UZ LEUVEN | LABORATORIUMGENEESKUNDE

CAT
Critically Appraised Topic

Implementation of the "BD OneFlow™ Lymphocyte Screening Tube" for the screening of non-Hodgkin lymphoma

Author: Jan Van Elslande
Supervisor: Nancy Boeckx
Search/methodology verified by: Pieter Vermeersch, Nancy Boeckx
Date: 19/03/2019

CLINICAL BOTTOM LINE

Flow cytometric immunophenotyping (FCI) plays an important role in the diagnosis and classification of chronic lymphoproliferative disorders (CLPD). However guidelines do not recommend to perform FCI on every sample. When FCI screening for CLPD is legitimately clinically indicated, guidelines recommend to start with an orienting screening panel (preferably in a single tube), followed by more extensive panels if the screening reveals a possibly pathologic population. The workflow in the university hospital Leuven (UZL) complies with the recommendations found in the available guidelines.

In UZL, a new screening tube for CLPD was implemented in 2016, the Euroflow BD OneFlow™ Lymphocyte Screening Tube (LST). The BD OneFlow™ LST replaced an in-house CLPD screening panel called the "screen non-Hodgkin lymphoma (NHL) panel". In this CAT it was determined that the implementation of the BD OneFlow™ LST resulted in an incremental cost and analysis time, but a more convenient workflow probably with less mistakes, increasing quality.

To estimate a possible clinical impact of this implementation, a total of 3320 screen CLPD samples were investigated over a study period of almost 6 years (01/2013 – 11/2018). The proportions of detected CLPD were compared between the period in which the "screen NHL panel" was used as the primary CLPD screen and the period in which the BD OneFlow™ LST was used for this purpose. It was shown that a higher proportion of monoclonal B-cell lymphocytosis (MBL) was detected using the BD OneFlow™ LST, both low- and high count types. This was accompanied by a drop in the proportion of samples classified as normal, and a slight decrease in the number of B-NHL detected.

CLINICAL/DIAGNOSTIC SCENARIO

Lymphoproliferative disorders (synonyms: lymphoid neoplasms, lymphomas) are a diverse group of neoplasms derived from hematopoietic cells designated to the lymphoid lineage. These cancers develop through the accumulation of clonogenic events in the genome (mutations, translocations, …) which lead to proliferative and/or survival advantage over their normal counterparts and an accumulation of clonal cells and their products [1–3].

The WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues of 2016 ("WHO 2016" for short), divides lymphomas into broad categories based on the maturity and lineage of the normal cell counterpart from which the tumor derived. A first distinction exists between the Hodgkin and non-Hodgkin lymphomas (NHL). Hodgkin lymphomas are usually a histologic diagnosis and rarely encountered with flow cytometric immunophenotyping (FCI); they are not further discussed here.
Within the NHL, a distinction is made between precursor lymphomas and mature lymphomas. The precursor lymphomas are characterized by proliferation of blasts, with a clinical presentation of acute leukemia. They are also not further discussed in this text.
Within the mature NHL (also referred as chronic lymphoproliferatieve disorders or CLPD), a distinction is made between mature B-cell, mature T-cell, and NK-cell lymphomas. A separate category is provided for the immunodeficiency-associated lymphoproliferative disorders. Within these broad categories, many distinctions are further made, based on clinical, histologic, immunophenotypic and genetic characteristics[1]. An overview of these disease entities is presented in Attachment I.

Mature NHL were 7th most common cancer in Belgium in 2016, with a combined incidence of around 20-30/100.000 persons per year across all ages[4]. The incidence of NHL increases with age and has a male predominance [5,6].

There is no single gold standard technique for the diagnosis of CLPD. The WHO 2016 emphasizes the importance of combining clinical information (presentation, course), morphology (cytology, histology), genetics (karyotyping, FISH and molecular diagnostics) and immunophenotype to reach a correct diagnosis [1].

FCI uses labelled antibodies to rapidly and sensitively assess protein expression (i.e. the immunophenotype) on individual cells in a mixed cell suspension [3]. This allows for a determination of lineage, maturation stage and clonality of a population of interest, essential information for the diagnosis and classification of hematologic neoplasms [3]. This technique first appeared in clinical laboratories in the 1980s and since then its use has expanded markedly. Now FCI plays a prominent role not only in diagnosis and classification of hematologic neoplasms, but also in therapeutic decision making, staging, estimation of prognosis and determination of residual disease, making it an essential component of modern hematopathology practice [1,7]. It has been shown that FCI 1) is more sensitive than morphology alone in detecting hematologic neoplasms 2) increases diagnostic concordance of cytology with pathology and 3) improves diagnostic accuracy by 10% to 45% depending on lymphoma subtype [2,7,8].

Not only has the use of immunophenotyping expanded since the 80's, so has its complexity. Current immunophenotyping protocols generally include ≥8 colour tubes. Over 360 cluster of differentiation (CD) molecules exist, so many different combinations of CD directed antibodies and fluorochromes can be made. Furthermore, these antibodies can be combined in many ways in diagnostic/follow-up panels. Several different instruments exist, each having their own laser and detector characteristics. Finally, the complex dataset generated by a flow cytometer needs to be interpreted by an expert in this field. All these factors may lead to variability across laboratories, making it difficult to compare results in different settings[2,9–11].

The EuroFlow consortium attempted to reduce this variability and subjectivity by introducing a series of standardized flow cytometric approaches in hemato-oncology. They made detailed descriptions covering the entire FCI analysis process, from instrument settings to antibody panels, reagents, sample preparation protocols, data acquisition, data analysis and quality control [2,11,12]. Antibody panels were developed and prospectively tested for a range of hematologic neoplasms, consequently a diagnostic flowchart was suggested on how use these different panels [2].

In UZL, several of these panels were implemented in the last years. This CAT evaluated the implementation of one of these panels, the BD OneFlow™ lymphocyte screening tube (LST) which was introduced in July 2016.

The aim of this CAT was to estimate whether this new test panel resulted in an increased detection of mature lymphoid neoplasms, compared to the previously used in-house panel, which was called the "screen NHL panel". Furthermore, the impact of the BD OneFlow™ LST tube on the laboratory workflow (TAT, hands-on time and cost) was estimated, as well as a possible clinical impact of this new tube. The appraisal starts with an overview of existing guidelines regarding the clinical indications for FCI as well as instructions for the construction of flowcytometric panels. This CAT will not go into detail on technical aspects of instrumentation and software settings used in FCI, for these subjects referral will be made to the detailed EuroFlow standard operating procedure (SOP) [12] and other guidelines on these matters [10,11,13].

QUESTIONS

1) *What is the role of flowcytometric immunophenotyping on blood and bone marrow samples in the diagnosis of mature non-Hodgkin lymphomas? What do current guidelines recommend regarding screening panels to diagnose these diseases?*
2) *Question 2: What is the impact on the laboratory of the implementation of the BD OneFlow™ LST regarding cost, hands-on time, turnaround time?*
3) *Question 3: What is the (possible) clinical impact of the implementation of the BD OneFlow™ LST? Is there an increased/decreased detection of NHL?*

SEARCH TERMS

Criteria for study inclusion/exclusion:

- *Inclusion: both of the following*
  - *Flowcytometric immunophenotyping*
  - *non-hodgkin lymphoma diagnosis*
- *Exclusion: one or more of the following*
  - *Body fluids*
  - *Treatment*
  - *Non-human*
  - *Histopathology (lymph node biopsies, FNAC, …)*
  - *Genetics*
  - *Full text not available*
  - *Non-English full text*

- *More recent articles were preferred over older ones*

Search strategy

Only the databases and search words which were used are presented here. For full details of the search strategy see attachment 5.

1) *MeSH Database (PubMed): MeSH term: ""Immunophenotyping"[Mesh]", "Lymphoproliferative Disorders"[Mesh], "Lymphoma"[Mesh]*
2) *PubMed Clinical Queries*
    a. *"Immunophenotyping"[Mesh]) AND "Lymphoproliferative Disorders"[Mesh]*
    b. *"Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening*
    c. *"Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening*
    d. *"Lymphoma/diagnosis"[Mesh]*
3) *Databases*
    a. *Pubmed (Medline; from 1966)*
        i. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh]*
        ii. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh]*
        iii. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND LST*
        iv. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening panel*
        v. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND screening AND guideline*
        vi. *"Lymphoma/diagnosis"[Mesh]) AND screening AND flow cytometric AND immunophenotyping*
        vii. *"lst tube"*
        viii. *Similar articles section and referred articles*
    b. *SUMSearch (http://sumsearch.uthscsa.edu/)*
        i. *"non-hodgkin lymphoma AND flowcytometric" – diagnosis, human only*
        ii. *"non-hodgkin lymphoma" – diagnosis, human only*
    c. *The National Institute for Clinical Excellence (http://www.nice.org.uk/)*
        i. *"Lymphoma AND diagnosis": 65*
    d. *Cochrane (http://www.update-software.com/cochrane*
        i. *"Lymphoma"*
        ii. *"immunophenotyping AND lymphoma"*
4) *UpToDate Online version 12.2 (2004)*
    a. *"non-Hodgkin lymphoma"*

RELEVANT EVIDENCE/REFERENCES

Here the references are presented in order of appearance, for a sorting based on reference type, see attachment 5.

1. The International Agency for Research on Cancer. *WHO classification of tumours of haematopoietic and lymphoid tissues*. (2016).
2. Van Dongen, J. J. M. *et al.* EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia* **26**, 1908–1975 (2012).
3. Seegmiller, A. C., Hsi, E. D. & Craig, F. E. The current role of clinical flow cytometry in the evaluation of mature B-cell neoplasms. *Cytometry B. Clin. Cytom.* **29**, 20–29 (2018).
4. Belgian Cancer Registry — Tabellen op jaarbasis. Available at: https://kankerregister.org/Statistieken_tabellen_jaarbasis. (Accessed: 5th March 2019)
5. Smith, A. *et al.* Lymphoma incidence, survival and prevalence 2004–2014: sub-type analyses from the UK's Haematological Malignancy Research Network. *Br. J. Cancer* **112**, (2015).
6. Bowzyk Al-Naeeb, A., Ajithkumar, T., Behan, S. & Hodson, D. J. Non-Hodgkin lymphoma. *BMJ* **362**, k3204 (2018).
7. Davis, B. 2006 Bethesda International Consensus Recommendations on the Flow Cytometric Immunophenotypic Analysis of Hematolymphoid Neoplasia: Medical Indications. *Cytom. Part B …* **85**, 77–85 (2007).
8. The Non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood* **89**, 3909–18 (1997).
9. Preijers, F. W. M. B., Huys, E., Favre, C. & Moshaver, B. Establishment of harmonization in immunophenotyping: A comparative study of a standardized one-tube lymphocyte-screening panel. *Cytom. Part B - Clin. Cytom.* **86**, 418–425 (2014).
10. Kern, W. *et al.* Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. *Leukemia* **25**, 567–574 (2011).
11. Glier, H. *et al.* Standardization of 8-color flow cytometry across different flow cytometer instruments: A feasibility study in clinical laboratories in Switzerland. *J. Immunol. Methods* 0–1 (2017).

doi:10.1016/j.jim.2017.07.013
12. Kalina, T. *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* **26**, 1986–2010 (2012).
13. L., W. B. 2006 Bethesda International Consensus Recommendations on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry: Optimal Reagents and Reporting for the Flow Cytometric Diagnosis of Hematopoietic Neoplasia. *Clin. Cytom.* (2007). doi:10.1002/cyto.b
14. Rajab, A., Axler, O., Leung, J., Wozniak, M. & Porwit, A. Ten-color 15-antibody flow cytometry panel for immunophenotyping of lymphocyte population. *Int. J. Lab. Hematol.* **39**, 76–85 (2017).
15. Craig, F. E. & Foon, K. A. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* **111**, 3941–3967 (2008).
16. Greig, B., Oldaker, T., Warzynski, M. & Wood, B. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: Recommendations for training and education to perform clinical flow cytometry. *Cytom. Part B Clin. Cytom.* **72B**, S23–S33 (2007).
17. Davids, M. Approach to the adult with lymphocytosis or lymphocytopenia - UpToDate. Available at: https://www.uptodate.com/contents/approach-to-the-adult-with-lymphocytosis-or-lymphocytopenia?search=lymphocytosis&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1. (Accessed: 31st January 2019)
18. Freedman, A., Friedberg, J. & Aster, J. Clinical presentation and diagnosis of non-Hodgkin lymphoma - UpToDate. (2018). Available at: https://www.uptodate.com/contents/clinical-presentation-and-diagnosis-of-non-hodgkin-lymphoma?search=clinical presentation and diagnosis of non-hodgkin lymphoma&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1. (Accessed: 31st January 2019)
19. Braylan, R. C., Orfao, A., Borowitz, M. J. & Davis, B. H. Optimal number of reagents required to evaluate hematolymphoid neoplasias: results of an international consensus meeting. *Cytometry* **46**, 23–7 (2001).
20. Bain, B. J., Barnett, D., Linch, D., Matutes, E. & Reilly, J. T. Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. *Clin. Lab. Haematol.* **24**, 1–13 (2002).
21. Johansson, U. *et al.* Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms. British Committee for Standards in Haematology. *Br. J. Haematol.* **165**, 455–88 (2014).
22. Kroft, S. H. & Harrington, A. M. Flow Cytometry of B-Cell Neoplasms. *Clin. Lab. Med.* **37**, 697–723 (2017).
23. Craig, J. W. & Dorfman, D. M. Flow Cytometry of T cells and T-cell Neoplasms. *Clin. Lab. Med.* **37**, 725–751 (2017).
24. NomenSoft - RIZIV. Available at: https://www.riziv.fgov.be/nl/toepassingen/Paginas/NomenSoft.aspx. (Accessed: 19th March 2019)
25. van der Velden, V. H. J. *et al.* Optimization and testing of dried antibody tube: The EuroFlow LST and PIDOT tubes as examples. *J. Immunol. Methods* (2017). doi:10.1016/j.jim.2017.03.011
26. Hedley, B. D., Keeney, M., Popma, J. & Chin-Yee, I. Novel lymphocyte screening tube using dried monoclonal antibody reagents. *Cytom. Part B - Clin. Cytom.* **88**, 361–370 (2015).
27. Rajab, A. & Porwit, A. Screening bone marrow samples for abnormal lymphoid populations and myelodysplasia-related features with one 10-color 14-antibody screening tube. *Cytom. Part B - Clin. Cytom.* **88**, 253–260 (2015).
28. Swerdlow, S. H., Campo, E. & Pileri, S. A. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* **127**, (2016).
29. Rawstron, A. C. *et al.* Different biology and clinical outcome according to the absolute numbers of clonal B-cells in monoclonal B-cell lymphocytosis (MBL). *Cytom. Part B - Clin. Cytom.* **78**, 19–23 (2010).
30. Ghia, P. *et al.* General population low-count CLL-like MBL persists over time without clinical progression, although carrying the same cytogenetic abnormalities of CLL. *Blood* **118**, 6618–6625 (2011).
31. Jain, N. *et al.* Prevalence and Economic Burden of Chronic Lymphocytic Leukemia (CLL) in the Era of Oral Targeted Therapies. *Blood* **126**, (2015).
32. Shanafelt, T. D. *et al.* Brief report: Natural history of individuals with clinically recognized monoclonal B-cell lymphocytosis compared with patients with Rai 0 chronic lymphocytic leukemia. *J. Clin. Oncol.* **27**, 3959–3963 (2009).

Question 1: what is the role of flowcytometric immunophenotyping on blood and bone marrow samples in the diagnosis of mature non-Hodgkin lymphomas? What do current guidelines recommend regarding screening panels to diagnose these diseases?

Background

For the screening of CLPD, initially two 6-colour tubes were used, the "sIgB" and "TBNK", together called the "screen NHL panel". This was used until august 2016 and then the switch to the BD OneFlow™ EuroFlow LST was made. The BD OneFlow™ LST is an 8-colour 12 antibody tube, developed by the EuroFlow consortium. More details on the time periods and number of tubes are provided in the appraisal of Question 2 and 3 (cf. below).

The markers used in these tubes are shown in Table 1. Note that some different antigens (e.g. CD4 and CD20) are conjugated with the same fluorochrome. This is possible because in vivo, these antigens are never found together on the same cells, neither in normal settings nor in the case of a malignant cell population [14].

Table 1: labelled monoclonal antibodies used in the screen NHL panel and the BD OneFlow™ EuroFlow LST tube. Shaded antibodies are provided in a pre-manufactured cocktail (BD Biosciences). sIg: surface immunoglobulin.

	Screen NHL panel		
Tube	sIgB tube	TBNK tube	**BD OneFlow™ LST**
Markers	CD5-APC	CD4–PE-CY7	CD45-V500-C
	CD10-PE-CY7	CD8–APC-H7	CD19/TCR-γδ-PE-cy7
	CD20-APC-H7	CD16/CD56-PE	CD5-PerCP-Cy5.5
	sIgKappa-FITC	CD19-APC	CD3-APC
	sIgLambda-PE	CD45-PerCP	CD20/CD4-V450
	CD19-PerCP	CD3-FITC	CD8/sIglambda-FITC
			CD56/sIgkappa-PE
			CD38–APC-H7

Another difference between the screen NHL panel and the BD OneFlow™ LST is the number of events which is analysed. For the TBNK tube (part of the screen NHL panel), 10.000 events are analysed within the leukogate (based on CD45-SSC). For the sIgB (also part of the screen NHL panel) 5.000 events within the B-cell gate (based on CD19-SSC) are analysed. For the BD OneFlow™ LST, a total of 100.000 events are collected, but there is no specific gate that these events have to be situated in. An example of a sample analysed using the BD OneFlow™ LST is shown in Attachment 2.

How the diagnostic flowchart for CLPD is followed in the university hospital Leuven (UZL) depends on the information provided with the test request. If no clinical information is provided (other than the screening request for CLPD), a screening panel is used. If specific information is available (e.g. lineage of the tumour determined by histology on a tissue biopsy, morphology suggestive for a certain pathology), more specific panels are used. Here we focus on the screening situation (
Figure 5).

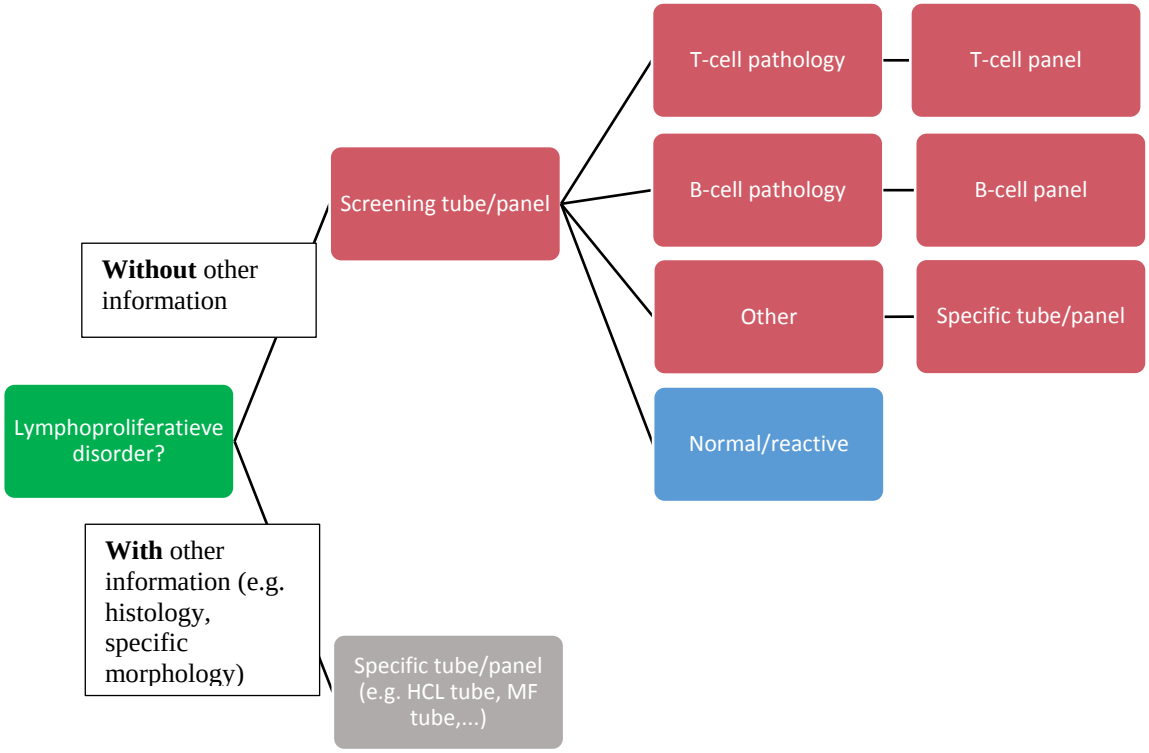


Figure 1: UZL workflow for tube/panel decision. MF: mycosis fungoides, HCL: hairy cell leukemia.

It is beyond the scope of this text to discuss every role and aspect of FCI in CLPD. To answer the questions asked in this CAT, we will mainly focus on the clinical indications, panel composition and flowcharts for the screening /diagnosis of NHL proposed by the different guidelines and compare these with previous and current practices in UZL as described in the above text.

Clinical indications

As mentioned in the "Clinical/diagnostic scenario" section, flow cytometric immunophenotyping (FCI) is an integral component of modern hematopathology practice. However, this does not mean that it is diagnostically productive and cost-effective in all clinical situations[15]. In 2006, international consensus recommendations were made to guide the clinical settings in which FCI can be considered (Table 2)[7].

Table 2: medical indications for flow cytometric immunophenotyping as recommended by the Bethesda guidelines of 2006. Adapted from [7].

Indications	**No indications**
Lymphadenopathy, organomegaly (especially hepatosplenomegaly), tissue infiltrates (especially skin, mucosa and bone)	Isolated anaemia
Bi- or pancytopenia without clear explanation	Isolated thrombocytosis, neutrophilia or basophilia
Unexplained lymphocytosis, monocytosis, eosinophilia	Polyclonal hypergammaglobulinemia
Atypical cells / blasts observed by morphology (absolute indication)	
Paraprotein and/or plasmacytosis in blood and/or bone marrow	
Monitoring • Staging • Prognostication • Minimal residual disease (MRD) • Follow-up (progression? Relapse? Disease acceleration?)	

The article does not provide information on which reagents, procedures and panel(s) of antibodies that are to be used in these different clinical situations [7]. However, these subjects were covered in two separate guidelines released by this group[13,16].

The UpToDate pages on "Clinical presentation and diagnosis of non-Hodgkin lymphomas" and "Approach to the adult with lymphocytosis or lymphocytopenia" describe FCI as the preferred test for determining clonality as it is rapid and cost-effective. The ability to determine lineage and maturation stage makes it crucial for the diagnosis and sub classification of most forms of NHL. However, it is not recommended as a first line screening test for every patient with lymphocytosis, the indications presented on this website are summarized in Table 3 [17,18].

Table 3: indications for flowcytometric immunophenotyping as recommended by UpToDate anno 2019.

Urgent indications	**Less urgent indications**
Blasts in the peripheral blood (precursor lymphoid neoplasm?)	ALC >5.000/µL, unless recent viral infection, asplenia, medication that can explain this finding
Absolute lymphocyte count (ALC) >30.000/µL, without a known diagnosis	Unexplained ALC >4.000/µL for >1 month
	Rising ALC
	Atypical lymphocytes on peripheral blood smear suggestive for malignancy
	Cytopenias
	Hepatosplenomegaly
	Lymphadenopathy

More guidelines on the different aspects of FCI exist besides the three documents released by the Bethesda International Recommendations in 2006. In the 1990's, practitioners already recognized a need for consensus statements regarding different aspects of flow cytometry, and several different organizations made an attempt at this [7]. Table 4 presents a number of guidelines and their scopes, published since 2000. Note that guidelines specifically focusing on disease entities outside of the scope of this CAT are not included.

Table 4: Important guidelines regarding flow cytometric immunophenotyping for non-Hodgkin Lymphomas published since 2000.

Organisation	**Main subject(s) scoped**	**Year**
ISAC 2000 [19]	Panel construction	2001
British Committee for Standards in Haematology (BCSH) [20]	Sample collection and conditions, panel construction	2002
Bethesda International Consensus Recommendations (3 guidelines)	1. Optimal reagents and reporting (including panel construction) 2. Medical indications 3. Training and education	2006
European LeukemiaNet Work Package 10 [10]	Panel construction	2011
EuroFlow consortium (2 guidelines) [2,12]	1. Instrument settings and protocols for sample preparation and quality control 2. Panel design	2012
BCSH [21]	Instrumentation, panel design (basic) and validation, reagent handling, pre-analytical variables, data acquisition, analysis and reporting, training of staff, validation procedures and auditing.	2014

Mature B-cell neoplasms

Mature B-cell neoplasms constitute the vast majority of NHL. In our study 94.6% of patients with abnormal lymphocytes concerned B-cell CLPD. Of these, chronic lymphocytic leukemia (CLL) and monoclonal B-cell lymphocytosis (MBL) with CLL phenotype accounted for 57.3%.

FCI can differentiate normal B-cells from neoplastic B-cells in two ways. One is the assessment of immunoglobulin light chain class restriction, the other is identifying aberrant antigen expression[3,15,22].

Neoplasms of B-cells usually consist of a clone origination from a single neoplastic B-cell, therefore it's cells express only one class of the immunoglobulin light chains, either kappa or lambda. If the population is sufficiently large, this will result in an altered kappa/lambda ratio. However, small populations admixed with normal polyclonal B-cells can be missed if only the ratio is evaluated. Therefore, small populations should be assessed for distinct (clustered) light scatter characteristics or an aberrant phenotype such as the presence of antigens not normally expressed or a weaker/absent expression of normal antigens. The most frequent abnormal expression is CD5 positivity combined with light chain restriction. This finding points to a possible diagnosis of mantle cell lymphoma (MCL) or CLL. Note that small populations of normal B-cells can be CD5 positive in healthy individuals, but these will have a polyclonal light chain expression. Another abnormal expression pattern is CD10 combined with a mature marker CD20. Although both markers can be expressed in B-cells depending on their maturation stage, they should not be expressed together in a large subset of B-cells. This finding can suggest the presence of a follicular lymphoma (FL) or diffuse large B-cell lymphoma (DLBCL), again when combined with light chain restriction. Weaker expression of light chains, CD20, CD79b and/or CD22 is also abnormal, and can suggest a CLL, especially when combined with the previously mentioned CD5 positivity. An abnormally strong expression of CD20 on the other hand can be indicative for a MCL or hairy cell leukaemia (HCL). The latter usually presents with a higher side scatter, as well as a specific unique phenotype (strongly positive for CD11c, CD103 and CD25) [3,15,22] .

To screen for B-cell neoplasms, guidelines suggest that a screening panel should include several backbone markers to gate for B-cells, surface immunoglobulin kappa (sIgKappa) and surface immunoglobulin lambda (sIgLambda) to assess clonality as well as some specific markers that could be abnormally expressed. It should not, however, provide a complete characterization of the immunophenotype needed for classification. This is done by subsequent testing, when the screening suggests a presence of an abnormal population [3,15,22].

The screen NHL panel as well as the BD OneFlow™ LST contain backbone markers CD45 (mature population vs blasts?), CD19 (pan B-cell marker used for B-cell gating) and CD20 (normally expressed on mature B-cells). Furthermore, both panels asses clonality by determination of sIgKappa and sIgLambda expression, as well as CD5, the most common aberrantly expressed marker encountered with FCI regarding B-CLPD. The screen NHL panel contains one extra B-cell marker, namely CD10. In the BD OneFlow™ LST, CD38 is determined instead (Table 1). Both markers have their advantages and disadvantages, these are not further discussed in this text [3,15,22]. It can be concluded that both the screen NHL panel and the BD OneFlow™ LST comply with the recommendations for screening of B-cell CLPDs as proposed in the guidelines presented in Table 4.

Mature T- and NK neoplasms

These two entities are considered together because of similar biological and clinical characteristics. Both are rare entities, in our study population comprising only 5.4% of patients with abnormal lymphocytic populations.

It is more difficult to identify phenotypically abnormal T- or NK cells than abnormal mature B cells. FCI usually represents only part of the work-up for these diseases, with a final diagnosis often requiring assimilation of information from multiple sources, e.g. genetics, histology and clinical course. The main reason for this is the absence of a clear surrogate marker for clonality such as the restricted light chain expression of B-cell neoplasms [15,22,23].

Mature T- and NK cell neoplasms can be detected through identification of aberrant marker expression and/or a restriction of CD4/CD8 (T-cells). The diminished expression of 1 or more pan T-or NK cell markers is the most common feature of T- and NK cell neoplasias[23]. CD5, surface CD3 (sCD3) and CD7 are the most frequently lost antigens. However, there are some (small) cell populations present in healthy individuals which can also be negative for CD7 and/or CD5 (especially in reactive conditions). More frequently than a complete lack of staining for a specific antigen, T- and NK cell neoplasms can exhibit a weaker staining with certain antibodies. This is even harder to differentiate with normal/reactive cells, especially for the NK cells, which in normal situations can demonstrate somewhat variable intensity staining for e.g. CD2, CD7, CD16 and CD57. T-cell subset restriction is another possible indicator for T-cell neoplasm, e.g. a CD4 over CD8 predominance (or vice versa) or a TCR-γδ over TCR-αβ predominance. However, these markers are not as potent as sIgkappa/sIgLambda restriction observation in B-cells. This is because many reactive conditions can cause a skewed CD4/CD8 or TCRγδ/TCR αβ ratios. Also the genes for CD4 and CD8 do not demonstrate allelic exclusion [15,23].

Flow cytometric evaluation of the T-cell receptor (TCR) V-β expression is a more specific test to demonstrate a clonal presence of a specific β-chain of the TCR. This test however, also has its limitations. The first is that only around 75-80% of β-chain variants is examined, the second is that TCR-γδ positive neoplasms cannot be examined with this technique, since these cells to not possess β-chains [15]. Finally, this test is expensive and laborious, making it impossible to perform on every sample suspected of lymphoproliferative disease.

The screen NHL panel and BD OneFlow™ LST both contain backbone markers and sCD3 (T-cells). NK cells are defined by exclusion as cells within the lymphocyte gate (based on CD45-SSC) that are not-CD3 and not-CD19.

Specific markers for aberration in both panels are CD5 and CD56. The screen NHL also contains CD16, which in the BD OneFlow™ LST is exchanged for TCR-γδ (Table 1). Both of these markers have their own advantages and disadvantages, not further discussed in this text. In conclusion, both the screen NHL panel and the BD OneFlow™ LST comply with the guidelines for T- and NK-cell screening presented in Table 4.

EuroFlow consortium

Although there are several guidelines which provide recommendations regarding panel composition, only the EuroFlow guideline validated the proposed marker combinations in a prospective study with reference samples [2]. Another difference is that the EuroFlow guidelines cover the entire process of FCI, whilst many guidelines focus on only one or several parts of it (Table 4). Furthermore, the EuroFlow consortium provided a detailed, ready to use SOP to implement their recommendations [12]. This explains why many guidelines were only partially successful in reducing variability between laboratories, while the EuroFlow method has proven to provide datasets which are interlaboratory sufficiently comparable to develop software tools for automatic classification, and allowing for the organization of multicentric FCI studies [2,11,12].
The EuroFlow consortium also proposed a diagnostic flowchart for hematologic neoplasm screening and classification (Figure 2). The BD OneFlow™ LST has a prominent role in this, as a first screening tool when a CLPD is suspected and to guide further testing using more extensive panels for full characterization of a pathologic population.

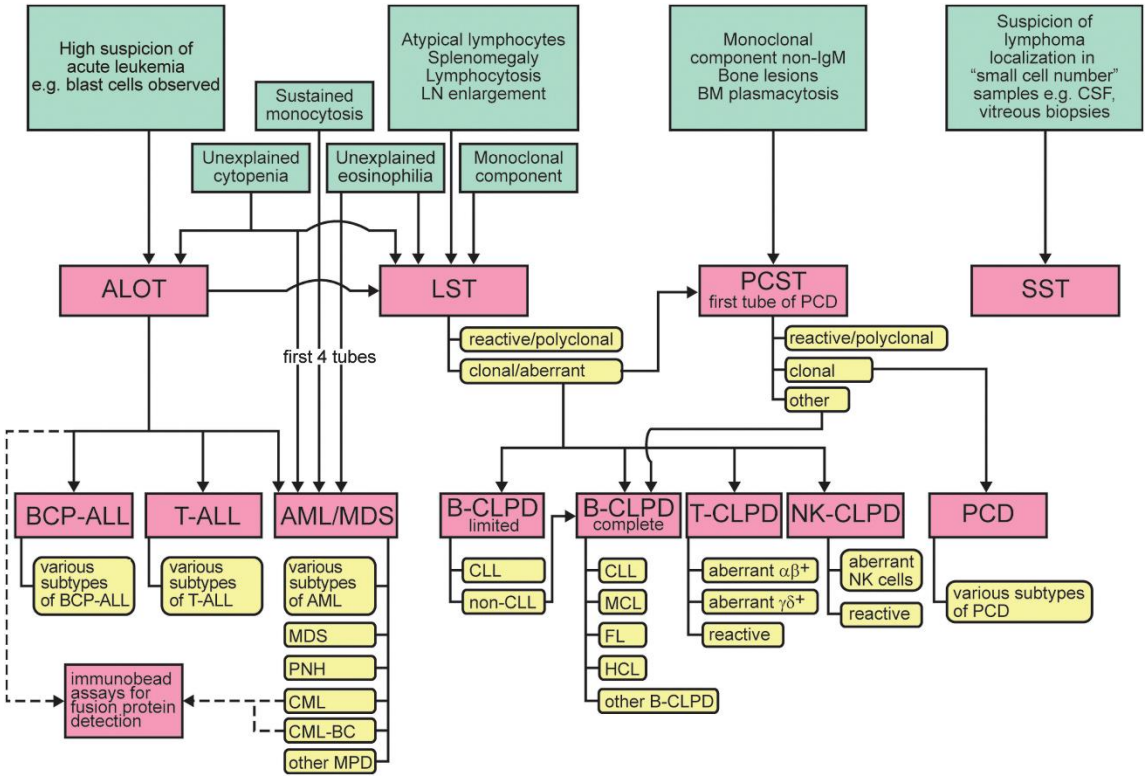


Figure 2: diagnostic flowchart for hematological neoplasms as proposed by the EuroFlow consortium in 2012 (adapted from [2])

In UZL, the ALOT, LST and PCST BD OneFlow™ tubes are all used in the screening settings proposed by this flowchart. However, the specific characterization panels (e.g. B-CLPD panel, T-CLPD panel) are not implemented as of this moment, instead custom panels are used for this purpose.

Guidelines: conclusion

In conclusion, FCI has an essential role in the diagnosis, classification and monitoring of CLPD. It is however, neither necessary nor cost-effective to perform FCI on every sample (Table 2). Furthermore, when FCI is legitimately clinically indicated, it is neither necessary nor cost-effective to perform a full characterization of all lymphocytes in the sample. Rather, a rapid screening step (preferably in a single tube) is recommended to differentiate whether or not a pathologic population is present and to what lineage it belongs (Figure 2). The workflow in UZL follows such a strategy (Figure 1). Finally, the markers included in the formerly used screen NHL panel and the currently used BD OneFlow™ LST comply with the guidelines regarding panel composition for CLPD screening situations (Table 1, Table 4).

Question 2: What is the impact on the laboratory of the implementation of the BD OneFlow™ LST regarding cost, hands-on time, turnaround time?

Background

We retrospectively queried all analysis of the last 6 years that were performed in our laboratory in the setting of screening for a lymphoproliferative disease. From 01/2013 onwards, all screenings for a CLPD were done using our "screen NHL panel". From august 2016 onwards, the BD OneFlow™ LST was implemented, initially on a FACSCanto II system (BD Biosciences) until 08/2018 and later on a FACSLyric instrument (BD Biosciences).

Sample preparation and staining

All three tubes (sIgB, TBNK and BD OneFlow™ LST) require a different sample preparation. An overview of the different steps is presented in Table 5. This sample preparation can be done entirely manually, or assisted by a Lyse Wash Assistant or LWA (BD Biosciences) for steps 4-7. Note that when using the LWA, one can save hands-on time, but the processing-time required by the LWA is longer than when the samples are processed manually.

The TBNK sample preparation is the shortest, because no wash step is required before adding the different monoclonal reagents. Washing is necessary for the sIgB and BD OneFlow™ LST tubes because immunoglobulin light chains are being targeted and the presence of circulating immunoglobulins interfere with the binding of the specific antibodies.

The final volume for all samples is 400 µL.

Table 5: summarized workflow for the different tubes.

Workflow steps	**Screen NHL panel**		
	TBNK	sIgB	**BD OneFlow™ LST**
1. Labeling of tubes	X	X	X
2. Washing • Add CellWASH • 5 min centrifugation • Removal of supernatans	No	2 times	3 times
3. Pipetting of monoclonal reagents	Single/combined (Table 1)	Single/combined (Table 1)	Not necessary (dry tube)
4. Incubation (room T°)	10 min	10 min	30 min
5. Lysis • Lysis buffer • 10 min incubation • 5 min centrifugation	15 min	15 min	15 min
6. Washing (cf. step 2)	1 time	1 time	1 time
7. Resuspension • 0,4mL CellFix/CellWASH	X	X	X

Impact on hands-on time

It is difficult to measure the exact hands-on time used for these tubes, as this is influenced by many factors. There are many other tubes simultaneously being prepared, influencing the workflow at every level, e.g. the use of a centrifuge can be delayed by other samples, the same applies to the use of e.g. the LWA.

Two surrogate markers were measured to attempt a quantification of the hands-on time. The analysis time (=from the start of sample preparation to printing of the final result), and the turn-around time (TAT, this is the time from reception of the sample to final validation).
The analysis time was registered over a period of 6 weeks (October-November 2018) by the technicians performing the routine analyses. The average times were compared using a Z-test for the BD OneFlow™ LST and sIgB, and a t-test assuming unequal variances (tested with an F-test) for comparisons with the TBNK tube. Excel was used for these calculations.

Table 6: analysis time for the different tubes. Note that the time required to perform a screen NHL panel is not the sum of the times required for the TBNK and sIgB tubes separately, since most steps can be done simultaneously. However the total time to perform a "screen NHL panel" could not be measured since these are no longer performed.

	Screen NHL panel			
	TBNK (n=11)	sIgB (n=47)	**BD OneFlow™ LST** (n=49)	p-value (all comparisons)
Analysis time (SD)	0:57 (0:13)	1:38 (0:35)	1:56 (0:26)	p<0.001

It is clear that the time to perform a TBNK tube is the shortest, followed by sIgB and subsequently the BD OneFlow™ LST. Note that we can most likely explain these findings because of the different steps in the workflow (Table 5). This workflow can also be summarized according to the number of steps required (Table 7). Though the BD OneFlow™ LST requires more wash steps and incubation/centrifugation time, less pipetting actions are required (one vs. nine for the screen NHL panel). Also only one sample acquisition and sample gating needs to be performed since only one tube is analysed, compared with two tubes for the screen NHL panel.

Table 7: summary of total number of steps and incubation/centrifugation time required in the preparation of samples

	Screen NHL panel		
	TBNK	sIgB	**BD OneFlow™ LST**
Pipetting actions	4 (sample + antibodies)	5 (sample + antibodies)	1 (only the sample)
Total wash steps	1	3	4
Total incubation and centrifugation time	30 minutes	40 minutes	55 minutes
Sample acquisition on flow cytometer, data analysis and printout	1 time	1 time	1 time

These factors taken into consideration, there is probably not a great difference in hands-on time between both approaches, since the extra hands-on time spent on some steps is won at other steps.

The TAT was determined as total time measured from reception of the sample, to final validation of the result. Only after final validation, the results and conclusion become visible for the clinicians who requested the test.

Table 8: turn- around time (TAT) in the different study periods.

	Screen NHL period (n= 2171)	**BD OneFlow™ LST period** (n=1102)	Difference
TAT (SD)	1 day 17 hours (1 day 8 hours)	2 days (3 days 4 hours)	6 hours (p<0.01)

In the BD OneFlow™ LST period, the TAT was on average 6 hours longer compared to the first and second screen NHL period (p<0.01).

The fact that the time to perform a FCI analysis takes around 1-2 hours (Table 6) but the time from reception until final validation takes 1-3 days (Table 8) suggests that the analysis time is not the rate limiting step in the process of FCI. The rate-limiting step is probably the (assistant) clinical biologist who needs to decide which panels need to be performed for every case before analysis can start, as well as interpret the data generated by the FCI analysis. Also FCI is not routinely performed on Saturdays and Sundays, which can lead to a long TAT for samples received on Friday afternoons.

Impact on cost

The NHL screen panel and BD OneFlow™ LST both analyse 12 cell markers, the payment of the RIZIV thus remains the same before and after the introduction of the BD OneFlow™ LST.

One marker can be charged to the RIZIV 555730/55741 (B500), the other eleven with 556474/556485 (B400). This results in a summed B-value of 4900. At the current coefficient (B = 0,033021), this equals 161.8 euros if performed for another centre (100% of B-value). When performed for outpatients, only 25% (40,5 euros) can be charged, supplemented by a maximum fixed cost ("forfeit") of 37.9 euros (since sum of B-values >3500), resulting in a total of 78.4 euros. When the patient is hospitalized in the form of day hospital: a fixed cost of 56.05 euros (F15) is added, adding up to a total of 134.5 euros. For hospitalized patients, only the 25% (40.5 euros) can be charged, the rest of the financing happens through the intake forfeit and a forfeit per day, however this is shared with all other laboratory tests performed for that patient that day [24].

Hands-on time (hourly rate for laboratory technician) won't differ much, as discussed earlier, so the difference in cost comes down to the reagent costs.

Table 9: theoretical reagent cost for the different analysis, as well as the difference between the BD OneFlow™ LST period and the two screen NHL periods

	Screen NHL panel		
	sIgB	TBNK	**BD Oneflow™ LST**
Reagent cost per analysis (excl. tax)	+/- 20 euros	+/- 20 euros	+/- 60 euros

Table 9 provides an overview of the theoretical reagent cost for the different analyses, based on the catalog price.

Since the reimbursement did not change and technician working time is approximately equal, a consequence of the BD OneFlow™ LST tube is an incremental laboratory cost for CLPD screening.

Impact on the laboratory: literature review and conclusion

The BD Oneflow™ LST tube contains 12 different monoclonal antibodies. Pipetting of all these antibodies individually would be time consuming and prone to operational mistakes, e.g. accidental omission or switching of an antibody. If detected in time, this leads to wasted antibodies and a waste of time (since a rerun is often necessary), increasing cost [25]. If not detected, this can lead to a potential misinterpretation of patient samples, possibly with clinical impact [26]. The BD Oneflow™ LST is a commercially available tube containing all antibodies in a dried format, requiring only the sample to be added.

For this project, it was not possible to quantitatively asses the number of mistakes avoided with the implementation of the BD Oneflow™ LST, since no queryable logging system exists for this parameter. However, based on our own experience, mistakes are estimated to happen at least once every one or two weeks for manually pipetted tubes. Other studies estimated the proportion of mistakes while pipetting antibodies at 1-4% of samples [25].

Dry, ready-to-use tubes (such as the BD Oneflow™ LST) thus clearly have an advantage over manual pipetting in terms of avoided mistakes, possibly increasing efficiency and quality [25]. Another advantage is reduced staining variability between different samples due to a longer stability of dried versus liquid reagent mixes, generating more robust data. This increased reproducibility can, according to some studies, allow for better detection of small changes in the expression levels of individual markers and immunophenotypic profiles [25].

Regarding of comparability, many studies have shown excellent agreement between dried and liquid reagents [14,25–27]. With the implementation of the BD Oneflow™ LST tube, an in-house validation study was also performed to confirm this, showing good correlations between the screen NHL panel and BD Oneflow™ LST tube. A similar validation was performed to compare results obtained with the BD Oneflow™ LST tube performed on the FACS Canto II system compared to the FACS Lyric flow cytometer, with similar results.

Another advantage of the BD Oneflow™ LST tube is the standardized approach, creating interlaboratory comparable data and reducing subjectivity. This allows for multicentric studies and development of software databases that can aid in the identification of cell populations. E.g. a locally acquired data file can be compared with EuroFlow databases to assist in identifying pathologic populations and ease their classification into specific disease entities [2,11]. This is already possible today using Infinicyt™ software.

In conclusion, the disadvantages of the BD Oneflow™ LST compared to the previously used screen NHL panel are a higher cost and longer analysis time. The advantages are a more convenient workflow which is less prone to mistakes, possibly resulting in an increased quality of results. The hands-on time is probably about equal. The TAT increased, but it is unclear whether this has to do with the implementation of the BD OneFlow™ LST rather than other factors. Finally, an increased standardization offers several other advantages, from interlaboratory comparability to development of smarter software tools.

Question 3: What is the (possible) clinical impact of the implementation of the BD Oneflow™ LST? Is there an increased/decreased detection of NHL?

Background

In order to assess the clinical impact of the BD OneFlow™ LST tube, first an analysis of the total number of FCIs was made. This was followed by determining the number of tubes related to CLPD. Eventually, the scope of this

CAT was to investigate the screen NHL panel and BD OneFlow™ LST tube, since these are the tubes used in CLPD screening setting. Other analyses were excluded (Figure 3).

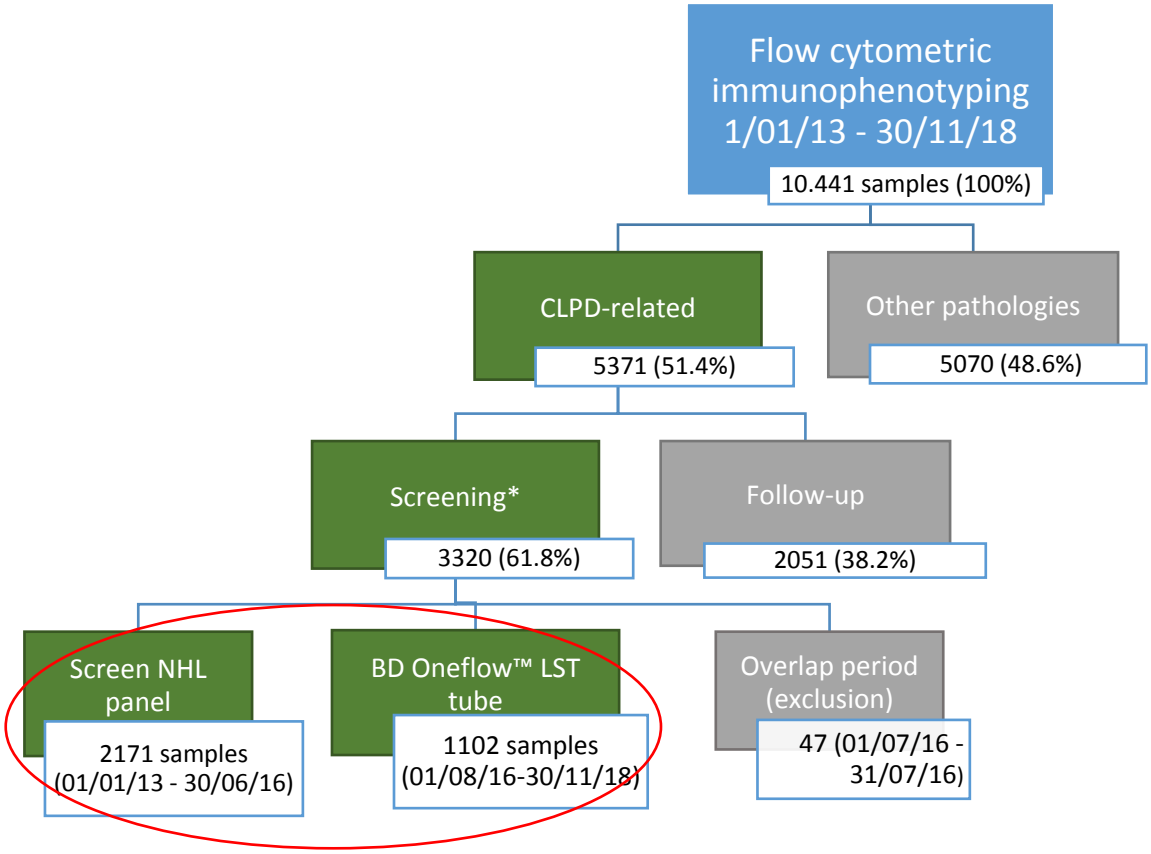


*Figure 3: the red oval delineates the study groups and periods on which this study focused. The samples in the grey boxes were excluded from analysis. CLPD: chronic lymphoproliferative disorder. *without specific information*

Total number of flowcytometric immunophenotypings performed

In the period 01/01/2013 – 30/11/2018 (5,9 years), 10.441 FCIs were performed. Most of these concerned bone marrow (BM) and peripheral blood (PB) samples, 51% and 43% respectively. 6% of the analyses were performed on body fluid samples (cerebrospinal, pleural, ascites). The average number of FCIs performed per month was 145.4 (SD 17.9), this is graphically depicted in Figure 4. This figure shows that the total number of flows stays approximately constant over time, with a slight increase in the number of analyses performed on body fluids over the years.

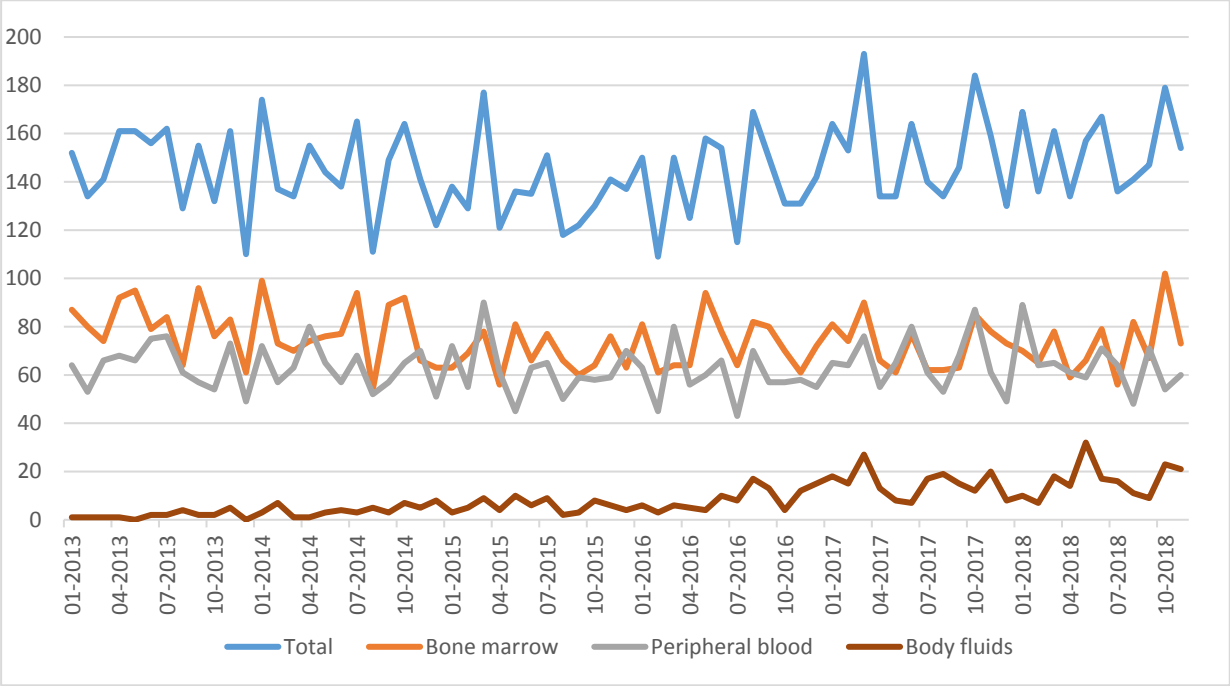


Figure 4: average number of flow cytometric immunophenotypings per month (all tubes and panels), different sample types.

Number of immunophenotypings performed regarding chronic lymphoproliferative disorders

51.4% of all the FCI analyses (n= 5371) were related to the screening and follow-up of CLPD (Figure 3). This corresponds to approximately 70.5 analyses per month (SD 14.0) A graph was constructed of the number of screen NHL panels (sIgB plus TBNK), BD OneFlow™ LST, single sIgB and single TBNK tubes (used in follow-up of CLPD) per month (
Figure 5), since these are the four principal tubes used regarding CLPD.
Figure 5 shows that the total of these four panels (green line) remains approximately constant over time. The number of screen NHL panels however, suddenly drops right before January 2015, after which this number is again approximately constant over time but at a lower average number of tubes per month, compared to the period before January 2015. In July 2016, the switch between the screen NHL panel and the BD OneFlow™ LST tube is made.

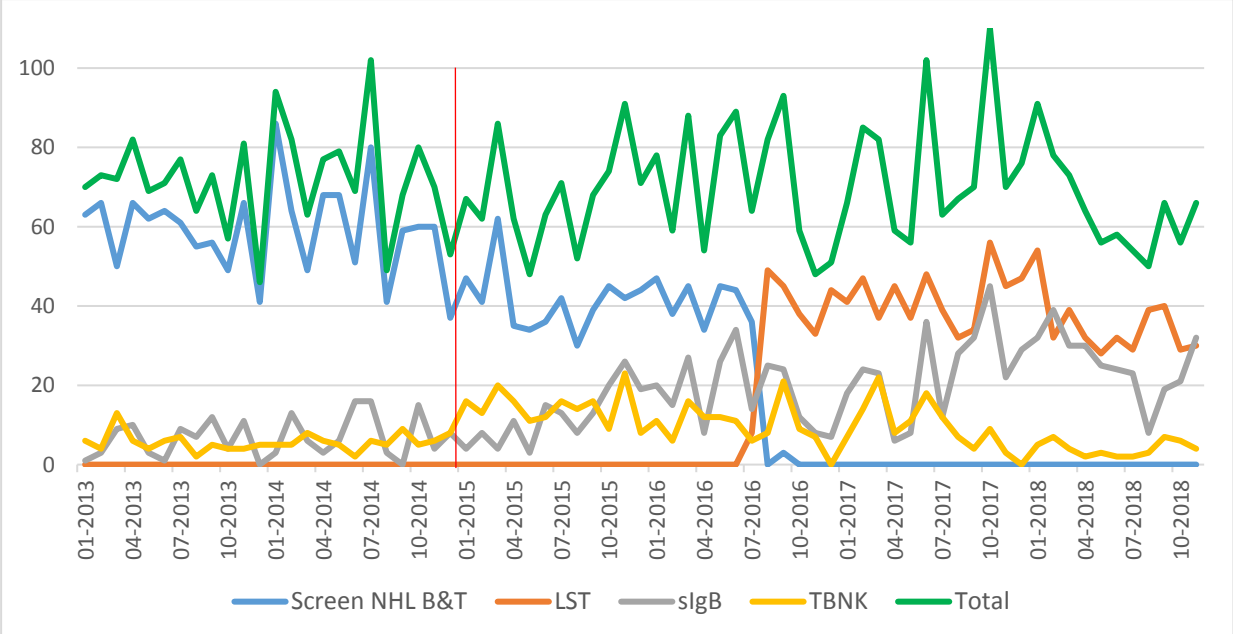


Figure 5: number of chronic lymphoproliferative disorder tubes used per month (screen NHL panels (TBNK plus sIgB), BD OneFlow™ LST and single TBNK/sIgB tubes). Screening and follow-up samples are both shown in this graph. The red line shows the point at which the number of screen NHL panels suddenly decreases, after which it is again stable at a lower number of analyses performed per month.

The number and proportion of screen NHL panels performed per month is different between the period 01/2013-12/2014 and 01/2015-06/2016 ($p<0.01$). The number and proportion of BD OneFlow™ LST tubes was comparable to the number and proportion of screen NHL panels between 01/2015 – 06/2018 ($p = 0,3$), but not with the entire

study period of the NHL panel (01/2013 – 06/2016, p <0.001). Therefore, screen NHL period was split into two periods which were assessed separately. A first screen NHL period dating from 01/2013 to 12/2014 and a second dating from 01/2015 to 06/2016. This is visually and numerically depicted in Figure 6 and Table 10, respectively. The sudden drop in screen NHL panels can probably be attributed to changes in intralaboratory guidelines for panel usage in different clinical situations, resulting in a more restricted use of this screening panel.

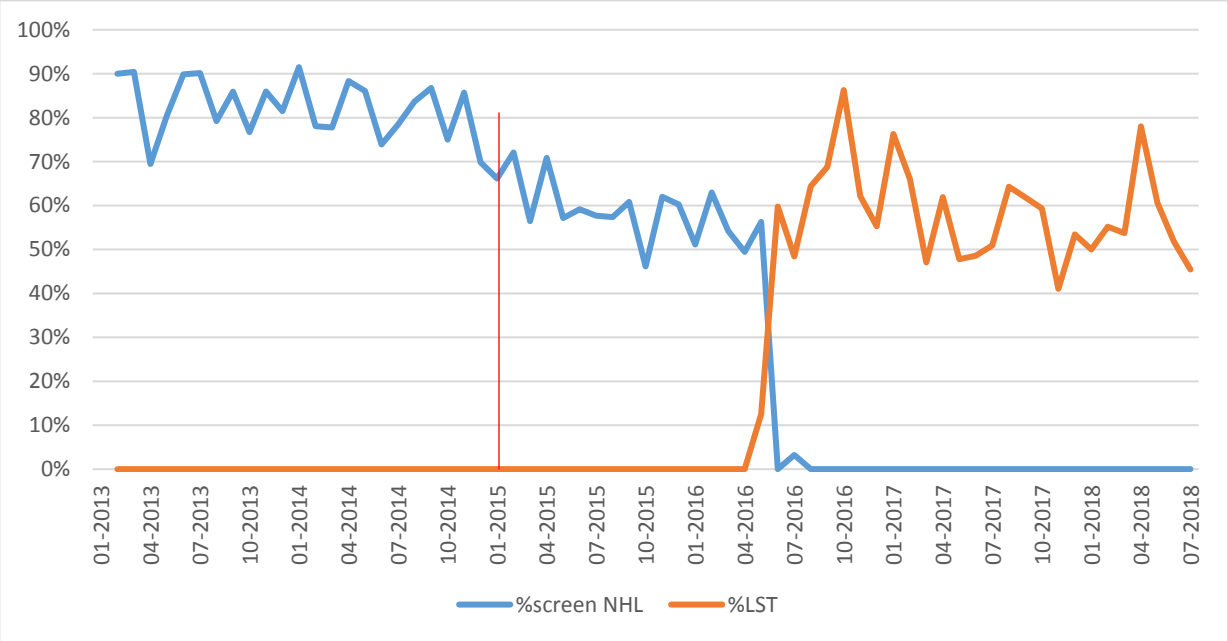


Figure 6: proportion of the screen NHL panel and BD Oneflow™ LSTs performed, calculated as % of all performed CLPD tubes (sIgB, TBNK, screen NHL and BD OneFlow™ LST). In January 2015 (red line), the proportion of screen NHL panels drops and then again remains approximately constant at a lower average.

Flowcytometric immunophenotyping for the screening of CLPD

In this study, we wanted to examine whether the BD OneFlow™ LST tube has an increased detection rate for CLPD compared to the former used screen NHL panel. Therefore, follow-up samples (n=2051) were excluded from analysis and 3320 diagnostic samples were further examined (Figure 3).

These could be split into three separate study periods. The NHL screen panel was used as primary screen for CLPD from 01/01/2013 to 30/06/2016 (42 months) and 2171 of these analyses were performed. This period was split into two periods, a first part from 01/01/2013 to 31/12/2014 (24 months, n=1421) and the second from 01/2015-06/2018 (18 months, n=750), based on the proportion of screening panels used compared to the total number of CLPD screens (cf. Figure 6, Table 10). The BD OneFlow™ LST period dated from 01/08/16 to 30/11/2018 (28 months) and comprised 1102 analyses (Figure 3). One month of overlap (July 2016) was excluded from analysis.

The characteristics of the study populations are presented in Table 10. As was suggested by Figure 5 and Figure 6, the proportion (and number) of screening tubes used in the first screen NHL period (before 01/01/2015) is significantly higher than the two subsequent periods. The proportion of external analysis increased significantly between the first screen NHL period and the BD OneFlow™ LST period (p<0.01).

Table 10: population characteristics of the three study periods. N/A: non-applicable, CLPD: chronic lymphoproliferative disorder, SD: standard deviation, CI: confidence interval, NS: non-significant at p = 0.01

	Screen NHL period 1	**Screen NHL period 2**	**BD OneFlow™ LST period**	**Difference (99% CI)**		
Time window	01/01/2013 - 31/12/2014 (24 months)	01/01/2015 – 30/06/2016 (18 months	01/08/2016- 30/11/2018 (28 months)	Screen NHL 1 vs 2	Screen NHL 1 vs LST	Screen NHL 2 vs LST
n (screening tubes)	1421	750	1102	N/A	N/A	N/A
Age (SD)	60.1 (18.4)	61.8 (18.2)	61.8 (18.4)	NS	NS	NS
Male / female	54.1% / 45.9%	54.4% / 45.6%	50.1% / 49.9%	NS	NS	NS
Total number of FCI/month (SD), all tubes	142.4 (17.6)	137.8 (16.3)	151.4 (18.2)	NS	NS	NS

Average CLPD tubes / month (SD)	71.7 (12.9)	70.3 (13.3)	69.7 (15.9)	NS	NS	NS
Average CLPD screening tubes per month	59.3 (11.5)	41.7 (6.9)	39.3 (7.8)	17.6 (10.2 – 25.0)	20 (12.9-27.1)	NS
Proportion of CLPD tubes	82.7%	59.9%	57.6%	23% (19.0-27.6)	26% (22.4-29.8)	NS
Proportion external analyses	39.8%	45.1%	49.8%	NS	10% (5,0 – 15,2)	NS

Estimation of detection rate

As the number of BD OneFlow™ LST tubes performed were not the same as the number of screen NHL tubes, it is difficult to interpret absolute numbers of detected diseases. Therefore, the proportion of detected diseases were examined.

To classify the different disease entities detected with the screening tubes, the conclusions of the FCI protocols were interpreted and classified according to specific criteria. Simplified criteria are depicted in Table 11. The exact words and phrases that were looked for are shown in Attachments 3&4.

Table 11: simplified criteria used for classification of conclusions of flowcytometric immunophenotyping reports. NHL: non-Hodgkin lymphoma.

Normal	**Reactive**	**Dubious**	**B-NHL**	**MBL**	**T-NHL**	**Other**
No evidence for monoclonal B- ,T- , or NK-pathology.	Disturbed CD4/CD8 ratio without significant aberrant phenotype AND/OR Increased number of NK cells AND/OR Weaker expression CD7 on T-cells without abnormalities of other markers	Disturbed kappa/lambda ratio AND/OR Markedly disturbed CD4/CD8 ratio AND/OR Minimal invasion not excludable AND/OR Weaker expression of CD5 on T-cells without abnormalities of other markers (except weaker CD7 expression)	Monoclonal B-cells >5.000/µL	Monoclonal B-cells <5.000/µL	Evidence for the presence of a T-NHL	Blasts OR Non reliable interpretation OR Plasmacells

To reduce manual work, two excel formulas were written to recognize words or combinations of words in the conclusion protocols (Attachment 3). This allowed for automatic classification of 91.6% (4920/5371) of the conclusions. The remaining 8.4% (451/5371) were classified manually. The following categories were also reviewed manually after automatic classification: “other”, “reactive”, “dubious”, “probably normal”, “MBL”, “B-NHL” and “T-NHL”.

Since the gold standard for lymphoma diagnosis is a combination of clinical information, morphology, histologic and genetic information we cannot determine a real sensitivity of our test, since a contingency table cannot be made

[28] . However, we make the assumption that the incidence of lymphomas won't drastically change in a couple of years, and since the number of FCIs remained approximately constant (cf. Figure 4,
Figure 5 and Table 10) and the patient characteristics were comparable (Table 10), we can (to some extent) attribute changes in the proportions of detected diseases to the implementation of the BD OneFlow™ LST tube.

Proportions were compared using z-test statistics (since n>30).

Table 12: proportions of detected diseases during the different study periods. NHL: non-Hodgkin lymphoma, MBL: monoclonal B-lymphocytosis, CI: confidence interval, NS: non-significant at p = 0.01 level.

	Screen NHL period 1	**Screen NHL period 2**	**BD OneFlow™ LST period**	**Difference (99% CI)**		
Time window	01/01/2013 - 31/12/2014 (24 months)	01/01/2015 – 30/06/2016 (18 months	01/08/2016-30/11/2018 (28 months)	Screen NHL 1 vs 2	Screen NHL 1 vs LST	Screen NHL 2 vs LST
Normal	65.2%	57.2%	56.9%	8.0% (2.3-13.6)	8.3% (3.2-13.3)	NS
B-NHL	17.8%	20.5%	15.1%	NS	NS	5.5% (0.7-10.2)
MBL	11.4%	14.3%	20.0%	NS	8.6% (4.8-12.4)	5.7% (1.2-10.2)
Reactive	1.3%	3.9%	3.0%	1.9% (0.6-4.5)	1.7% (0.1-3.2)	NS
Dubious	2.1%	1.1%	2.4%	NS	NS	NS
T-NHL	1.8%	1.9%	2.0%	NS	NS	NS
Other	0.4%	1.2%	0.7%	NS	NS	NS

A significant decrease (around 8%) in the number of "Normal" samples between the first screen NHL period and the two succeeding periods can be noted. This could be explained by the decrease in the number (and proportion) of screen NHL panels used between these periods (Figure 6, Table 10). Indeed, when a screening tube is used more selectively, less "Normal" results are expected, since the pre-test probability increases.
With the implementation of the BD OneFlow™ LST, there was a significant increase in the proportion of detected MBLs of 7.5% and 5.6% compared to the screen NHL period 1 and 2, respectively. However, the proportion of detected B-NHL decreased around 5% compared to the second screen NHL period, the exact meaning of this is unclear. A marginal increase (around 2%) is noted in the number of samples classified as "Reactive".

Estimation of clinical impact

Monoclonal B-cell lymphocytosis

In this study we observed a significantly higher detection of MBLs with the introduction of the BD OneFlow™ LST. However, the detection of a MBL often leads to counselling difficulties since the clinical meaning of this entity is not always clear. Therefore, a short overview of MBL is presented here.

The diagnostic criteria for CLL have changed considerably in the last 20 years. In 2008, the entity of monoclonal B-cell lymphocytosis (MBL) was recognized by the WHO 2008 classification. In the most recent edition (2016), MBL is defined as the presence of a monoclonal B-cell population, with an absolute count <5.000 cells/µL. Additionally, the patient should not have any detectable clinical signs of lymphoproliferative disorder, e.g. lymphadenopathy, organomegaly and cytopenias. MBL is classified in different categories based on the immunophenotype. Three categories are defined: CLL phenotype, atypical CLL phenotype and non-CLL type (Table 13). The CLL phenotype is the most common variant, accounting for approximately 75% of all MBL cases [1].

*Table 13: diagnostic criteria for monoclonal B-cell lymphocytosis (MBL), chronic lymphocytic leukaemia (CLL) and other B-cell non-Hodgkin lymphomas (B-NHL).*the Catovsky score is based on the expression patterns of the monoclonal population regarding following antigens: CD5 (one point if positive), surface immunoglobulin light chain (one point if weakly expressed), CD23 (one point if positive), FMC7 (one point if negative) and CD79b (one point if weak/negative expression). Adapted from [1] and [29].*

	Low-count MBL			High-count MBL			CLL		Non-CLL B-NHL
	Non-CLL	Atypical CLL	CLL	Non-CLL	Atypical CLL	CLL	Atypical	Typical	
Concentration of monoclonal B-cells	<500/µL (Median 1/µL[29]) AND No clinical symptoms indicative for lymphoproliferative disorder			500-5.000/µL (Median 2.900/µL[29]) AND No clinical symptoms indicative for lymphoproliferative disorder			>5.000/µL with or without clinical symptoms OR <5.000/µL with clinical symptoms		>5.000/µL with or without clinical symptoms OR <5.000/µL with clinical symptoms
Catovsky score*	0-2	3	4-5	0-2	3	4-5	3	4-5	0-2
Prevalence	3-12% (>20% if >60 years, up to 50-75% if >90 years [30])						0.04 - 0.05% [31]		Depends on subtype
Risk of progression to treatment requiring disease	Very rare			1-2% per year			Rai A: 5-70%, depending on risk factors		

The WHO classification distinguishes two entities of MBL, low-and high-count, based on a cut-off of 500 monoclonal B-cells per µL [1]. Other studies describe the low-count MBLs as "population screening MBL's" as these are usually detected in healthy persons during screenings with high-sensitive FCI. Most of these persons have monoclonal B-cell counts of <50/µL. Progression in these low-count MBLs is rare, and this entity can also disappear without treatment (10-55% over 3 years in one study, depending on the phenotype) [30]. The high-count MBLs however, do pose a risk of progression to treatment-requiring disease. The main predictive factor for this is the concentration of monoclonal B-cells in the peripheral blood. According to some studies, the concentration of monoclonal B-cells can be interpreted as a continuous variable, with a threefold risk increase for progression per increase in monoclonal B-cells concentration of 1000/µL [32]. Another factor associated with worse prognosis is a positive expression of CD38 and/or ZAP70 [1,32].

To estimate clinical impact of an increased detection of MBLs, it was also determined how many low-and high count MBLs were incrementally detected, with calculation of the p-value (using z-statistics) for the statistical differences between the different periods (Figure 7).

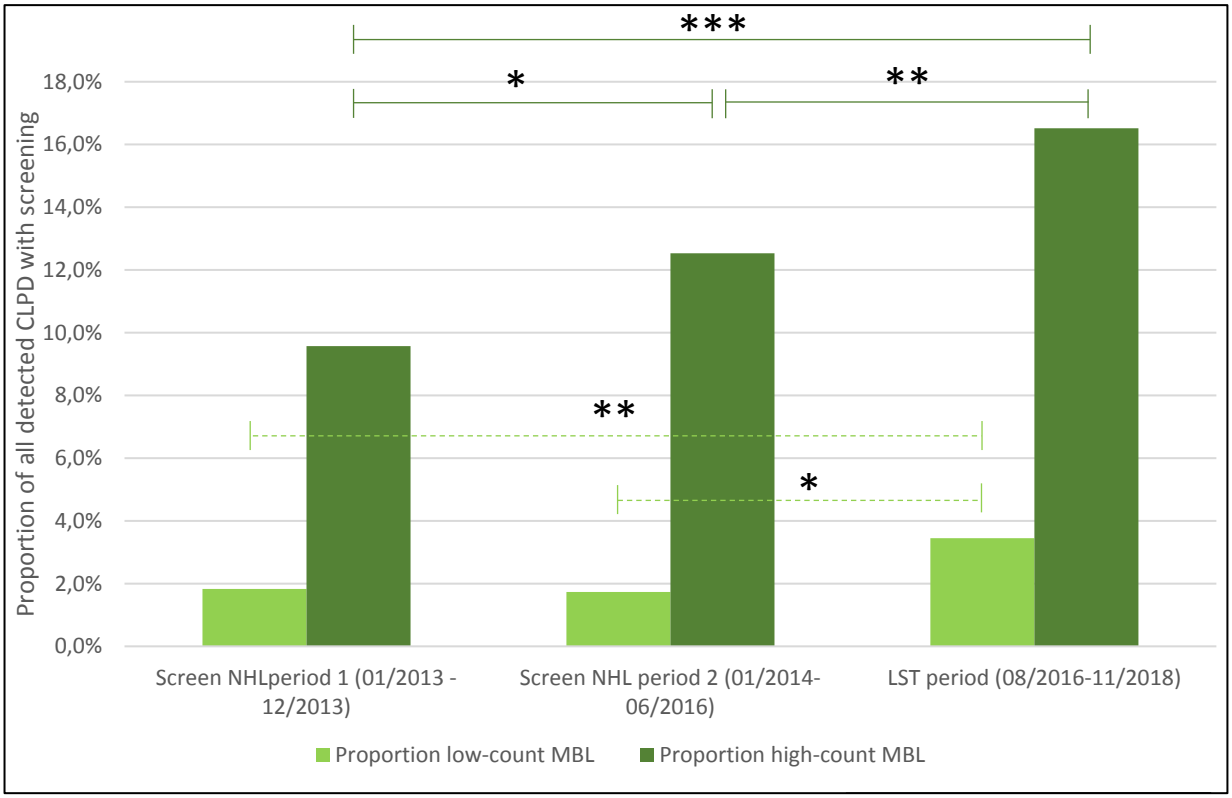


*Figure 7: proportion of monoclonal B-cell lymphocytosis (MBL) detected during the three study periods. The dashed light green lines compare the proportions of low-count MBLs, the solid dark green lines the proportions of high-count MBLs. CLPD: chronic lymphoproliferative disease *: p<0.05, ** p<0.01, *** p<0.001*

Estimation of clinical impact: conclusions

Figure 7 shows that the proportion of high and low-count MBLs detected increased significantly with the implementation of the BD OneFlow™. A higher detection of low-count MBLs could be explained by the higher number of events analysed with the BD OneFlow™ LST, allowing for a lower LOD and LOQ (limit of detection and quantification). However, low-count MBLs do not have clinical impact since these do not require clinical follow-up. The proportion of high-count MBLs also increased significantly, these do have clinical significance, as indicated in
Table 13. However, between the screen NHL 2 and BD OneFlow™ LST period, less B-NHLs were found (Table 12). This can lead to a hypothesis that in the BD OneFlow™ LST period, some samples that were classified as high-count MBL would have been classified as B-NHL with the screen NHL panel. This might be explained by extra washing steps, which could result in an extra loss of pathologic cells. This could lead to a reduced absolute number of monoclonal B-cells close below the cut-off for B-NHL, namely 5.000/µL.
Other possibilities to explain these findings are statistical coincidence (the border of the CI is close to zero), a change in the test request behaviour, or an actual drop in the incidence of B-NHL between these two periods.

COMMENTS

TO DO/ACTIONS

1) Further implementation of EuroFlow-recommended tubes, panels and software.

ATTACHMENTS

- Attachment 1: WHO 2016 classification of mature lymphoproliferative disorders.
- Attachment 2: example of an analysis using the BD OneFlow™ LST with the Euroflow LST template
- Attachment 3: details on the process of classifying the protocols into the 7 categories used in the text (Normal, B-NHL, T-NHL, MBL, reactive, dubious and others)
- Attachment 4: the words/phrases screened for in the conclusions of the FCI protocols to classify results into categories.
- Attachment 5: details regarding search strategy.

Attachement 1: WHO 2016 classification of mature lymphoproliferative disorders (adapted from [28])

Mature B-cell neoplasms	**Mature T and NK neoplasms**	**Hodgkin lymphoma**
Chronic lymphocytic leukemia/small lymphocytic lymphoma • Monoclonal B-cell lymphocytosis*	T-cell prolymphocytic leukemia	Nodular lymphocyte predominant Hodgkin lymphoma
	T-cell large granular lymphocytic leukemia	Classical Hodgkin lymphoma • Nodular sclerosis classical Hodgkin lymphoma • Lymphocyte-rich classical Hodgkin lymphoma • Mixed cellularity classical Hodgkin lymphoma • Lymphocyte-depleted classical Hodgkin lymphoma
B-cell prolymphocytic leukemia	Chronic lymphoproliferative disorder of NK cells	
Splenic marginal zone lymphoma	Aggressive NK-cell leukemia	
Hairy cell leukemia	Systemic EBV positive T-cell lymphoma of childhood*	
Splenic B-cell lymphoma/leukemia, unclassifiable • Splenic diffuse red pulp small B-cell lymphoma • Hairy cell leukemia-variant	Hydroa vacciniforme–like lymphoproliferative disorder*	
	Adult T-cell leukemia/lymphoma	**Immunodeficiency-associated lymphoproliferative disorders**
	Extranodal NK-/T-cell lymphoma, nasal type	Plasmacytic hyperplasia PTLD
Lymphoplasmacytic lymphoma	Enteropathy-associated T-cell lymphoma	Infectious mononucleosis PTLD
Waldenström macroglobulinemia	Monomorphic epitheliotropic intestinal T-cell lymphoma*	Florid follicular hyperplasia PTLD*
Monoclonal gammopathy of undetermined significance (MGUS), IgM*	Indolent T-cell lymphoproliferative disorder of the GI tract*	Polymorphic PTLD
Heavy-chain diseases • m heavy-chain disease • g heavy-chain disease • a heavy-chain disease	Hepatosplenic T-cell lymphoma	Monomorphic PTLD (B- and T-/NK-cell types)
	Subcutaneous panniculitis-like T-cell lymphoma	Classical Hodgkin lymphoma PTLD
	Mycosis fungoides	**Histiocytic and dendritic cell neoplasms**

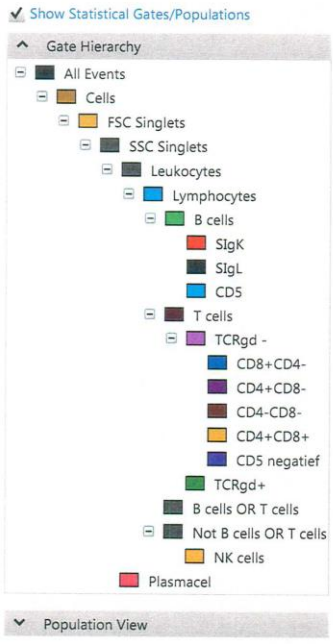
Plasma cell neoplasms
• Monoclonal gammopathy of undetermined significance (MGUS), IgG/A*
• Plasma cell myeloma
• Solitary plasmacytoma of bone
• Extraosseous plasmacytoma
• Monoclonal immunoglobulin deposition diseases*
• Plasma cell neoplasms with associated paraneoplastic syndrome
  ○ POEMS syndrome
  ○ TEMPI syndrome
	Sézary syndrome	Histiocytic sarcoma

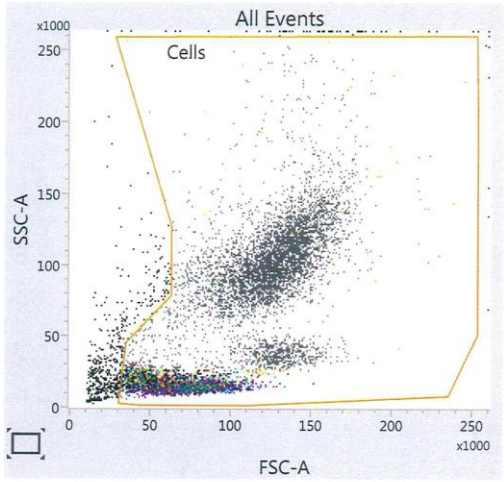
	Primary cutaneous CD301 T-cell lymphoproliferative disorders • Lymphomatoid papulosis • Primary cutaneous anaplastic large cell lymphoma	Langerhans cell histiocytosis
		Langerhans cell sarcoma
		Indeterminate dendritic cell tumor
	Primary cutaneous gd T-cell lymphoma	Interdigitating dendritic cell sarcoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)	Primary cutaneous CD81 aggressive epidermotropic cytotoxic T-cell lymphoma	Follicular dendritic cell sarcoma
	Primary cutaneous acral CD81 T-cell lymphoma*	Fibroblastic reticular cell tumor
Nodal marginal zone lymphoma • Pediatric nodal marginal zone lymphoma	Primary cutaneous CD41 small/medium T-cell lymphoproliferative disorder*	Disseminated juvenile xanthogranuloma
	Peripheral T-cell lymphoma, NOS	Erdheim-Chester disease*
Follicular lymphoma • Testicular follicular lymphoma • In situ follicular neoplasia*	Angioimmunoblastic T-cell lymphoma	
	Follicular T-cell lymphoma*	

• Duodenal-type follicular lymphoma* • Pediatric-type follicular lymphoma*	Nodal peripheral T-cell lymphoma with TFH phenotype*	
	Anaplastic large-cell lymphoma, ALK-positive	
Large B-cell lymphoma with IRF4 rearrangement*	Anaplastic large-cell lymphoma, ALK-negative*	
Primary cutaneous follicle center lymphoma	Breast implant–associated anaplastic large-cell lymphoma*	
Mantle cell lymphoma • Leukaemic non-nodal mantle cell lymphoma • In situ mantle cell neoplasia*		
Diffuse large B-cell lymphoma (DLBCL), NOS		
Germinal center B-cell type*		
Activated B-cell type*		
T-cell/histiocyte-rich large B-cell lymphoma		
Primary DLBCL of the central nervous system (CNS)		
Primary cutaneous DLBCL, leg type		
EBV1 DLBCL, NOS*		
EBV1 mucocutaneous ulcer*		
DLBCL associated with chronic inflammation		
Lymphomatoid granulomatosis		
Primary mediastinal (thymic) large B-cell lymphoma		
Intravascular large B-cell lymphoma		
ALK-positive large B-cell lymphoma		
Plasmablastic lymphoma		
Primary effusion lymphoma		
HHV8-associated lymphorliferative disorders		
Burkitt lymphoma		
Burkitt-like lymphoma with 11q aberration*		
High-grade B-cell lymphoma, • with MYC and BCL2 and/or BCL6 rearrangements* • High-grade B-cell lymphoma, NOS* • B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma		

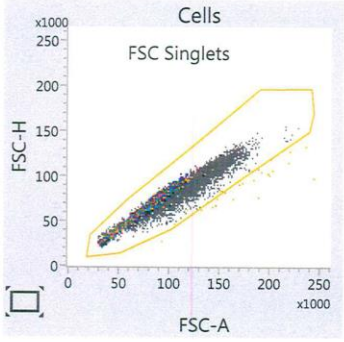
Attachment 2: example of an analysis using the BD OneFlow™ LST with the EuroFlow LST template

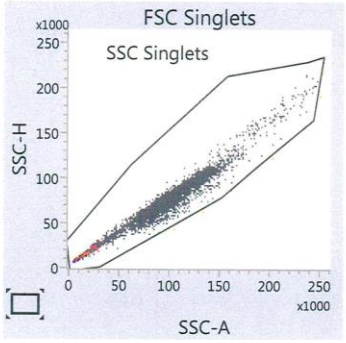
A

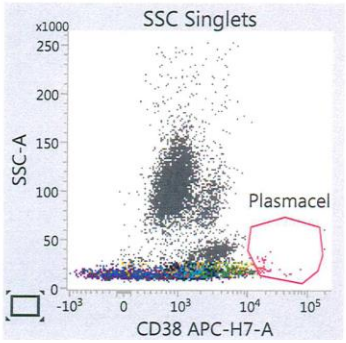




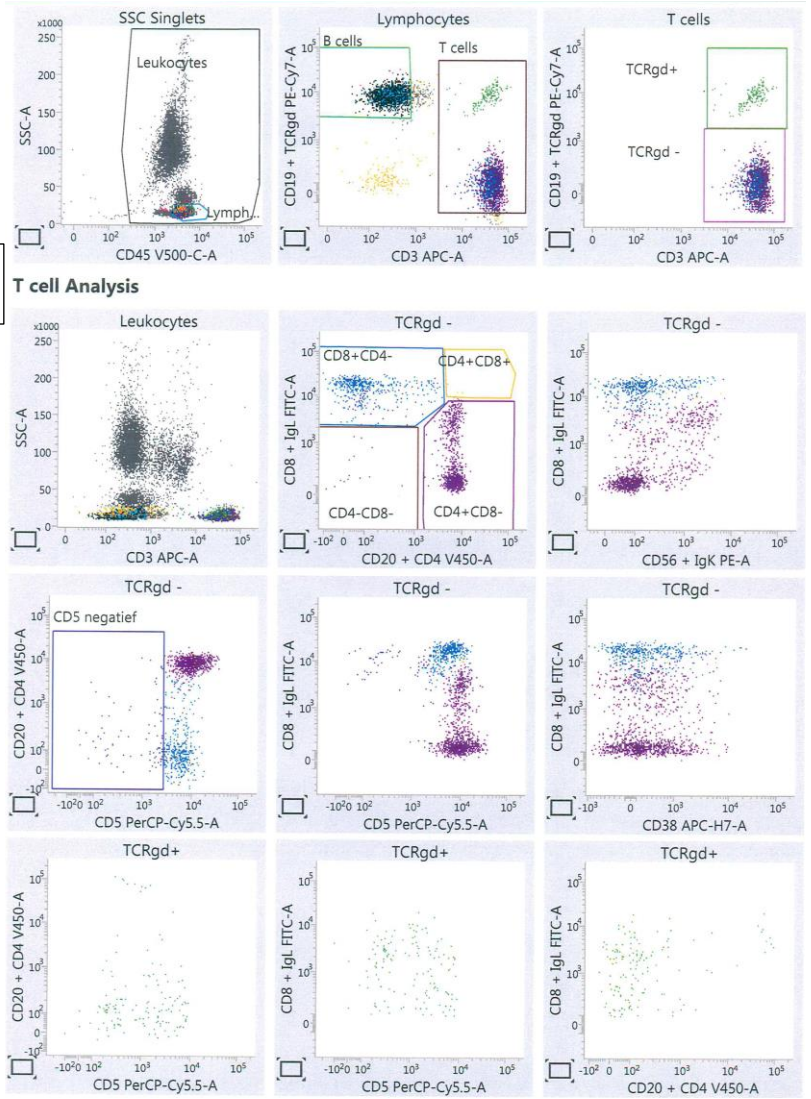
LymphoSum	**95.5**
Tcell Ratio	**2.3**
Bcell Ratio	**0.0**



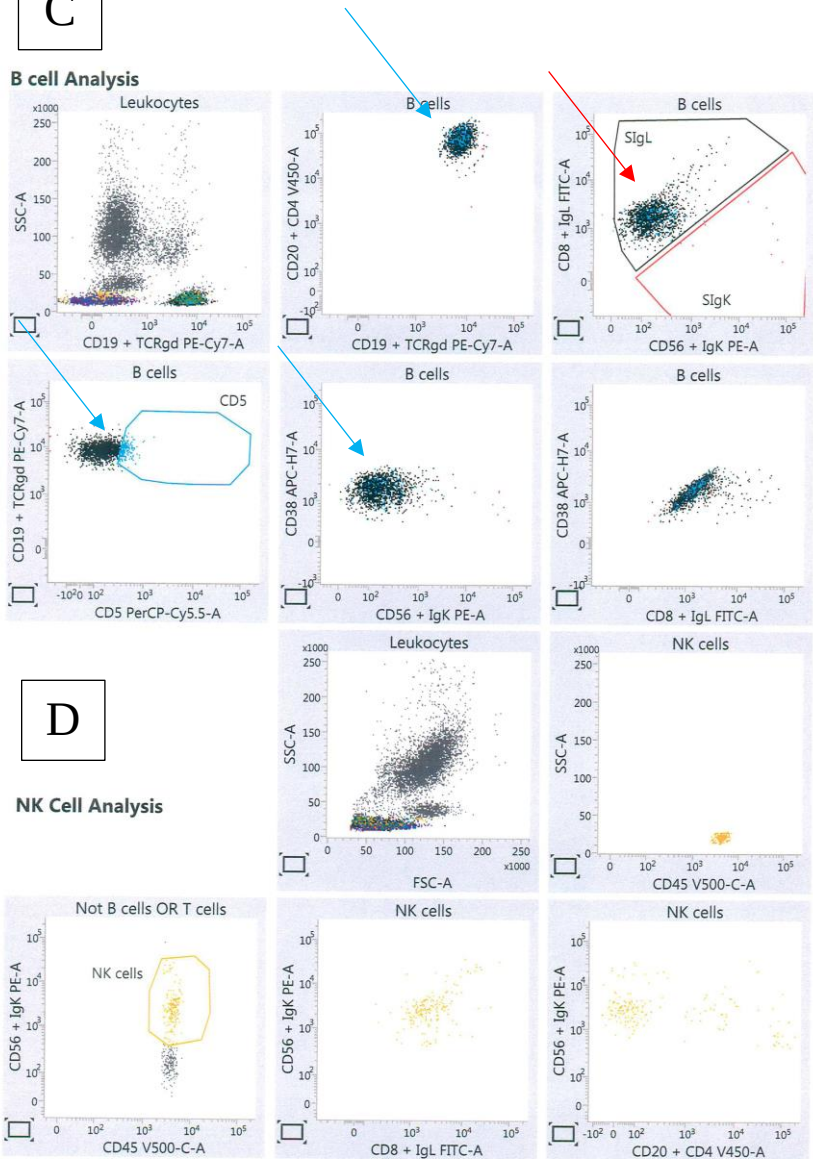




B



C



The first gating separates cells from debris and (non-lysed) red blood cells based on forward scatter (FSC) – sideward scatter (SSC) characteristics. Subsequently, doublets are filtered out using area and height characteristics of FSC and SSC. Within the remaining singlets, a leukocyte gate is defined on the basis of CD45 expression. Within this CD45-SSC plot, lymphocytes are delineated, usually well separated from other leukocyte populations (part A). The abnormal presence of a population of blasts can sometimes be observed, as a $CD45^{dim}$ population with low SSC. The lymphocytes are further investigated using a differential gating with backbone markers CD19 (B-cells), CD3 (T-cells) and non-CD19, non-CD3 (NK-cells). Then eight plots are dedicated to plots examining the T-cells (part B), five to the B-cells (part C) and three to the NK cells (part D). In this example, a surface immunoglobulin lambda restricted population is visible within the population of B-cells (red arrow). This population is CD20 normally positive and negative for CD5 and CD38 (blue arrows). This finding is suggestive for the presence of a B-non Hodgkin lymphoma.

Attachment 3: details on the process of classifying the protocols into the 7 categories used in the text (Normal, B-NHL, T-NHL, MBL, reactive, dubious and others).

Automatic classification

First excel formula

=IFERROR(IF(FIND("Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een monoclonaal";AA119);"Normaal");IF(FIND("**Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een monoclonale**";AA119);"Normaal"));IF(FIND("**Immunofenotypering toont geen argumenten voor de aanwezigheid van een residuele**";AA119);"Normaal (FU)"));IF(FIND("**onvoldoende evidentie voor een onderliggend monoclonaal**";AA119);"Normaal"));IF(FIND("**onvoldoende argumenten voor de aanwezigheid van een monoclonaal**";AA119);"Normaal"));IF(FIND("**Immunofenotypering toont een normale verdeling binnen de lymfocytenpopulatie met een normale CD4/CD8-verhouding**";AA119);"Normaal"));IF(FIND("**Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een residuele monoklonale**";AA119);"Normaal (FU)"));IF(FIND("**Immunofenotypering toont geen argumenten voor de aanwezigheid van een monoclonaal**";AA119);"Normaal"));IF(FIND("**onvoldoende argumenten voor een monoclonaal**";AA119);"Normaal"));IF(FIND("**Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een residuele monoclonale**";AA119);"Normaal (FU)"));IF(FIND("**toont de aanwezigheid van een monoclonale B-cel**";AA119);"B-NHL"));IF(FIND("**toont de aanwezigheid van een persisterende monoclonale B-cel**";AA119);"B-NHL(FU)"));IF(FIND("**toont de aanwezigheid van een monoclonale B**";AA119);"B-NHL"));IF(FIND("**toont de aanwezigheid van kleine een monoclonale B-cel**";AA119);"B-NHL"));IF(FIND("**toont de aanwezigheid van een kleine monoclonale B-cel**";AA119);"B-NHL"));IF(FIND("**Echter aanwezigheid van een monoclonale B-cel**";AA119);"B-NHL"));IF(FIND("**toont geen argumenten voor de aanwezigheid van een monoclonale B-cel**";AA119);"Normaal"));IF(FIND("**toont een persisterende monoclonale B-cel**";AA119);"B-NHL(FU)"));IF(FIND("**toont de blijvende aanwezigheid van een monoclonale B-cel**";AA119);"B-NHL(FU)"));IF(FIND("**toont de persisterende aanwezigheid van een aberrante CD3+ T-cel**";AA119);"T-NHL(FU)"));IF(FIND("**toont de persisterende aanwezigheid van een aberrante T-cel**";AA119);"T-NHL(FU)"));IF(FIND("**passend bij gekend T-cel lymfoom**";AA119);"T-NHL(FU)"));IF(FIND("**toont de persisterende aanwezigheid van de gekende aberrante T-cel**";AA119);"T-NHL(FU)"));IF(FIND("**passend bij het gekende T-cel lymfoom**";AA119);"T-NHL(FU)"));IF(FIND("**toont een monoclonale B-cel**";AA119);"B-NHL"));IF(FIND("**blasten**";AA119);"**blasten**"));IF(FIND("**toont de aanwezigheid van een monoclonale CD5+ B-cel**";AA119);"B-NHL"));IF(FIND("**onvoldoende argumenten voor de aanwezigheid van";AA119);"Normaal (wss**)"));IF(FIND**("toont een verlaagd aantal polyclonale";AA119);"Normaal**"));IF(FIND("**toont de aanwezigheid van een monoclonale T-cel";AA119);"T-NHL**"));IF(FIND("**compatibel met T-cel lymfoom**";AA119);"T-NHL"));IF(FIND("**toont de aanwezigheid van een residuele monoclonale B-cel**";AA119);"B-NHL(FU)"));IF(FIND("**sterk verdacht voor T-cel lymfoom**";AA119);"T-NHL"));IF(FIND("**toont de aanwezigheid van een kleine residuele pathologische populatie T**";AA119);"T-NHL(FU)"));IF(FIND("**MBL**";AA119);"MBL"));IF(FIND("**beeld best passend bij een B-NHL** ";AA119);"B-NHL"));IF(FIND("**zonder aantoonbare monoclonaliteit";AA119);"Normaal**"));IF(FIND("**reactioneel?**";AA119);"Reactioneel"));IF(FIND("**onvoldoende om met zekerheid**";AA119);"Dubieus";));IF(FIND("**toont de aanwezigheid van een aberrante T-cel**";AA119);"T-NHL"));IF(FIND("**Te volgen**";AA119);"Dubieus"));IF(FIND("**eactionele veranderingen?**";AA119);"Dubieus"));IF(FIND("**reactief?**";AA119);"Reactioneel"));IF(FIND("**normale verdeling van de lymfoide subpopulaties**";AA119);"Normaal"));IF(FIND("**passend bij gekend Sézary**";AA119);"T-NHL"));IF(FIND("**persisterende aanwezigheid van een monoclonale B-cel**";AA119);"B-NHL(FU)"));IF(FIND("**geen duidelijke aberrante T-cel**";AA119);"Normaal"));IF(FIND("**te volgen**";AA119);"Dubieus"));IF(FIND("**passend bij gekende CLL**";AA119);"B-NHL(FU)"));IF(FIND("**verdacht voor minimale invasie**";AA119);"Dubieus"))

Second excel formula

Because excel does not allow for more than 64 different levels of arguments, it was needed to construct a second formula.

=IFERROR(IF(FIND("**persisterende aberrante CD4+**";AA408);"T-NHL");IF(FIND("**normaal aantal T-LGL's**";AA408);"Normaal"));IF(FIND("**niet uit te sluiten**";AA408);"Dubieus"));IF(FIND("**hairy cellen (+/-**";AA408);"B-NHL"));IF(FIND("**passend bij het gekende B-NHL**";AA408);"B-NHL"));IF(FIND("**normale verdeling**";AA408);"Normaal"));IF(FIND("**normale verhouding";**AA408);"Normaal"));IF(FIND("**persisterende monoclonale CD5+**";AA408);"B-NHL"));IF(FIND("**onvoldoende evidentie voor de aanwezigheid**";AA408);"Normaal"));IF(FIND("**beeld kan passen bij**";AA408);"Dubieus"));IF(FIND("**kleine residuele monoclonale B-cel**";AA408);"B-NHL"));IF(FIND("**passend bij het gekende Sézary**";AA408);"T-NHL"));IF(FIND("**niet met zekerheid**";AA408);"Dubieus"));IF(FIND("**verdacht voor T-NHL**";AA408);"T-NHL"));IF(FIND("**toont een kleine aberrante T-cel**";AA408);"T-NHL"));IF(FIND("**toont de aanwezigheid van een monoklonale B-cel**";AA408);"B-NHL"));IF(FIND("**passend bij invasie door een B-NHL**";AA408);"B-NHL"));IF(FIND("**passend bij het gekende T-cel**";AA408);"T-NHL"));IF(FIND("**onvoldoende argumenten voor invasie door B-NHL**";AA408);"Normaal"));IF(FIND("**van een monoclonaal B- of T-cel**";AA408);"Normaal"));IF(FIND("**lambda clonale B-cel**";AA408);"B-NHL"));IF(FIND("**CD5+ monoclonale B-cel**";AA408);"B-NHL"));IF(FIND("**passend bij recidief B-CLL**";AA408);"B-NHL"));IF(FIND("**toont de blijvende aanwezigheid van een monoklonale B-cel**";AA408);"B-NHL"));IF(FIND("**een populatie monoklonale B-lymfocyten (+/-**";AA408);"B-NHL"));IF(FIND("**overwicht aan sIglambda**";AA408);"Dubieus"));IF(FIND("**monoclonale B-celpopulatie (+/-**";AA408);"B-NHL"));IF(FIND("**niet te bevestigen of uit te sluiten**";AA408);"Dubieus"));IF(FIND("**sterk verdacht voor de aanwezigheid van een onderliggend monoclonaal T-cel**";AA408);"T-NHL"));IF(FIND("**monoclonale CD5+ B-cel**";AA408);"B-NHL"));IF(FIND("**passend bij de gekende NK-pathologie**";AA408);"T-NHL"));IF(FIND("**geen argumenten voor de aanwezigheid van**";AA408);"Normaal"));IF(FIND("**geen argumenten voor een residuele**";AA408);"Normaal"));IF(FIND("**Geen argumenten voor een monoclonaal B- of T-cel**";AA408);"Normaal"));IF(FIND("**sterk verdacht voor een onderliggend monoclonaal T-cel**";AA408);"T-NHL"));IF(FIND("**passend bij de gekende B-CLL**";AA408);"B-NHL"));IF(FIND("**kleine monoclonale B-celpopulatie**";AA408);"B-NHL"));IF(FIND("**Sézary**";AA408);"T-NHL"));IF(FIND("**monoclonale B-celpopulatie (**";AA408);"B-NHL"));IF(FIND("**beeld passend bij een B-NHL**";AA408);"B-NHL"));IF(FIND("**twee monoclonale B-cel populaties**";AA408);"B-NHL"));IF(FIND("**biclonale**";AA408);"B-NHL"));IF(FIND("**sterk verdacht voor een onderliggende T-cel**";AA408);"T-NHL"));IF(FIND("quasi geen B";AA408);"Normaal"));IF(FIND("**toont de aanwezigheid van een persisterende aberrante T-cel**";AA408);"T-NHL"));IF(FIND("passend bij invasie door het gekende T-cel";AA408);"T-NHL"));IF(FIND("**toont de aanwezigheid van een persisterende monoclonale T-cel**";AA408);"T-NHL"));IF(FIND("**compatibel met een monoclonaal T-cel**";AA408);"T-NHL"));IF(FIND("**sterk suggestief voor een B-NHL**";AA408);"B-NHL"));IF(FIND("**passend bij invasie door gekend T-cel**";AA408);"T-NHL"));IF(FIND("**toont de aanwezigheid van een populatie hairy cellen**";AA408);"B-NHL"));IF(FIND("**toont onvoldoende argumenten**";AA408);"Normaal"));IF(FIND("**monoclonale B-cellymfocytose**";AA408);"MBL"));IF(FIND("**sézary**";AA408);"T-NHL"));IF(FIND("**lymfocytensubsets**";AA408);"Normaal"));IF(FIND("**passen bij het gekende T-NHL**";AA408);"T-NHL"));IF(FIND("**passend bij een T-NHL**";AA408);"T-NHL"));IF(FIND("**CLL**";AA408);"B-NHL"));IF(FIND("**toont de aanwezigheid van een monoclonaal B-cel**";AA408);"B-NHL"));IF(FIND("**noch uit te sluiten**";AA408);"Dubieus"));IF(FIND("**polyklonaal**";AA408);"Normaal"));IF(FIND("**sterk suggestief voor een T-**";AA408);"T-NHL"));IF(FIND("**CD5+ monoklonale B-cel**";AA408);"B-NHL"));IF(FIND("**nbetrouwb**";AA408);"Onbetrouwbaar"))

Manual classification

With the two formulas mentioned above 91.6% (4920/5371) were classified automatically. The remaining 8.4% (451/5371) were classified manually.

After this automatic classification, several categories were reviewed manually to make sure correct classification took place. The categories “reactive” en “dubious” were checked together with prof. Boeckx.

The following rules were used to classify these results.

- Increased number of NK-cells: reactive unless >2000/µL
- (Mildly) disturbed CD4/CD8: reactive
- Weaker CD7 expression without loss other markers: reactive

- Weaker CD5 expression T-cells: dubious

Attachement 4: the words/phrases screened for in the conclusions of the FCI protocols to classify results into categories using the formulas in Attachment 3.

Normaal	B-NHL	T-NHL	Blasten	MBL	Dubieus	Reactioneel	Onbetrouwbaar
Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een monoclona(a)l(e)	toont de aanwezigheid van een monoc/klonale B-cel	toont de persisterende aanwezigheid van een aberrante CD3+ T-cel	blasten	MBL	Onvoldoende om met zekerheid	reactief?	nbetrouwb
Immunofenotypering toont geen argumenten voor de aanwezigheid van een residuele	toont de aanwezigheid van een persisterende monoclonale B-cel	toont de persisterende aanwezigheid van een aberrante T-cel		monoclonale B-cellymfocytose	Te volgen		
onvoldoende evidentie voor een onderliggend monoclonaal	toont de aanwezigheid van kleine een monoclonale B-cel	passend bij gekend T-cel lymfoom			eactionele veranderingen?		
onvoldoende argumenten voor de aanwezigheid van een monoclonaal	Echter aanwezigheid van een monoclonale B-cel	toont de persisterende aanwezigheid van de gekende aberrante T-cel			te volgen		
Immunofenotypering toont een normale verdeling binnen de lymfocytenpopulatie met een normale CD4/CD8-verhouding	toont een persisterende monoclonale B-cel	passend bij het gekende T-cel lymfoom			verdacht voor minimale invasie		
Immunofenotypering toont onvoldoende argumenten voor de aanwezigheid van een residuele monoklonale	toont de blijvende aanwezigheid van een monoclonale B-cel	toont de aanwezigheid van een monoclonale T-cel			niet uit te sluiten		
toont geen argumenten voor de aanwezigheid van een monoclonale B-cel	toont een monoclonale B-cel	compatibel met T-cel lymfoom			beeld kan passen bij		
onvoldoende argumenten voor de aanwezigheid van	toont de aanwezigheid van een monoclonale CD5+ B-cel	sterk verdacht voor T-cel lymfoom			niet met zekerheid		
toont een verlaagd aantal polyclonale	toont de aanwezigheid van een residuele monoclonale B-cel	toont de aanwezigheid van een kleine residuele pathologische populatie T			overwicht aan slglambda		
zonder aantoonbare monoclonaliteit	beeld best passend bij een B-NHL	toont de aanwezigheid van een aberrante T-cel			niet te bevestigen of uit te sluiten		

reactioneel?	persisterende aanwezigheid van een monoclonale B-cel	passend bij gekend Sézary			noch uit te sluiten		
normale verdeling van de lymfoide subpopulaties	passend bij gekende CLL	persisterende aberrante CD4+					
geen duidelijke aberrante T-cel	hairy cellen (+/-	passend bij het gekende Sézary					
normaal aantal T-LGL's	passend bij het gekende B-NHL	verdacht voor T-NHL					
normale verdeling	persisterende monoclonale CD5+	toont een kleine aberrante T-cel					
normale verhouding	kleine residuele monoclonale B-cel	passend bij het gekende T-cel					
onvoldoende evidentie voor de aanwezigheid	toont de aanwezigheid van een monoklonale B-cel	sterk verdacht voor de aanwezigheid van een onderliggend monoclonaal T-cel					
onvoldoende argumenten voor invasie door B-NHL	passend bij invasie door een B-NHL	passend bij de gekende NK-pathologie					
van een monoclonaal B- of T-cel	lambda clonale B-cel	sterk verdacht voor een onderliggend monoclonaal T-ce					
geen argumenten voor de aanwezigheid van	CD5+ mono(c/k)lonale B-cel	Sézary					
geen argumenten voor een residuele	toont de blijvende aanwezigheid van een monoklonale B-cel	sterk verdacht voor een onderliggende T-cel					
Geen argumenten voor een monoclonaal B- of T-cel	passend bij recidief B-CLL	toont de aanwezigheid van een persisterende aberrante T-cel					
toont onvoldoende argumenten	een populatie monoklonale B-lymfocyten (+/-	toont de aanwezigheid van een persisterende monoclonale T-cel					
lymfocytensubsets	monoclonale CD5+ B-cel	compatibel met een monoclonaal T-cel					
polyklonaal	monoclonale B-celpopulatie (+/-	passend bij invasie door gekend T-cel					
	passend bij de gekende B-CLL	sézary					
	monoclonale B-celpopulatie (	passen bij het gekende T-NHL					
	beeld passend bij een B-NHL	passend bij een T-NHL					
	twee monoclonale B-cel populaties	sterk suggestief voor een T-					
	biclonale						
	sterk suggestief voor een B-NHL						
	toont de aanwezigheid van een populatie hairy cellen						
	CLL						
	toont de aanwezigheid van een monoclonaal B-cel						

Attachment 5: details regarding search strategy.

1) *MeSH Database (PubMed): MeSH term: ""Immunophenotyping"[Mesh]", "Lymphoproliferative Disorders"[Mesh], "Lymphoma"[Mesh]*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
    a. *"Immunophenotyping"[Mesh]) AND "Lymphoproliferative Disorders"[Mesh]: 1 systematic review, not suitable for this study.*
    b. *"Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening: 8 systematic reviews, none suitable for this study.*
    c. *"Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening: 217 systematic reviews, 3 selected for abstract review, none suitable to answer the questions asked in this study (1 used for background information: Accuracy of flow cytometry and cytomorphology for the diagnosis of meningeal involvement in lymphoid neoplasms: A systematic review)*
    d. *"Lymphoma/diagnosis"[Mesh]: 74 systematic reviews: non suitable for this study.*
3) *Databases*
    a. *Pubmed (Medline; from 1966)*
        i. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh]: 7755 results → narrowing search*
        ii. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND screening: 3583 results → narrowing search*
        iii. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND LST: 1 result (Establishment of harmonization in immunophenotyping: A comparative study of a standardized one-tube lymphocyte-screening panel.)*
        iv. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND lymphocyte screening panel: 108 results: 1 duplicate (cf. above), 10 studies selected for abstract review, 4 retained (Novel lymphocyte screening tube using dried monoclonal antibody reagents, Quality assessment program for EuroFlow protocols: summary results of four-year (2010-2013) quality assurance rounds., Recommendations of the SFH (French Society of Haematology) for the diagnosis, treatment and follow-up of hairy cell leukaemia., Immunophenotyping of selected hematologic disorders–focus on lymphoproliferative disorders with more than one malignant cell population*
        v. *"Immunophenotyping"[Mesh] AND "Lymphoproliferative Disorders"[Mesh] AND screening AND guideline: 36 results, 1 new article retained*
        vi. *"Lymphoma/diagnosis"[Mesh]) AND screening AND flow cytometric AND immunophenotyping: 136 results, 3 retained (Flow Cytometry of B-Cell Neoplasms., Flow Cytometry of T cells and T-cell Neoplasms, Flow cytometry and its use in the diagnosis and management of mature lymphoid malignancies.)*
        vii. *"lst tube": 25 results, 1 retained (Diagnostic utility of the lymphoid screening tube supplemented with CD34 for Ogata score calculation in patients with peripheral cytopenia), 4 duplicates*
        viii. *Similar articles section and referred articles: EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols., Flow cytometric assessment of T-cell chronic lymphoproliferative disorders., Screening bone marrow samples for abnormal lymphoid populations and myelodysplasia-related features with one 10-color 14-antibody screening tube., Immunophenotyping of selected hematologic disorders focus on lymphoproliferative disorders with more than one malignant cell population, Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms., Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10*
    b. *SUMSearch (http://sumsearch.uthscsa.edu/)*
        i. *"non-hodgkin lymphoma AND flowcytometric" – diagnosis, human only: 9 results, none suitable*
        ii. *"non-hodgkin lymphoma" – diagnosis, human only: 10 guidelines found, 3 selected for abstract review, 1 retained (2 others: no acces to full text) (2006 Bethesda International ConsensusRecommendations on the ImmunophenotypicAnalysis of Hematolymphoid Neoplasiaby Flow Cytometry: Optimal Reagentsand Reporting for the Flow CytometricDiagnosis of Hematopoietic Neoplasia)*
    c. *National Guideline Clearinghouse (http://www.ngc.org/) – not used*
    d. *Institute for Clinical Systems Improvement (http://www.icsi.org) – not used*
    e. *The National Institute for Clinical Excellence (http://www.nice.org.uk/)*
        i. *"Lymphoma AND diagnosis": 65 results, 1 guideline retained: Non-Hodgkin's lymphoma: diagnosis and management*
    f. *Cochrane (http://www.update-software.com/cochrane*

i. *"Lymphoma": 48 cochrane reviews, none suitable for this analysis*
ii. *"immunophenotyping AND lymphoma": 0 cochrane reviews, 138 trials, 1 retained (An approach to diagnosis of T-cell lymphoproliferative disorders by flow cytometry)*

g. *Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm) – not used*

4) *Guidelines clinical laboratory medicine*
   a. *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/) – nothing found with terms "non-Hodgkin lymphoma", "immunophenotyping", "flowcytometric"*
   b. *International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp) – nothing found with term "Lymphoma"*
   c. *Westgard QC (http://www.westgard.com) – not applicable*
   d. *Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/) – not used*

5) *UpToDate Online version 12.2 (2004)*
   a. *"non-Hodgkin lymphoma": 6 relevant pages: Clinical presentation and diagnosis of non-Hodgkin lymphoma, Overview of non-Hodgkin lymphoma in children and adolescents, Evaluation, staging, and response assessment of non-Hodgkin lymphoma, Overview of the pathobiology of the non-Hodgkin lymphomas, Approach to the adult with lymphocytosis or lymphocytopenia, Approach to the child with lymphocytosis or lymphocytopenia*

References classified as type

Guidelines and Recommendations (most recent topics on top))

   a. *Greig, B., Oldaker, T., Warzynski, M. & Wood, B. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: Recommendations for training and education to perform clinical flow cytometry. Cytom. Part B Clin. Cytom. 72B, S23–S33 (2007).*
   b. *Bain, B. J., Barnett, D., Linch, D., Matutes, E. & Reilly, J. T. Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. Clin. Lab. Haematol. 24, 1–13 (2002).*
   c. Davis, B. 2006 Bethesda International Consensus Recommendations on the Flow Cytometric Immunophenotypic Analysis of Hematolymphoid Neoplasia: Medical Indications. *Cytom. Part B ...* **85**, 77–85 (2007).
   d. Johansson, U. *et al.* Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms. *Br. J. Haematol.* **165**, 455–488 (2014).
   e. *Kern, W. et al. Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. Leukemia 25, 567–574 (2011).*

2) *Systematic Reviews and Meta-analyses*
   a. *None available*

3) *Reviews*

   a. Kroft, S. H. & Harrington, A. M. Flow Cytometry of B-Cell Neoplasms. *Clin. Lab. Med.* **37**, 697–723 (2017)
   b. Craig, J. W. & Dorfman, D. M. Flow Cytometry of T cells and T-cell Neoplasms. *Clin. Lab. Med.* **37**, 725–751 (2017).
   c. Seegmiller, A. C., Hsi, E. D. & Craig, F. E. The current role of clinical flow cytometry in the evaluation of mature B-cell neoplasms. *Cytometry B. Clin. Cytom.* **29**, 20–29 (2018).
   d. Craig, F. E. & Foon, K. A. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* **111**, 3941–3967 (2008).
   e. de Tute, R. M. Flow cytometry and its use in the diagnosis and management of mature lymphoid malignancies. *Histopathology* **58**, 90–105 (2011).

4) *Original Articles*

   a. Van Dongen, J. J. M. *et al.* EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia* **26**, 1908–1975 (2012).
   b. Kalina, T. *et al.* Frequent issues and lessons learned from EuroFlow QA. *J. Immunol. Methods* 0–1 (2018). doi:10.1016/j.jim.2018.09.008
   c. Kalina, T. *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* **26**, 1986–2010 (2012).
   d. van der Velden, V. H. J. *et al.* Optimization and testing of dried antibody tube: The EuroFlow LST and PIDOT tubes as examples. *J. Immunol. Methods* (2017). doi:10.1016/j.jim.2017.03.011
   e. Kalina, T. *et al.* Quality assessment program for EuroFlow protocols: Summary results of four-year (2010-2013) quality assurance rounds. *Cytom. Part A* **87**, 145–156 (2015).
   f. Preijers, F. W. M. B., Huys, E., Favre, C. & Moshaver, B. Establishment of harmonization in immunophenotyping: A comparative study of a standardized one-tube lymphocyte-screening panel. *Cytom. Part B - Clin. Cytom.* **86**, 418–425 (2014).
   g. Hedley, B. D., Keeney, M., Popma, J. & Chin-Yee, I. Novel lymphocyte screening tube using dried monoclonal antibody reagents. *Cytom. Part B - Clin. Cytom.* **88**, 361–370 (2015).
   h. Rajab, A. & Porwit, A. Screening bone marrow samples for abnormal lymphoid populations and

myelodysplasia-related features with one 10-color 14-antibody screening tube. *Cytom. Part B - Clin. Cytom.* **88**, 253–260 (2015).

i. Glier, H. *et al*. Standardization of 8-color flow cytometry across different flow cytometer instruments: A feasibility study in clinical laboratories in Switzerland. *J. Immunol. Methods* 0–1 (2017). doi:10.1016/j.jim.2017.07.013

j. Muyldermans, A., Florin, L., Devos, H., Cauwelier, B. & Emmerechts, J. Diagnostic utility of the lymphoid screening tube supplemented with CD34 for Ogata score calculation in patients with peripheral cytopenia. *Hematology* **8454**, (2018).

k. The Non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. Blood 89, 3909–18 (1997).

l. Shanafelt, T. D. et al. Brief report: Natural history of individuals with clinically recognized monoclonal B-cell lymphocytosis compared with patients with Rai 0 chronic lymphocytic leukemia. J. Clin. Oncol. 27, 3959–3963 (2009).

m. Call, T. G. et al. Incidence of Chronic Lymphocytic Leukemia and High Count Monoclonal B-cell Lymphocytosis using the 2008 Guidelines. Cancer 120, 2000–2005 (2014).

n. Lauria, F. et al. Differentiating chronic lymphocytic leukemia from monoclonal B-lymphocytosis according to clinical outcome: on behalf of the GIMEMA chronic lymphoproliferative diseases working group. Haematologica 96, 277–283 (2010).

5) Reference Works, Handbooks and Databases

a. The International Agency for Research on Cancer. *WHO classification of tumours of haematopoietic and lymphoid tissues*. (2016).

6) Posters, "grey literature", presentations

a. Davids, M. Approach to the adult with lymphocytosis or lymphocytopenia - UpToDate. Available at: https://www.uptodate.com/contents/approach-to-the-adult-with-lymphocytosis-or-lymphocytopenia?search=lymphocytosis&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1. (Accessed: 31st January 2019)

b. Freedman, A., Friedberg, J. & Aster, J. Clinical presentation and diagnosis of non-Hodgkin lymphoma - UpToDate. (2018). Available at: https://www.uptodate.com/contents/clinical-presentation-and-diagnosis-of-non-hodgkin-lymphoma?search=clinical presentation and diagnosis of non-hodgkin lymphoma&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1. (Accessed: 31st January 2019)

c. Jose, S., Road, P. & Laoghaire, D. BD OneFlow ™ LST 20 tests per kit — Catalog No . 658619. (2014).

d. Biosciences, B. D. BD OneFlow ™ LST Application Guide. (2014).

e. Belgian Cancer Registry — Tabellen op jaarbasis. Available at: https://kankerregister.org/Statistieken_tabellen_jaarbasis. (Accessed: 5th March 2019)

f. NomenSoft - RIZIV. Available at:https://www.riziv.fgov.be/nl/toepassingen/Paginas/NomenSoft.aspx. (Accessed: 19th March 2019)