNSL) involving the neuraxis rather than the leptomeninges or dura, histopathological diagnosis is mandatory in planning treatment, consisting of systemic chemotherapy with or without whole brain radiotherapy. Histopathological diagnosis is performed using stereotactic needle biopsy, which is limited by the accessibility for biopsy (e.g. location of the lesion or unexpected bleeding,…).[10]

In biopsy inaccessible lesions other diagnostic features have proved more important. Neuro-imaging features are often suggestive but not diagnostic, because atypical locations and features are common. In PCNSL, conventional tomography (CT), MRI, and Fluorodeoxyglucose Positron-emission tomography/CT (PET/CT) contribute to radiographic evaluation for initial diagnosis, prognosis, response, and surveillance. PET/CT contributes mainly in the staging, pretreatment prognosis, and therapeutic monitoring of PCNSL. In secondary CNS involvement, MRI is particularly useful in parenchymal involvement (in 1/3 of the patients with secondary CNS lymphoma), because of lower sensitivity of CSF analysis in the absence of leptomeningeal disease.[13]

Conventional cytomorphological analysis (CC) of CSF is still considered the gold standard for diagnosis of leptomeningeal disease.[7] Cytological examination of CSF for malignant cells is highly specific (>95%), but lacks sensitivity.[11,14] Sensitivity between 40 and 80%[15], and false negative results between 20 and 60%[5,11] have been reported. Furthermore, some diagnostic challenges in cytology have been discussed: interpretation may be difficult due to paucity of cells, resemblance between benign, reactive and malignant cells[11], and profound apoptotic effect due to corticosteroids (vanishing lymphoma) may cause diagnostic difficulties.[10]

Therefore flow cytometric immunophenotyping (FCM) is a suitable, objective method used to identify and quantify small cell populations with an aberrant expression pattern[6,16] in addition to CC. In the prospective trial of Benevolo *et al.,* FCM showed a better sensitivity than CC for the detection of occult leptomeningeal disease in aggressive lymphoma and in patients with increased risk of CNS dissemination.[5] This suggests the advantage that FCM allows an earlier detection of LM before clinical onset and positive CC.[4] While CC requires at least 5% neoplastic cells for detection, a detection level from 0.2% of neoplastic cells for FCM has been described.[6]

Although flow cytometry has proved useful, it has been challenged by paucity of cells in cerebrospinal fluid and rapid cell loss after sample collection.[17] Hence, there has been a growing interest in pre-analytical improvements to increase cell yield, including stabilising agents such as TransFix (Cytomark, Buckingham, UK), RPMI-1640 cell culture media, serum-containing medium,…; and analytical innovations, f.e. detection of human soluble CD19 (Immunostep, Salamanca, Spain) and use of the ‘small sample tube’ (SST), an enhanced version of the Lymphoid Screening Tube (LST) combination designed by EuroFlow™ consortium.

Questions

1. How important are laboratory diagnostics in leptomeningeal invasion by lymphoproliferative disorders?

2. What is the added value of flow cytometric characterisation of cerebrospinal fluid cells?

3. Flow cytometry of cerebrospinal fluid: need for (pre-)analytical quality improvement?

Search Terms

1) *MeSH Database (PubMed): MeSH term: "meningeal carcinomatosis", "lymphoma",*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters: "leptomeningeal metastases", "lymphoma", "transfix", "soluble CD19", "diagnostics", "cns lymphoma", "flow cytometry", "immunophenotyping",*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/): "guideline leptomeningeal metastases", Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm): "leptomeningeal", "meningeal carcinomatosis", "cns invasion", "lymphoproliferative disorder", "lymphoproliferative process", "cns metastasis", "non-Hodgkin lymphoma"*
4) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp), National Diabetes Information Clearinghouse (NDIC; http://diabetes.niddk.nih.gov/), Westgard QC (http://www.westgard.com), Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/)*
5) *UpToDate Online version 23.7 (2015)*

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5) *Reference Works, Handbooks and Databases*

6) *Posters, "grey literature", presentations*

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- Presentation General annual meeting BHS 2014: Flow cytometric analysis of cerebrospinal fluid – Cancer research center, Ibsal University, University hospital of Salamanca (Spain).

Appraisal

1. How important are laboratory diagnostics in leptomeningeal invasion by lymphoproliferative disorders?

Clinical biology

1. Biochemistry

First, elevated serum LDH levels have been described as a risk factor for CNS disease in NHL.[9] Van Besien *et al.* and Hollender *et al.* have stated, in respectively a prospective and retrospective study, increased serum LDH level as an independent predictor for CNS recurrence in high grade lymphoma.[2,18] In contrast, Quijano *et al.* found similar serum LDH levels in FCM+ versus FCM- patients.[11] On the one hand, these differences might be due to the different study populations: newly diagnosed large cell and immunoblastic lymphoma patients with symptomatic CNS disease in Van Besien *et al.* and patients with low-grade and high-grade lymphoma in Hollender *et al.*[2,18] On the other hand, CNS disease was respectively diagnosed by CC, brain biopsy or symptoms and radiological findings and by history, symptoms, CT, MRI, CC and histology[2,18], instead of confirmed by CC and/or FCM in Quijano *et al.*[11]

Second, serum Beta-2 microglobulin levels have found to be significantly higher among FCM+ patients.[11] Mavlight *et al.* described statistically significant higher CSF Beta-2 microglobulin levels in patients with lymphoma or leukemia than in those without. The CSF level was also significantly higher than the serum level in patients with CNS involvement.[19] Kantarjian *et al.* found a significant lower survival in ALL patients with serum Beta-2 microglobulin levels >4 mg/dL.[20]

Third, the probability of CSF localisation of hematologic malignancy is higher in patients with elevated CSF protein concentration.[8,12] Fourth, hypoalbuminemia (<35 g/dL) is associated with an increased probability of CNS recurrence within 5 years in high grade NHL.[18] Furthermore, lowered CSF glucose concentration (<40 mg/dL) has been proposed as additional risk factor[1], tumour markers such as Beta-glucuronidase, CSF levels of soluble CD27, LDH iso-enzyme 5[9] and chemokine ligand CXCL13 and interleukin (IL) 10[21] as possible indicators. In patients with PCNSL, CXCL13 plus IL-10 is highly specific for the diagnosis of CNS lymphoma.[21] Human soluble CD19 biomarker in CNS will be discussed in the third section.

Although several biochemical markers have been evaluated, their diagnostic use is restricted by limited sensitivity and specificity.[9]

2. Cytology

Commonly in CSF fluids with low cellularity (<5 WBC/µL), WBC differentiation by microscopy is not performed. However, when malignancy is suspected, WBC differentiation might be useful to perform in paucicellular CSF samples. Cytomorphological examination is performed on cytospin preparations stained with May-Grunwald-Giemsa.[14] Although cytology is highly specific, it has limited sensitivity because of paucicellularity of CSF and morphological resemblance of malignant and reactive cells.[4]

Detection of malignant cells is dependent on the spread of leptomeningeal disease. Positive cytology varied from 38% in focal disease to 66% in disseminated disease.[4,22] Because of the low detection rate, several lumbar punctures may be performed. But even after repeated lumbar punctures 10% to 20% false negative results have been found compared to neuroimaging studies (gadolinium-enhanced MRI of the brain and spine).[4,23]

Cytology is still method of choice, but several studies have suggested increased sensitivity in combination with FCM.[6] Detection of ≥5 WBC/µL in CSF is even proposed as a predictive factor of a positive FCM test.[5]

3. Flow cytometry

Polychromatic flow cytometry allows the detection of a large spectrum of cellular characteristics, where differentiation is based on granularity and volume of cells and immunophenotyping using surface membrane, cytoplasmic, and nuclear antigens.

In case of suspected leptomeningeal spread of hematologic malignancy, the presence of a monoclonal population and characteristic phenotype can be assessed by using the appropriate antibody panel according to the suspected diagnosis.

Malignant cells frequently appear in very small numbers in order of 0.01% in CSF. Therefore background fluorescence signals should be minimised and sufficient cells are required to analyse the lymphocyte subpopulations. This requires a sufficient CSF volume (at least 5mL) and adequate pre-analytical preservation of cells.[14]

Anatomic pathology

In contrast to secondary lymphoma, most PCNSL are diagnosed by stereotactic biopsy. Less frequently PCNSL are diagnosed by flow cytometric analysis. In general, surgical resection is avoided because of the risk of neurological sequelae and no added therapeutic value.[24] Therefore stereotactic biopsy remains the gold standard. Regarding the biopsy, morphology and immunophenotyping are required.[25]

2. What is the added value of flow cytometric characterisation of cerebrospinal fluid cells?

Flow cytometric analysis is particularly useful for the detection of small clonal cell counts of B-cell lymphocytes, easily separated by their cell characteristics (see above). It is a relatively rapid method and appropriate for abnormal lymphoid cells in CSF, especially when cytological differentiation between normal and abnormal lymphocytes is difficult.[16]

Increased sensitivity

In combination with a multicolour fluorescent antibody labelling, flow cytometry is a highly specific and sensitive technique to detect malignant cells at very low cell counts in CSF. Several studies found a superior sensitivity of flow cytometry compared with CC (*Table 1*). Besides better sensitivity, a raise in specificity has been described from 94% (CC) to 97% (FCM).[4]

Hedge *et al.* studied 51 patients with aggressive B-NHL with increased risk of leptomeningeal dissemination. High risk was defined as DLBCL with either ≥2 extra nodal sites and increased LDH or bone marrow involvement, BL or AIDS-related lymphoma. In 11 of 51 (22%) occult CSF involvement was detected, all were detected by flow cytometry and only 1 was diagnosed by CC ($p=.002$; median percent tumour cells [range]: 7 [0.2-99]). The single patient diagnosed by cytology had the highest concentration of tumour cells (99% of all CSF cells).[6]

In a prospective, multicentre study, 27 (22%) of 123 patients with aggressive B-NHL at high risk of CNS relapse had a positive result on FCM, while only 7 of 123 were positive by CC (6%) and three other cases being suspicious (2%).[11]

Alvarez *et al.* analysed CSF from 114 DLBCL patients at diagnosis (n=95) or at relapse (n=19), 14 (12%) samples were FCM+ and 1 (<1%) sample was CC+ and FCM+. Only 25% was FCM+ before relapse.[26]

Prospectively Benevolo *et al.* analysed 174 patients with aggressive B-NHL. Eighteen of 174 patients (10%) were FCM+ and only 7 (4%; $p<.001$) were CC+. In 11 patients discordance was found between FCM and CC (FCM+/CC-).[5]

Similar findings were observed in a retrospective study of 219 patients. Leptomeningeal spread was detected in 60 (27%) patients either by FCM, CC or both. In 44 of 60 (73%) patients the first sample was positive by FCM and in 19 of 60 (32%) by CC.[12]

A 2-fold to 3-fold more sensitive detection by FCM compared to CC in patients with newly diagnosed aggressive B-NHL was found in an Italian study. CNS disease was found by FCM in 11 (26%) patients, while only 4 (9.5%) were detected by CC.[27]

Schinstine *et al.* evaluated CSF of 32 patients with ambiguous morphological results ("atypical" or "suspicious" CSF), followed for 1 year. Subsequent analysis of CSF for CC and FCM revealed haematological malignancy in 19 of 32 patients (59%; FCM+) and CC was positive in 9 of 19 (47%; FCM+/CC+) patients.[28]

Cell count at diagnosis

In the prospective, multicentre study of Quijano *et al.*, a higher absolute count and the percentage of neoplastic B cells were identified by FCM ($p<.0001$) in patients with FCM+/CC+ compared to FCM+/CC- CSF samples. Furthermore a clear cut-off could be determined between FCM+/CC- and FCM+/CC+ (plus suspicious) patients. For FCM+/CC+ patients, the CSF evaluation shows an infiltration typically higher than 20% and ≥1 malignant B lymphocyte/μL (*Figure 1*).[11]

Progression free survival

In the prospective study of Benevolo *et al.* (n=174) the two-year progression free survival (PFS) was

60% (95% CI, 52-67) in the whole patient population, with a median time of progression of 10 months (IQR, 5-19). FCM- patients showed a significantly higher two-year PFS: 62% (95% CI, 54-70) versus 39% (95% CI, 17-60; P=.019) in FCM+ patients. The group of patients with FCM+/CC+ showed a significantly higher risk of progression compared with the FCM-/CC- group.[5]

3. Flow cytometry of cerebrospinal fluid: need for (pre-)analytical quality improvement?

Flow cytometry has been proven useful and more sensitive in detecting monoclonal cell populations in haematological malignancies However, it has some limitations regarding, f.e. low cell count, limited sample volume, poor cell stability,… in diagnosing CNS invasion of lymphoma. To address these limitations, (pre-)analytical quality improvement is required.

Limitations

1. Low cell count and limited sample volume

Flow cytometry is limited by the low cell count in CSF (normal <5 leucocytes/μL). In the study of Subira *et al*., clear identification of B, T CD4+, T CD8+ lymphocytes could be performed in all CSF samples with more than 5 cells/μL (n=10). However, monocytes were only clearly detected in 50% of these samples.[29] De Graaf *et al.* found a variation of minimal required CSF cells between 100 gated lymphocytes in lymphocyte subset characterisation and 1000 cells in suspected CSF localisation of lymphoma.[14] Furthermore, to perform the analysis sufficient CSF volume is needed. Several studies reported obtained CSF volumes between 0.5 and 4 mL (median volume 2 mL).[11,30] A total CSF volume of 2 mL should be sufficient for flow cytometry, but a minimum of 5 mL[14] and 10 mL[4] has been proposed to detect low numbers of rare cells.[4,14]

2. Poor cell stability

Rapid cell decline in CSF will be significant within 1 hour of specimen collection.[31] Dux *et al.* studied spontaneous decay of cells in native CSF and phosphate buffered saline (PBS). After 90 minutes, the number of lymphocytes reduced to 65% in native CSF, while monocytes and granulocytes reduced more rapidly towards 10% (*Figure* 2).[32] De Graaf *et al.* described similar results: 56% lymphocyte survival after 5 hours in native CSF compared to serum-containing medium (measured by FCM).[33]

3. Peripheral blood contamination

Blood contamination may occur due to traumatic lumbar puncture or CNS bleeding, hence peripheral blood (PB) contamination should be excluded. In case of suspected traumatic puncture, the added WBC can be either calculated by following formula:

$$WBC\ added = \frac{WBC\ blood}{RBC\ blood}\ x\ RBC\ csf$$

Or it can be determined by counting the number of RBC per WBC (>1000 RBC/WBC means blood contamination).[31] In addition to the added WBC, WBC differentiation can be taken into account and compared to reference values (*Table* 2).[14,34,35] White blood cell count reference values were

determined in neonates and young infants: the median CSF WBC count was 3/µL (95thpercentile: 19/µL) in infants ≤28 days, and 2/µL (95thpercentile: 9/µL) in infants from 29 to 56 days.[36]

In case of secondary LM, a small population of malignant cells in contaminated CSF sample can only be considered as CNS involvement if the concomitant blood sample is negative.[31]

4. Monoclonal B-cell population

Detection of B-cell populations with monotypic immunoglobulin light chain expression in CSF is used to diagnose CNS invasion of B-NHL. Monoclonality of the B-cell population is assessed by determination of surface light chain expression on CD19+ B-lymphocytes and use of the light chain ratio or kappa/lambda ratio. [14]

Rare-event analysis is susceptible to carry-over of cells from previous experiments. Therefore extensive cleaning and washing is required between experiments. Removal of free immunoglobulins (Igs) by dilution of the sample with washing buffer can prevent binding of free Igs by CSF samples.[31]

Normal range of the ratio vary between laboratories. A kappa/lambda ratio of 2 was described to have a specificity of 92.3% and a sensitivity of 73.1%. Consequently, approximately 10% of patients with a ratio above 2 have a monoclonal B-cell population, but no B-NHL. An increased kappa/lambda ratio is only suggestive for monoclonal B-cell populations, but further analysis with additional markers should be performed for diagnosis of B-NHL invasion in the CNS. Use of additional markers may increase specificity f.e. abnormal intensities of CD19 and CD20.[14] Furthermore, Vafaii *et al.* described three cases of monoclonal B-cell populations in patients with multiple sclerosis.[37] Besides, detection of monoclonal B-cell population in CSF doesn't necessarily indicate symptomatic CNS disease.[14]

Quality improvement

1. Pre-analysis

a. Preventing cell decay and use of fixatives

An important challenge is the significant spontaneous cell decay within 30 min after sampling.[14] Therefore, samples should be transported at ambient temperature to the laboratory immediately and processed within 1 hour after lumbar puncture, or otherwise stabilised.[31] For a maximal cell yield for analysis, CSF samples have to be centrifuged at low speed level (15min x 200*g*, 4°C).[14] CSF samples are typically transferred to the laboratory without stabilisation medium. Nevertheless, use of stabilisation media may reduce CSF cell loss.[4]

Several media have been investigated: f.e. FCM buffer (phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA), 5% fetal calf serum (FCS) and 0.02% NaN_3)[32], Earle's balanced salt solution with human serum albumin[38], RPMI 1640 (with 25mM HEPES, 1mM L-Glutamine, 2% penicillin/streptomycin, 5% heat-inactivated fetal bovine serum (FBS) and 2,500 IU heparin)[17,33] and Transfix.[11,39]

In FCM buffer, after 90 min, 90% of the lymphocytes could survive (*figure 2*).[32] Second, addition of Earle's balanced salt solution with human serum albumin to CSF prevents cell loss during 24 hours after sampling.[38] Third, de Graaf *et al.* observed a preservation of CSF cells with RPMI 1640 until at

least 5 hours after sampling.[32] This stabilising feature was confirmed by Greig *et al.* In this study, using non-stabilised CSF yielded only sufficient viable cells in 30% of the samples versus 94% in stabilised CSF.[17]

Alternatively, TransFix might be used to stabilise leucocytes and leucocyte antigens and consequently increases the time window between withdrawal and FCM analysis. Leucocytes could be preserved at high median numbers in non-infiltrated samples, preventing deterioration of cells in CSF for hours and even days.[11] De Jongste *et al.* analysed a total of 99 CSF samples for LM in native CSF, CSF with medium and CSF with TransFix. After 18 hours of storage, use of TransFix significantly increased the detection of LM as compared to native CSF (*Table 4*). On the other hand, detection rates in both conditions were the same after 30 minutes (*Table 3*).

Besides LM detection, impact on relative numbers of leukocytes and subsets and fluorescence intensities were studied. After 30 minutes, the median leucocytes in CSF with TransFix was 1.4 times higher and after 18 hours, 2.3 times higher than in native CSF. The preservation of lymphocytes and monocytes were also significantly better in CSF with TransFix than native CSF. No significant difference was found for granulocytes (*Figure 3*). *Figure 4* shows differences measured in fluorescence intensities.[39]

b. Support protocol for sample collection

Support protocol for sample collection proposed by Kraan *et al.*[31]

Sample should be processed within 1 hour or stabilised in RPMI containing 5% FBS or TransFix:

1) Collect ≥ 2 mL of CSF by lumbar puncture and place sample at 4°C.
2) For RPMI: Collect the CSF sample directly into a sterile 15 mL tube containing 2 mL RPMI with 5% FBS stabilisation medium and store up to 18 hours at 4°C.
3) For TransFix: Collect the CSF sample directly into a tube containing 0.4 mL TransFix stabilisation medium (ratio 1/5) and store up to 48 to 72 hours at 4°C.

2. Analysis

Because of low cell numbers in CSF, a two-step approach is suggested. First, one-third of the sample should be analysed using a "screening" tube, which may lead to a certain pathology. If the first step is inconclusive, sensitivity can be increased by repeating the screening using the remaining sample volume and the same reagent mix. Second, if a pathological population is detected with the screening tube, a complete phenotypic characterisation should be performed adapted to the initial phenotype found by screening and the number of cells.[14,31] Identification of cytoplasmic antigens on malignant cells in CSF should be reserved for limited cases as increased cell loss is associated with intracellular staining.[31] *Figure 5* shows a proposed flow of CSF sample analysis by immunophenotypic analysis of white blood cells with multiple monoclonal antibodies to surface antigens.

Furthermore an appropriate choice of antibody panel is very important. Several panels suitable for the identification of aberrant, leukemic, or lymphoma cells in CSF have been designed. Six-colour staining panels used in the prospective, multicentre study of Quijano *et al.* and proposed by Kraan *et al.* can be found in *table 5-6*.[11,31] The EuroFlow group also designed an 8-colour immunophenotyping panel for lymphoma screening in 'small samples' from CSF. This 13-parameter SST labelling is a correctly titrated panel and suitable for a complete phenotyping of the most relevant leukocyte

populations in CSF. For a more detailed phenotyping of the aberrant B- or T-cell populations, additional markers are required.[40]

Besides the conventional immunophenotyping, a kit, based on microbeads, can be used to detect human soluble CD19 (sCD19). So far, only Muniz *et al.* evaluated the contribution of sCD19 protein levels in CSF of patients suspected for LM (measured by ELISA and FCM). CSF levels of 13 B-cell associated markers were evaluated in 91 patients with DLBCL and 22 patients with BL. They found an association between higher sCD19 CSF levels and a greater frequency of neurological symptoms in DLBCL and BL, and parenchymal CNS lymphoma in DLBCL. Especially in combination with FCM, sCD19 appeared to be a strong predictor of event-free and overall survival in DLBCL and BL.[41]

4. Conclusion

Since long, CC is considered the gold standard for diagnosing CSF invasion by lymphoma. However, combining CC with other laboratory diagnostics, can significantly improve sensitivity. Flow cytometry has shown to significantly increase sensitivity, in particular in aggressive B-NHL, but there is still need for (pre-) analytical quality improvement, such as preventing cell decay by using a stabilising medium and the use of immunophenotyping panels, specifically designed for detection of CNS invasion.

To do/actions

- Contact clinicians (hematology/neurology) for try-out of Transfix or RPMI
- Adjustment of internal protocol for sample collection and preservation

Attachment

Table 1. Comparison of FCM and CC for detection of malignant lymphocytes in CSF of patients with hematological malignancies (adapted from Ahluwahlia *et al.*[4])

Study	Number	Positive FCM (%)	Positive CC (%)	Study population
Hedge *et al.* 2005[6]	51	11 (22%)	1 (2%)	High risk CNS disease: – DLBCL with either ≥2 extranodal sites and elevated LDH or BM involvement – BL – Aids-related lymphoma
Quijano *et al.* 2009[11]	123	27 (22%)	7 (6%); suspicious in 3 (2%)	High risk CNS disease: – Aggressive B-NHL with infiltration of extranodal sites (testis, breast, paranasal sinus, and/or BM), neurological symptoms or elevated LDH.
Alvarez *et al.* 2012[26]	114	14 (12%)	1 (<1%)	DLBCL patients at diagnosis (n=95) or at relapse (n=19)
Benevolo *et al.* 2012[5]	174	18 (10%)	7 (4%)	Aggressive B-NHL
Bromberg *et al.* 2007[12]	219	44 (73% of 60; 20% of 219)	19 (32% of 60; 9% of 219)	Patients with CSF evaluation for haematological malignancy: DLBCL (n=55), precursor B-lymphoblastic leukemia/lymphoma (n=37), BL (n=8), other B-NHL (n=50), AML (n=40), CML (n=7), other (n=22)
Di Noto et *al.* 2008[27]	42	11 (26%)	4 (9.5%)	High risk CNS disease: – DLBCL, Blastoid MCL, B-LBL, or T-LBL with either ≥2 extranodal sites and elevated
Schinstine *et al.* 2006[28]	32	19 (59%)	Repeat cytology: 9 (47% of 19)	Patients with initial 'atypical' or 'suspicious' CSF evaluation, followed during 1 year: ALL, B-cell lymphoma, BL, CLL, PCNSL, DLBCL, FL, gamma-delta T-cell lymphoma, HIV-NHL, HTLV-1 leukemia/lymphoma, Mycosis fungoides, T-cell lymphoma/neoplasm.

Table 2. Distribution of WBC subsets in CSF and lymphocyte subsets in CSF and PB. (adapted from de Graaf *et al.*[14,35] and Svenningsson *et al.*[34])

Subset	Absolute number of cells CSF[14,a]	Absolute number of lymphocyte subsets CSF[35,a]	Percentage cells/all WBC CSF[14]	Percentage cells/lymphocytes CSF[34]	Percentage cells/lymphocytes PB[34]
Leukocytes	1.12 (0.40–3.17)		100%		
Granulocytes	0.08 (0.02-0.43)		7%		
Monocytes	0.23 (0.08-1.11)		21%		
Lymphocytes	0.66 (0.16-1.88)		59%	100%	100%
T cells	0.62 (0.15-1.83)	0.46 (0.2-2.02)	55%	97%	74%
CD4+ T cells	0.44 (0.08-1.43)	0.34 (0.12-1.36)	55%	76%	63%
CD8+ T cells	0.13 (0.04-0.40)	0.13 (0.06-1)	39%	24%	37%
NKT cells	0.01 (0.00-0.06)	0.005 (0-0.037)	<1%	3.5%	6.7%
B cells	0.00 (0.00-0.06)	0.005 (0-0.034)	<1%	0.8%	14%
NK cells	0.01 (0.00-0.05)	0.011 (0.002-0.058)	<1%	2.2%	12%
Dendritic cells	0.04 (0.01-0.18)		4%		
Myeloid	0.02 (0.00-0.13)		2%		
Plasmacytoid	0.01 (0.00-0.03)		<1%		

[a]Medians (5th-95th percentiles) of absolute numbers 10^6/mL are given.

Table 3. Detection of LM after 30 minutes: TransFix vs. native CSF (reproduced from de Jongste *et al.*[39])

t = 30 minutes	Native: Positive	Suspicious	Negative	Total
TransFix™				
Positive	21	3	4	28
Suspicious	2	0	4	6
Negative	0	3	62	65
Total	23	6	70	99

$P = 0.23$ (McNemar test).

Table 4. Detection of LM after 18 hours: TransFix vs. native CSF (reproduced from de Jongste *et al.*[39])

t = 18 hours	Native: Positive	Suspicious	Negative	Total
TransFix™				
Positive	23	6	5	34
Suspicious	0	0	2	2
Negative	0	0	63	63
Total	23	6	70	99

$P = 0.005$ (McNemar test).

Table 5. Six –colour immunophenotyping panel used in the prospective, multicentre study of Quijano *et al.*[11,Δ]

Colour	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC Cy7
MoAb	CD8-Smlg lambda	CD56-Smlg kappa	CD4-CD19	CD3	CD20	CD45

Table 6. Six-colour immunophenotyping panel proposed by Kraan *et al.* (adapted)[31,Δ]

Colour	FITC	PE	PCX	PE Cy7	APC	APC Cy7
Unknown	CD8-Smlg lambda	CD56-Smlg kappa	CD4-CD19	CD3	CD20	CD45
B	Smlg lambda	CDX/IgX	CD19	CD10	Smlg kappa	CD45
T	CD5	CD7	CD45	CD4	CD8	CD3
AL	CD34	CD7	CD45	CD33	CD10	CD19
ALL	CD5	CD7	CD45	CD10	CD34	CD19

Table 7. Composition of SST for detection of lymphoid cells[40,Δ]

Colour	PacB	PacO	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC H7
MoAb	CD20	CD45	CD8-Smlg lambda	CD56-Smlg kappa	CD4	CD19	SmCD3 and CD14	CD38

ΔAbbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; APC, allophycocyanin; B, B-cell lymphoma; Cy5.5/7, cyanin 5.5/7; FITC, fluorescein isothiocyanate; H7, hilite 7; MoAb, monoclonal antibodies; PacB, Pacific Blue; PacO, Pacific orange; PE, phycoerythrin; PCX, PE-cyanin Cy5 or PE Cy5.5 or peridinin chlorophyll protein (PerCP) or PerCP Cy5.5; Sm, surface membrane; SST, small sample tube; T, T-cell lymphoma.

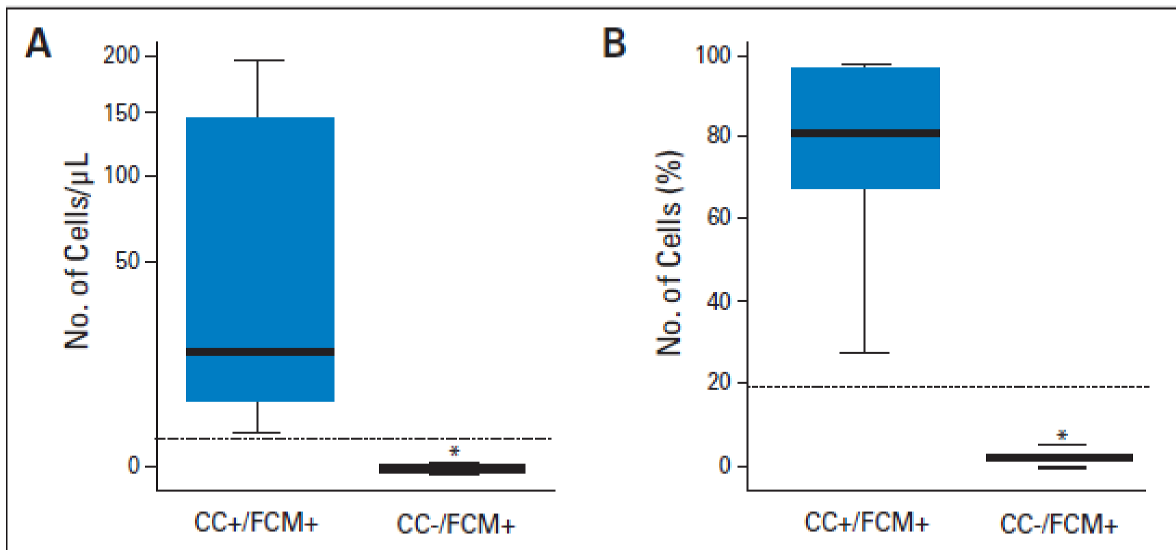

Figure 1. Comparative analysis of the (A) absolute and (B) relative numbers of neoplastic B cells in CSF samples being identified as containing neoplastic B cells by multiparameter flow cytometry (FCM) immunophenotyping, grouped according to the results of conventional cytology (CC). Absolute and relative counts of neoplastic B cells detected in FCM+/CC+ versus FCM+/CC- CSF samples were of 716 +/- 1,763 neoplastic B cells/µL (range, 0.9 to 4,712 cells/µL) and of 75% +/- 23% neoplastic B cells (range, 28% to 99%) versus 0.06 +/- 0.1 neoplastic B cells/µL (range, 0.001 to 0.5 cells/µL) and of 5% +/- 8% neoplastic B cells (range, 0.1% to 23%), respectively. Boxes extend from the 25th to the 75th percentiles; the line in the middle and vertical lines represent median values and 95% CIs, respectively. (*) P < .05 for comparisons of both absolute and relative numbers in cases showing a positive versus negative CC result. (reproduced from Quijano et al. 2009[11])

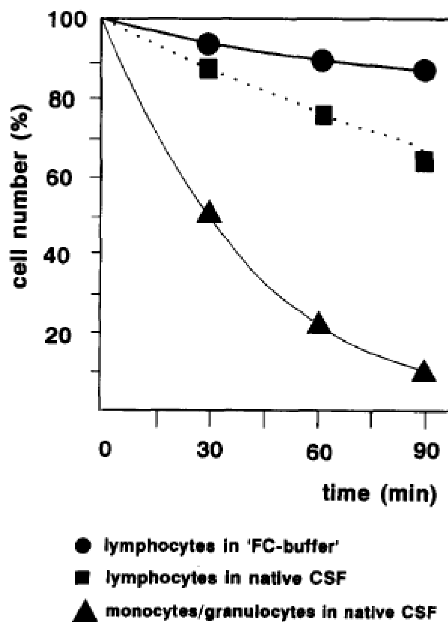

Figure 2. Spontaneous decay of cells in the native cerebrospinal fluid and in 'CSF-buffer'. (reproduced from Dux *et al.* 1994[32])

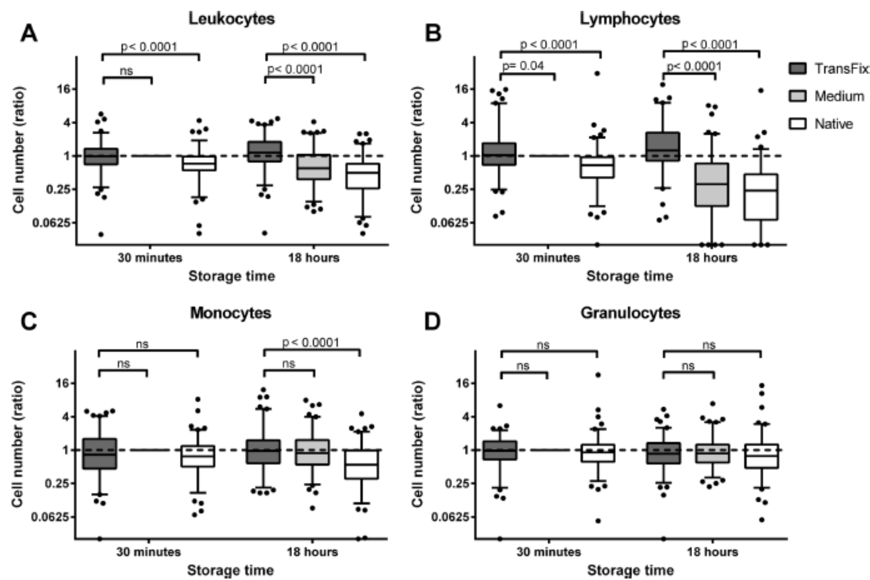

Figure 3. Relative numbers of leukocytes (A) and their subsets (B–D) after 30 minutes and 18 hours of storage in CSF with TransFix™, CSF with serum-containing medium, and native CSF. Relative numbers were calculated by dividing the absolute numbers (in cells/mL) by those in CSF with serum-containing medium at 30 minutes. A reference line is drawn at a relative cell number of 1, to indicate the cell number in serum-containing medium at 30 minutes. Boxes represent medians and quartiles, whiskers 5th and 95th percentiles. P values were calculated with the Wilcoxon signed rank test. ns, not significant. (reproduced from de Jongste *et al.*[39])

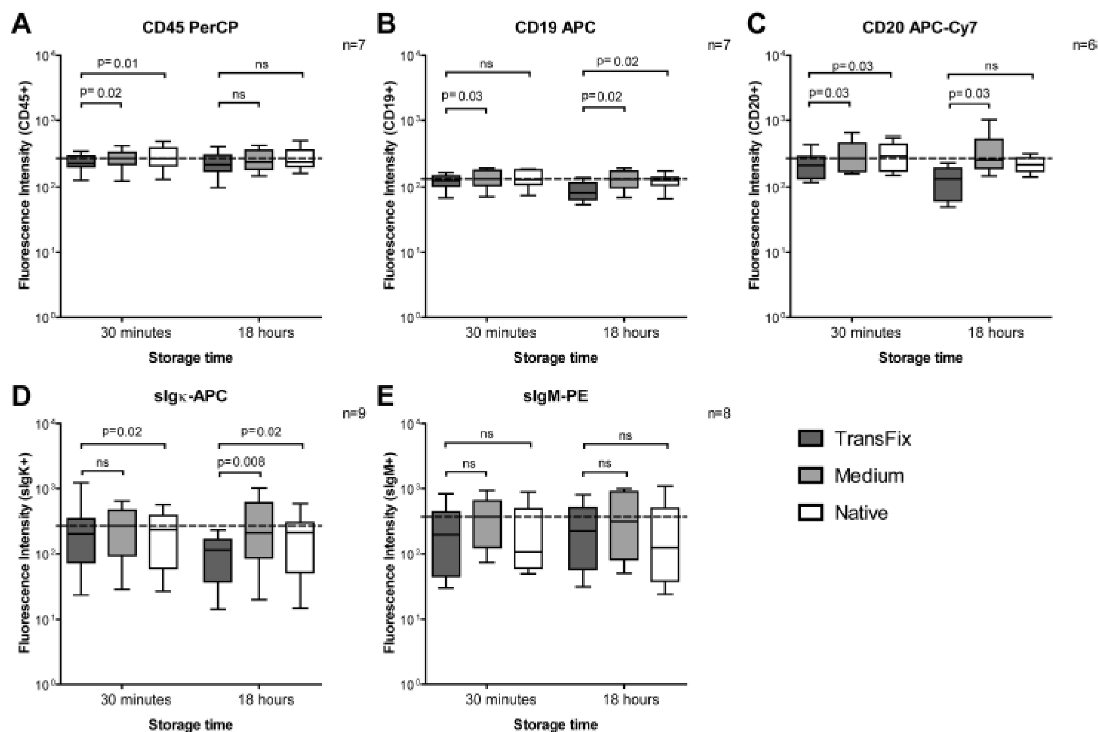

Figure 4. Fluorescence intensities after staining for the leukocyte marker CD45 (A), the B cell markers CD19 and CD20 (B, C), and the surface immunoglobulins kappa and M (D, E) in CSF with TransFix™, CSF with serum-containing medium, and native CSF after 30 minutes and 18 hours of storage. A reference line is drawn to indicate the median fluorescence intensity in serum-containing medium at 30 minutes. Boxes represent medians and quartiles, whiskers 5th and 95th percentiles. P values were calculated with the Wilcoxon signed rank test. ns, not significant. (reproduced from de Jongste *et al.*[39])

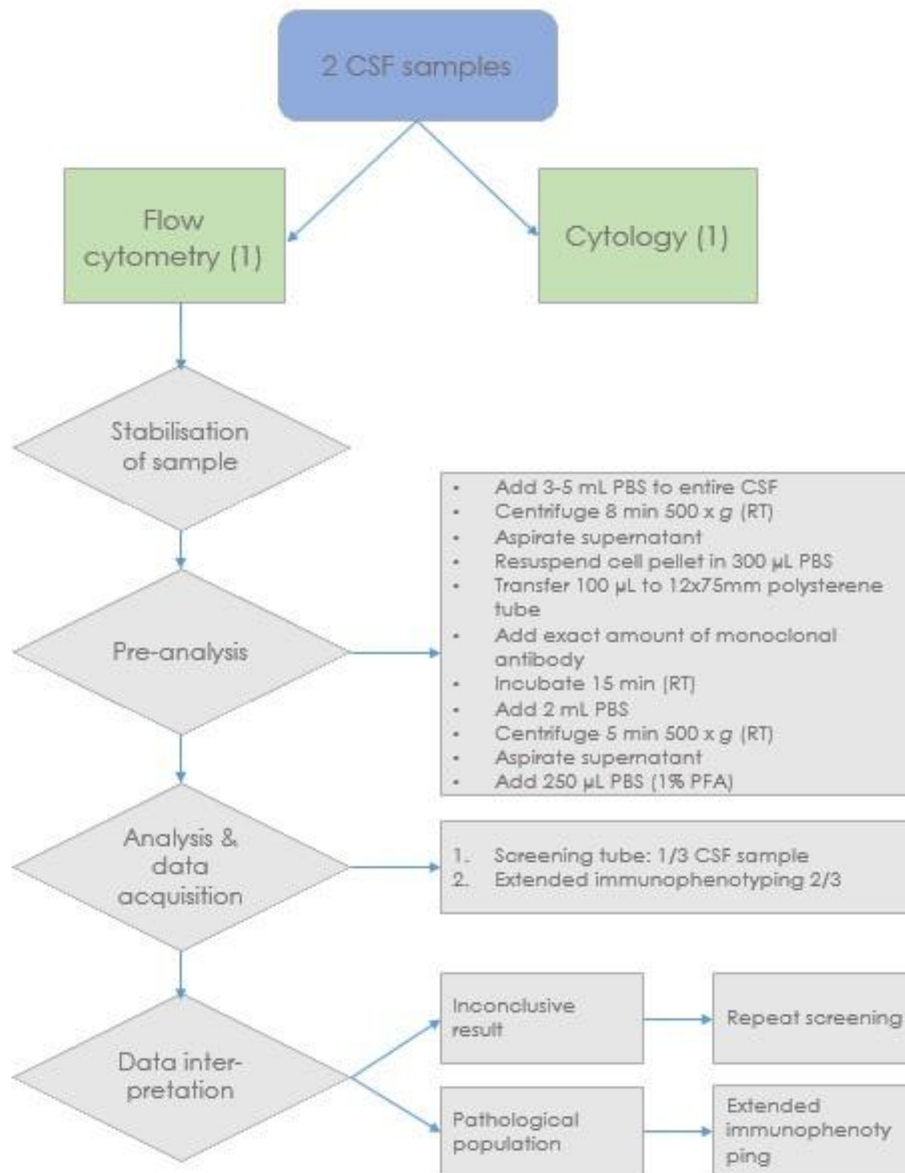

Figure 5. Flowchart of CSF sample analysis: cytology and immunophenotypic analysis of white blood cells with multiple monoclonal antibodies to surface antigens.[31]