Universitat de Girona
Facultat de Medicina

VALIDATION OF A 10-COLOUR FLOW CYTOMETRY PANEL COMPARED TO BONE MARROW BIOPSY TO ASSESS BONE MARROW INFILTRATION IN FOLLICULAR LYMPHOMA

A 5-year retrospective study

FINAL DEGREE PROJECT

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Vull agrair a la meva tutora clínica Dra. Natàlia Lloveras la seva ajuda i implicació en aquest projecte,

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INDEX OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

1. ABBREVIATIONS

BM	Bone marrow
BMA	Bone marrow aspirate
BMB	Bone marrow biopsy
BNLI	British National Lymphoma Investigation
CHOP	Cyclophosphamide, doxorubicin, vincristine, and prednisone
ChT	Chemotherapy
CT	Computed Tomography
CVP	Cyclophosphamide, vincristine, and prednisone
FC	Flow cytometry
FL	Follicular lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
GELF	Groupe d'Étude des Lymphomes Folliculaires
HL	Hodgkin lymphoma
HSCs	Hematopoietic stem cells
Ig	Immunoglobulin
LDH	Lactate dehydrogenase
LN	Lymph node
MHC	Major histocompatibility complex
NHL	Non-Hodgkin's lymphoma
OS	Overall survival
PET-CT	Positron Emission Tomography-Computed Tomography
PFS	Progression free survival
RT	Radiotherapy
TCR	T-cell receptor
WHO	World Health Organization

2. ABSTRACT

BACKGROUND:

Follicular lymphoma (FL) is the most prevalent indolent non-Hodgkin's lymphoma. It is formed by clonal germinal centre B-cells (GCB), cells with a specific immunochemistry. When FL reaches bone marrow, patients are staged in IV Ann-Arbor stage, and this can change the therapeutical behaviour. Bone marrow involvement is mainly detected in histologic slides of bone marrow biopsy, complemented by immunohistochemistry. In most cases, these cells can be detected by flow cytometry with specific monoclonal antibodies.

Flow cytometry is a quick, sensitive technique, easily performed on bone marrow aspirate cells. In the last 6-8 years it has evolved with better instruments and techniques used routinely, with very high sensitivity, and the ability to analyse up to 10-12 parametres in a single experiment. This technique can give a numerical proportion of pathologic B-cell vs normal mature B-cell or leukocytes of bone marrow, as well as it could give quantitative information of tumour load burden.

OBJECTIVE:

To compare the capacity of a systematized analysis of 10-colour flow cytometry (FC) panel compared to histology (Gold Standard) to detect bone marrow involvement in patients with follicular lymphoma diagnosed in the province of Girona between 2017-2021.

METHODS:

The design is a transversal retrospective study. Staging tests of 138 patients with FL diagnosed in Girona between 2017-2021 will be systematically revised to analyse the validity of bone marrow aspirate flow cytometry compared to the Gold Standard, bone marrow histology. Inadequate samples will be identified and excluded from the analysis.

KEYWORDS:

B-cell, follicular lymphoma, flow cytometry, bone marrow aspirate, Ann-Arbor stage IV, immunophenotype.

3. INTRODUCTION

3.1. DEFINITION

Follicular lymphoma is a tumour derived from germinal centre B-cells. It is a non-Hodgkin's lymphoma, and it is considered indolent.

Lymphomas are a heterogeneous group of cancers of the lymphatic system, and they can be divided in B-cell lymphomas, T-cell lymphomas, and NK-cell lymphomas. Lymphocytes can suffer an oncologic event during its division, and they can get out of control. In normal conditions, lymphocytes divide and die in an equilibrium, but when they have acquired some mutations the balance is broken, and they divide faster than they can be eliminated. In lymphomas, lymphocytes are not only abnormal in number, but also not normal themselves (1).

Lymphocytes are blood cells, and they are part of the human immune system, specifically the lymphatic system. They all come from the division of the same cell in the bone marrow, the lymphoblast, and then they have a maturation process in different lymph organs.

Lymphatic system is distributed throughout the body, and it engages a network of lymphatic vessels, organs, and tissues. The lymphatic system can be present in almost all parts of the body.

There are 2 principal types of cells in lymphatic tissues: B-lymphocytes and T-lymphocytes, and there are 2 types of **lymphatic organs**:

- **Primary**: bone marrow and thymus.
- **Secondary**: lymph nodes, spleen and lymphoid tissue associated to mucous membranes.

In the bone marrow there are hematopoietic stem cells (HSCs) to generate erythrocytes, leukocytes, and platelets. The HSCs can be divided in common myeloid progenitors, which will produce cells belonging to the innate immune system, or in common lymphoid progenitors, which will generate lymphocytes, which are the main cells of the

adaptative immune system. Lymphoid progenitors can be divided into T-cell precursors or into B-cell precursors. Each of them will develop in different organs to become mature cells (2).

3.2. CLASSIFICATION

Lymphomas are tumours of mature lymphoid cells whose principal origin is in lymph nodes. Considering the origin of the neoplastic cell, the World Health Organization (WHO) classify this type of tumours in **mature B-cell neoplasms**, mature T and NK neoplasms, Hodgkin lymphoma, posttransplant lymphoproliferative disorders and histiocytic and dendritic cell neoplasms.

An old classification is the one that divides lymphomas into Hodgkin lymphomas (LH) and non-Hodgkin's lymphomas (LNH). This division is based on the presence of Reed-Sternberg cells (CRS). Hodgkin lymphomas are composed of this CRS surrounded by inflammatory cells. **Non-Hodgkin's lymphomas** encompass a heterogeneous group of tumours. Depending on the type of the abnormal lymphocytes, we can distinguish B-LNH and T-LNH. Depending on their aggressivity, they can be divided into indolent, aggressive, and highly aggressive lymphomas *(figure 1)*. Aggressive lymphomas have an increased rate of proliferation, and indolent lymphomas have a decrease in apoptosis.

The term **mature B-cell neoplasms** includes all neoplasms of the B-lymphatic system, and it includes B-NHL and others, like chronic lymphocytic leukaemia/B-cell small lymphocytic lymphoma (different manifestations of the same neoplasm), and multiple myeloma (3,4).

- Indolent lymphomas: this type of lymphoma grows and spreads slower than the other types. Patients' survival is long, and they can have a good quality of life even without a specific treatment. These types of tumours can be transformed into an aggressive type of lymphoma. Indolent B-cell lymphomas encompass nodal marginal zone B-cell lymphoma, MALT-lymphoma, and follicular lymphoma, and indolent T-cell lymphomas include mycosis fungoides.

- Aggressive lymphomas: they are biologically more aggressive than indolent lymphomas and they can be lethal. Aggressive B-cell lymphomas encompass mantle cell lymphoma and diffuse large B-cell lymphoma, and aggressive T-cell lymphomas include peripheral T-cell lymphoma and anaplastic large cell lymphoma.
- Highly aggressive lymphomas: this type of lymphoma is the most aggressive, and it can be quickly lethal without an adequate treatment. Highly aggressive B-cell lymphomas include Burkitt lymphoma.

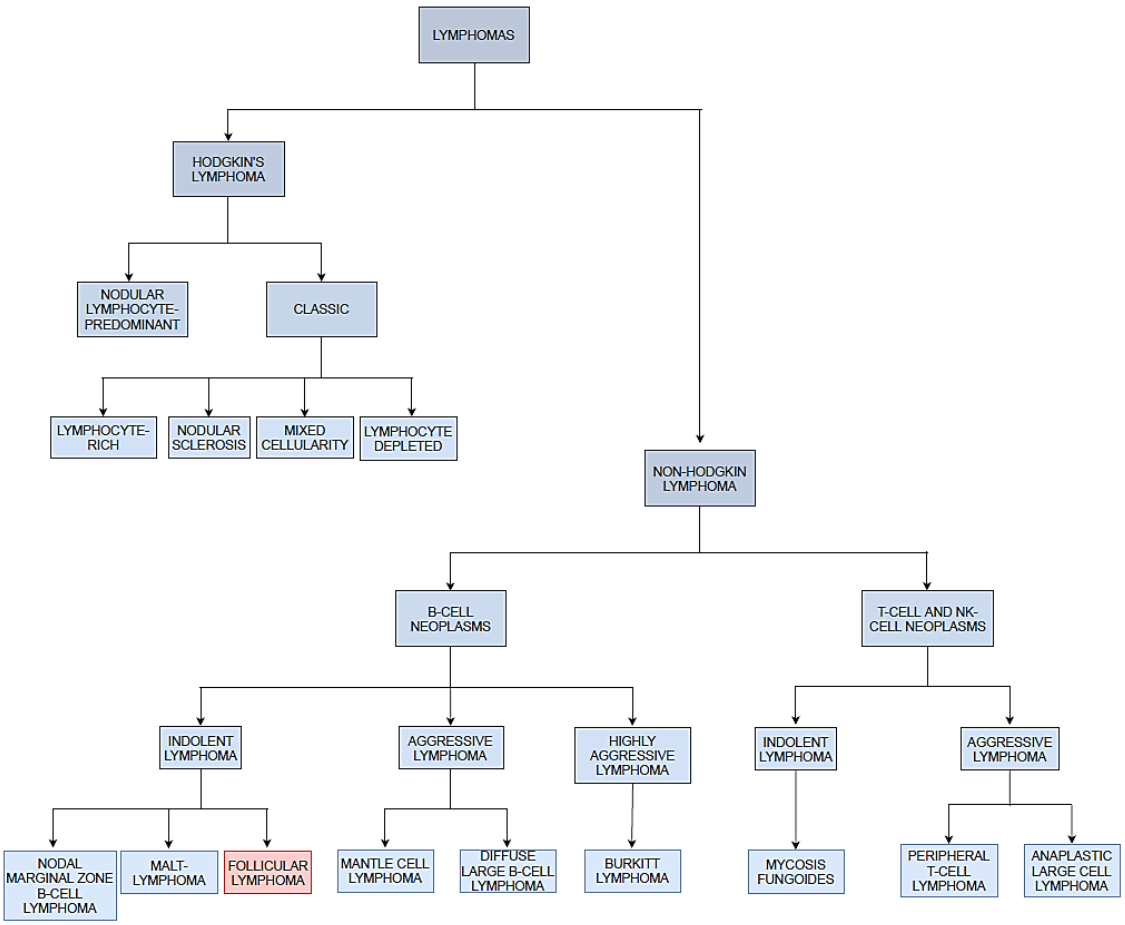

Figure 1. Lymphomas classification

3.3. EPIDEMIOLOGY

Follicular lymphoma is a subtype of non-Hodgkin's lymphoma that develops from B-cells of the germinal centre in the lymph nodes. Follicular lymphoma is the second most common type of NHL after the diffuse large B-cell lymphoma, and it is the fourth most

common type of mature B-cell neoplasms (*figure 2*). It represents 25% of all NHL and is the most common type of indolent lymphomas.

The number of patients diagnosed with FL each year has increased from 2-3/100.000 people in 1950 to 5-7/100.000 people presently. Diagnosed people are 60-65 years old on average, and it is slightly more prevalent in women, although some studies consider men and women are equally affected (5).

In Girona, between 2002 and 2013 287 FL were diagnosed, with a median age of 62 years. Global incidence of FL observed in Girona was of 3,45 cases/100.000 person-year; the incidence in women was of 3,69 (95% CI: 3,1-4,27), and in men 3,21 (95% CI: 2,67-3,76), a statistically insignificant difference (6).

In another study it was noticed that survival of FL in Girona is lower than the survival of FL reported by Surveillance, Epidemiology, and End Results Program (SEER) (7).

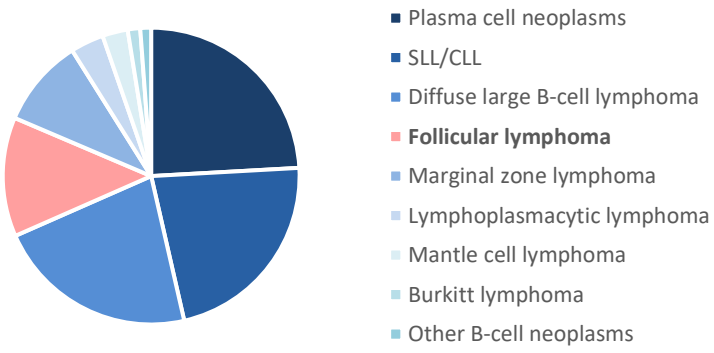

Figure 2. Number of diagnoses of mature B-cell neoplasms in Girona during 2002-2013 period. From (7)

3.4. RISK FACTORS

Nowadays, many FL risk factors have been identified, but the aetiology of FL is still poorly understood (8–10):

- Age: the incidence of FL increases with age, and it is very rare in young people.

- Gender: some studies have concluded that women have more risk to develop FL, unlike most NHL subtypes, that have a higher male sex ratio.
- Ethnicity: the incidence of FL is higher in Caucasian than in African-American.
- Diet: some studies have suggested an inverse relationship between linoleic acid, a polyunsaturated fatty acid, and NHL risk. It has been proved that vitamin D has a protective effect.
- Alcohol: it induces cancer in humans, and it also produces an immunosuppression, so it is an important etiological factor for lymphoid malignancies. Despite this fact, other studies have concluded that alcohol drinkers have a lower risk of developing FL.
- Tobacco: it has been also linked with the development of FL, as well as not to other type of lymphomas, but studies have discrepant results. Its risk is more important in women, and it is more related to duration than frequency of smoked cigarettes.
- Infections: some viruses have been related with the development of FL, such as Epstein-Barr virus (EBV), hepatitis C virus (HCV), human immunodeficiency virus type 1 (HIV-1), human T-cell lymphotropic virus type I (HTLV-1), and the herpesvirus associated with Kaposi sarcoma (KSHV). The one which has been related with more evidence with FL is hepatitis C virus.
- Environmental exposures: some types of chemicals, like those used to kill insects or hair dyes, can be related with the development of FL, but more research is needed to be able to secure this relationship.
- Congenital and acquired immunodeficiencies: solid organ transplantation, for example, includes an induction phase of immunosuppression.
- Autoimmunity: some studies have associated some autoimmune disorders with FL, such as Sjogren's syndrome, rheumatoid arthritis, autoimmune haemolytic anaemia, and aplastic anaemia.

3.5. CLINICAL PRESENTATION

Clinical presentation of follicular lymphoma is heterogeneous, and it is different according to the histologic grade and the extension of the tumour. The majority of cases have an indolent and slowly progressive clinical course, with long median survival, good response to initial treatment and a continuous pattern of recurrences and remissions.

Some common clinical characteristics of FL are the following (11,12):

- Asymptomatic: even in advanced stages.
- Peripheral lymphadenopathy: lymphatic node enlargement is one of the most frequent initial clinical findings. Two-thirds of patients with NHL present at least one lymphadenopathy. Lymphadenopathies are frequently painless, firm, and asymmetrically distributed, and they usually have a slow growth.
- Splenomegaly: LF can cause an abnormal enlargement of this organ. It appears in approximately 50% of the patients.
- Cytopenias: bone marrow is the most frequently affected extranodal site. If the lymphoma involves bone marrow, it can be asymptomatic or with symptoms derived from cytopenias.
    - Fatigue: patients can feel exhausted for no reason. It can be a symptom of anaemia.
    - Infections: due to the deficient immune system, patients with lymphoma can suffer more infections.
    - Spontaneous bleeding: they can be observed if there is a replacement of normal bone marrow by cancer cells, when the number of thrombocytes can be reduced. Like the previous symptoms, if the patient has spontaneous bleeding, we may think about a possible bone marrow affection.

- B-symptoms: they are fever for unknown reason, drenching night sweats and unwanted weight loss of more than 10% of body weight over 6 months. When a patient has this clinical presentation, we must suspect a transformation of the lymphoma to a more aggressive lymphoma.
- Other extranodal presentation: the affection of extranodal organs other than the bone marrow is very rare in FL.

3.6. PHYSIOPATHOLOGY

3.6.1. B-CELL DEVELOPMENT

To understand FL and NHL physiopathology, it is important to study how B-cell lymphocytes develop. Their development begins in the bone marrow, a primary lymphoid organ, where the B-cell precursor matures into a naive B-cell. This cell leaves the bone marrow circulating in the blood and goes to secondary lymphoid organs, where the second phase will take place.

Primary lymphoid organs

B-cells are derived from **hematopoietic stem cells** (HSC), and they first maturate in the bone marrow. Hematopoietic stem cells can be divided into **common lymphoid progenitor** and common myeloid progenitor, and B-cells are derived from the first type. Then, these cells become **pro-B cells**, and Ig heavy-chain gene rearrangement takes place in this phase. During rearrangements of Ig chains it takes a process called V(D)J recombination (V, D and J are gene segments) (13).

If it is successful, Ig heavy-chain is expressed in the cytoplasm, when B-cells will be named **pre-B cells**. Then, Ig light-chain rearrangement takes place, and it is also expressed, and pre-B cells become **immature B-cells** with Ig molecules on their surface: it is the B-cell antigen receptor (BCR).

Finally, after a last step of maturation, **naive B-cells** migrate to secondary lymphoid tissues. Naive B-cells express BCRs, which can recognise specific antigens.

Clusters of differentiation (CD) are surface molecules expressed on cells of the immune system, and they play key roles in immune cell-cell communication and sensing the environment. During their maturation B-cells go through different phases in which they acquire and lose the expression of several CDs. Early-stage B-cells express CD34, and these cells can express CD38 and CD10 too. Intermediate B-cells express CD38 but not CD34 and either CD20. A beginning of the loss of CD10 can be observed. Late-stage B-cells express CD20 and CD38, and CD10 is almost lost. Mature B-cells are fully CD20 but

lack CD38 and CD10. During these phases, CD45 has progressively more and more expression (*figure 3*) (14).

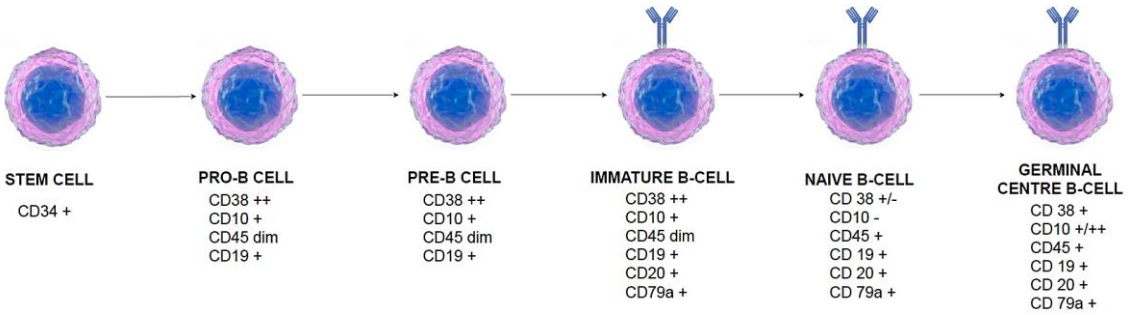

Figure 3. Immunophenotype of B-cells during their maturation

In summary, in bone marrow B-cells precursors proliferate and maturate, and they become immature and surface Ig-expressing B lymphocytes (*Annex I. Figure 10*). Cells leave bone marrow and circulate in blood to secondary lymphoid tissues, where they undergo further maturation (5).

Secondary lymphoid organs

B-cell activation occurs in the secondary lymphoid organs, such as spleen and lymph nodes. **Secondary lymphoid organs** can be divided into marginal zone, mantle zone and germinal centre; normal B-cell activation takes place in the **germinal centre**.

Germinal centre has a dark zone (DZ), where B-cells proliferate as centroblasts and undergo somatic hypermutation (HSM); centroblasts become centrocytes and migrate to the light zone (LZ), formed by B-cell no proliferating selected based on their affinity for the antigen. Centrocytes can back to be centroblasts or they can differentiate into memory B-cells or plasma cells (15).

In summary, in the secondary lymphoid organs there are the follicles, where naive B-cells are in contact with antigens. Following this binding of a T-dependent antigen, some mature B-cells can mature into plasma cells, and others undergo further proliferation and differentiation. B-cells clones of higher-affinity antigen-binding potential will be selected: when they are in contact with T-dependent antigen, some changes can take place to increase this potential, such as:

- B-cells may undergo receptor editing.
- Somatic hypermutation (SHM) can contribute to increase antibody affinity: some mutations can be introduced to the genes encoding the antibody-binding regions of the Ig receptor.
- It can be Ig class switching.

3.6.2. FL PATHOGENY

The **germinal centre** of the lymph node is the source of several types of lymphomas, like diffuse large B-cell lymphoma, Burkitt lymphoma, and follicular lymphoma (*figure 4*). Therefore, FL is defined as a proliferation of malignant germinal centre B-cells, mixt with several types of non-malignant cells, such as centroblasts (16).

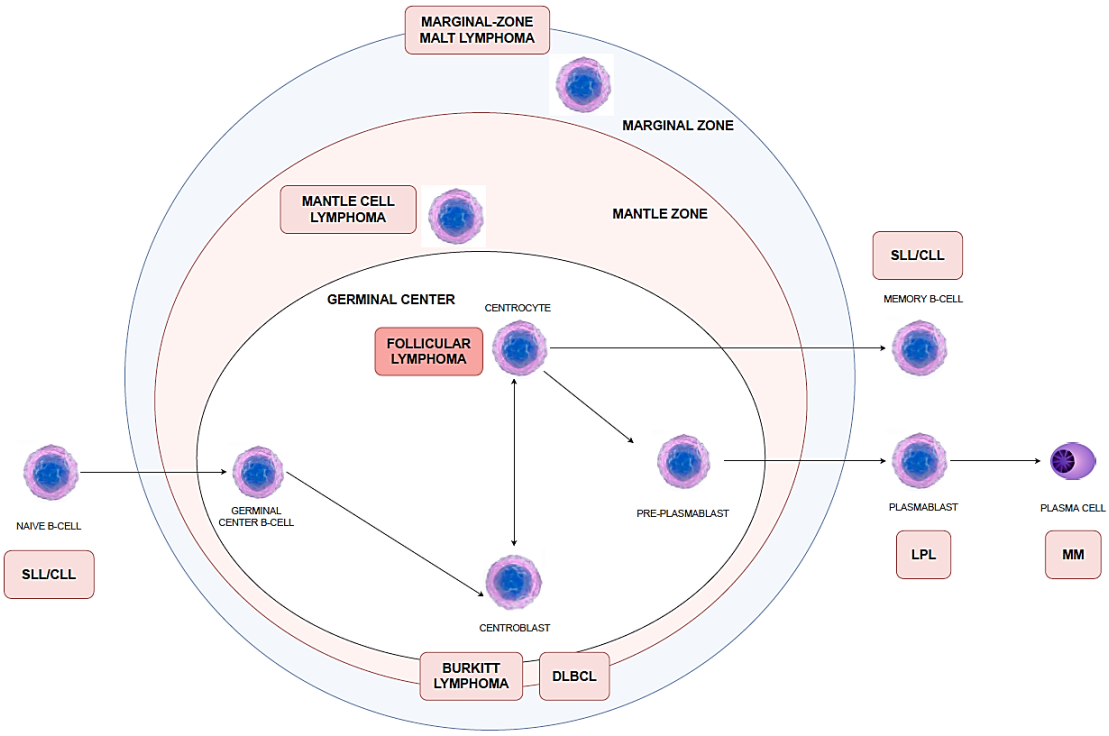

Figure 4. Origin mature B-cell neoplasms. SLL/CLL: small lymphocytic lymphoma/chronic lymphocytic leukaemia; DLBCL: diffuse large B-cell lymphoma; LPL: lymphoplasmacytic lymphoma; MM: multiple myeloma. Adapted from (1)

During the development process, cells can suffer **recombination** of gene segments: in normal cells, DNA would be repaired by several processes, but that recombination can

contribute to chromosome translocations in a lymphoma, and it can lead to the protooncogene activation. Depending on when the error occurs, we have different types of lymphomas, and tumour cells will have different characteristics (11).

The **t(14;18)(q32;q21) translocation** is the most common acquired non-random chromosomal translocation, and it is found in 80% of all cases of FL. In this translocation IgH locus and BCL2 locus are involved, and it leads to an overexpression of the anti-apoptotic protein BCL2 in the germinal centre cells. In normal conditions, germinal centre cells suffer an apoptosis unless they are activated to become plasma cells or memory cells. However, the overexpression of BCL2 favours the accumulation of secondary chromosomal alterations and the survival of these cells.

In FL, the hallmark **t(14;18)(q32;q21) translocation** occurs early in B-cell development, from an error in V(D)J recombination. All naive B-cells, with or without the translocation, go to the follicles and are selected for entry and proliferation in the germinal centre. As it has been explained, B-cells with t(14;18)(q32;q21) have a survival advantage for the expression of BCL2, an antiapoptotic protein that also favours the accumulation of secondary chromosomal alterations in the mutated cells.

This translocation has been detected also in a very low levels in high number of people with no evidence of lymphoma, so it shows that t(14;18)(q32;q21) translocation is not enough on its own for the formation of a follicular lymphoma and there are other necessaries mechanisms involved in the development and the progress of the lymphoma (16,17).

3.7. DIAGNOSIS

3.7.1. DIAGNOSTIC TESTS

FL diagnose should be based on several types of information (18,19):

- Anamnesis: B-symptoms must be asked to the patient, as well as other symptoms that the tumour can cause.

- Physical exploration: peripheral LNs, liver and spleen must be explored to detect possible lymphadenopathies, hepatomegaly, or splenomegaly, respectively.
- Eastern Cooperative Oncology Group (ECOG) evaluation: ECOG performance is a scale used to evaluate patient functionality and it can help in treatment decisions.
- Laboratory: it may include the following items:
  - Hemogram: patients may have normochromic normocytic anaemia due to ineffective erythropoiesis, or to a bone marrow infiltration. Patients may also have thrombocytopenia secondary to a bone marrow infiltration, or to a hypersplenism.
  - Routine blood chemistry: including immunoglobulin levels, lactate dehydrogenase (LDH), β2-microglobulin (B2M) and uric acid.
  - Liver and kidney functions.
  - Acute phase reactants (PCR, VSG): their values may be increased.

- Serology: hepatitis B, C and HIV serology.
- Lymph node (LN) biopsy: we will be able to do immunophenotype, cytogenetics and PCR-based techniques. Lymph node biopsy confirms a lymphoma diagnosis.

LYMPH NODE

HISTOLOGICAL CHARACTERISTICS

FL is a B-cell tumour derived from germinal centre cells. There is a replacement of the normal architecture by neoplastic follicles, uniform in size. Most cases show a uniform pattern of closely packed **follicles** in follicular nodules, but the architecture of FL can be variable: some FL can show a mixture of follicular and diffuse areas, and in a few cases we can see a completely diffuse architecture without of any identifiable follicular structures. Neoplastic follicles have vague borders and scant or absent mantle zones (*figure 5*).

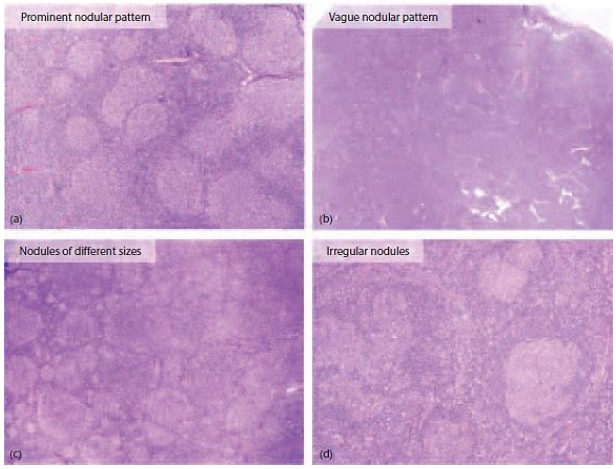

Figure 5. FL patterns of growth. From (20)

FL has a majority population of centrocytes and expresses germinal centre antigens, but it also contains other types of cells, such as centroblasts. In fact, FL is formed by a mixture of centrocytes (large non-cleaved cells) and centroblasts (large non-cleaved cells) (20–23).

Follicular lymphoma has been histologically classified in several ways according to the proportion of large centroblastic cells. The World Health Organization (WHO) classifies FL in 3 grades depending on the number of centroblasts per high-power field (HPF) (*table 1*).

Table 1. World Health Organization histological classification of FL (22)

Grade	Description
1	0-5 centroblasts/ high-power field
2	6-15 centroblasts/ high-power field
3A	>15 centroblasts/ high-power field, centrocytes present
3B	>15 centroblasts/ high-power field, centrocytes absent

A FL of grade 1, 2 or 3A is treated as an indolent tumour, and a FL of grade 3B is considered an aggressive lymphoma, so the distinction between FL of grade 3A or 3B is crucial (19).

IMMUNOCHEMISTRY

In **immunochemistry**, tumour cells of FL are like B-cells of the germinal centre, and they present the typical FL phenotype (*figure 6*).

- Positive for B-cell-associated antigens: **CD19, CD20, CD22 and CD79a**.
  - CD19: it is a common B-cell marker. It regulates B-cell development, activation, and differentiation.
  - CD20: it is a membrane receptor that B-lymphocytes acquire in their physiological development, so it is expressed in most types of B-cell lymphomas.
  - CD79a: it is expressed almost exclusively in B-cells. It is a transmembrane protein that functions as the B-cell receptor transducer.

- Positive for germinal centre B-cells (GCB) derivation: **CD10 and Bcl-6**.
  - CD10: it is a metallopeptidase that is expressed in early lymphoid progenitor cells and normal cells of the germinal centre. It supports the diagnosis of some B-cell lymphomas, such as follicular lymphoma but also Burkitt lymphoma.
  - Bcl-6: it acts as a transcriptional repressor, and it is frequently translocated or hypermuted in various lymphomas. It is in the germinal centre or post-germinal centre neoplastic cells.

- Positive for surface immunoglobulin: usually **IgM**, but IgG or IgA can be seen. Mature B-cell neoplasms most often show a single clone of cells (κ or λ).
- Negative for T-cell markers: such as CD3 and CD5.

Tumour cells can also express **Bcl-2** protein, which is useful to distinguish reactive from neoplastic follicles, because normal germinal centre cells are Bcl-2-negative and approximately 85% of FL cells are Bcl-2-positive. This protein regulates apoptosis, fundamental in the regulation of the physiological development of lymphocytes (21,22,24).

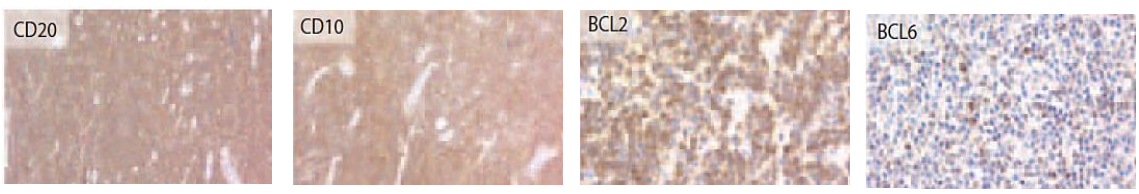

Figure 6. Immunohistochemistry: typical FL phenotypes. From (20)

3.7.2. STAGING TESTS

- Imaging:
  - CT of neck, chest, abdomen.
  - PET-CT: it used to be considered as an optional test as low-grade FL can have low fluorodeoxyglucose (FDG) uptake, and most CT lesions are negative with low Standardized Uptake Value (SUV). Recently some studies that have compared CT with PET-CT have concluded that PET-CT alters the stage of FL in many cases. On the other hand, it has been proved that in patients with FL and clinical signs of histological transformation PET-CT is useful to identify transformed lymph nodes (25).

- Bone marrow (BM): bone marrow is where the haematopoiesis takes place, and it is the most frequent extraganglionar site affected, leading to the designation of stage IV of Ann-Arbor stage, so its study is mandatory in FL staging. It is evaluated at diagnosis, and after treatment to monitor treatment response or unexplained cytopenias. To study bone marrow, two types of studies can be performed:
  - Bone marrow aspirate (BMA).
  - Bone marrow biopsy (BMB).

BONE MARROW ASPIRATE (BMA)

Bone marrow aspirate consists of obtaining a fluid portion of the bone marrow. The aspiration trocar and the needle are inserted until they reach the posterior iliac crest. Then the professional will aspirate with a syringe to obtain the fluid, and then the sample will be processed (26).

Cytological assessment is possible with flow cytometry (FC), but also immunophenotypic, cytogenetic, molecular genetics, and other specialised investigations (27,28).

FLOW CYTOMETRY

Flow cytometry is a semi-automated method that combines 2 basic approaches: cytometry and flow. It is an analysis technique that lets the clinic identify different cell

populations, as well as obtaining great information from them depending on the protein expressed. An advantage of FC in comparison to other tests is that it can count many cells and it can analyse many parameters, including immunological characterisation of cells. 8 and 10 colours flow cytometers now very frequently provide up to 10 or 12 parameters, which is extremely valuable in immunophenotyping (14).

The cytometer is composed of a fluid system, an optic system, and an electronic system (*figure 7*). Fluid system aligns cells one by one in a flow chamber towards the beam of light; cells are marked with fluorochrome, and monoclonal antibodies can be conjugated with this marker, which lets us know which type of cell we are analysing.

Optic system consists of lasers and filters; lasers illuminate the cells, which disperse the light according to its characteristics; cells are labelled with fluorochromes, so when the light is dispersed is detected by a detector that will detect the wavelength emitted by excitation of fluorochrome. Finally, this light signal will be traduced by the electronic system. We obtain many graphics which show which types of cells have been detected according to the antibodies pattern. Acquisition files are generated and they can be analysed or re-analysed at any time (29,30).

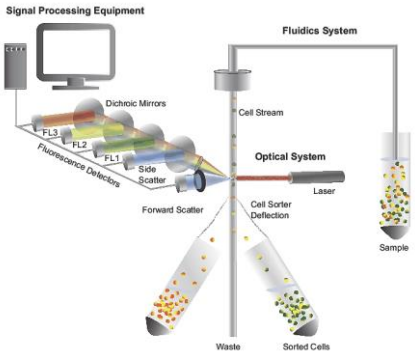

Figure 7. Schematic of a flow cytometry system. From https://www.newport.com/n/flow-cytometry

Many surface proteins and glycoproteins on hematologic cells have been studied, so thanks to flow cytometry cell population and its alterations can be identified. The markers for the study of FL include antibodies with specificities for CD20, bcl-2, CD10, bcl-6, CD23 and CD5. The immunostaining of bone marrow shows CD19, CD20 and BCL6 positives, and CD10 may be also positive but it is often downregulated (20).

In *Annex I (figures 10 and 11*) we can see the CD acquisition during B-cell maturation by FC, and how a follicular lymphoma with bone marrow infiltration is seen with FC.

Flow cytometry is considered a very useful tool to study NHL, but its utility in the staging and follow up of B-NHL has not been well established. Some authors conclude that this technique does not provide additional information to the histology, but others support that both tests should be practised in all patients diagnosed with FL because there can be cases of discordance (31).

Advantages and limitations of this test are summarized in *table 2*.

Table 2. Characteristics of the flow cytometry of BMA. Adapted from (32)

ADVANTAGES	LIMITATIONS
A small sample of volume is enough	Special training is required
Cellular subsets can be quantified	Fresh sample with live cells is required
Rapid	Some cells can be destroyed during the sample processing and analysis
Highly sensitive	

BONE MARROW BIOPSY (BMB)

Bone marrow biopsy consists of obtaining a small sample of bone marrow to study the bone marrow histology. A special wide needle is pushed into the bone, usually into the posterior iliac crest, as in bone marrow aspiration. The professional has to rotate it to remove a sample of bone, which contains bone marrow inside. The sample will be processed, and bone marrow histology will be studied. Before the biopsy a local anaesthetic is injected. This procedure is so painful for most patients, so it can lead to a bias because the sample may not be good enough to be evaluated (33).

HISTOLOGY

Bone marrow biopsy allows us to evaluate bone marrow architecture and cellularity, and also to identify cells by immunohistochemistry. The bone marrow of patients with

stage IV FL typically shows a **paratrabecular pattern**, but nodular or interstitial infiltrates may be also seen (*figure 8*) (20).

Advantages and limitations of this test are summarized in *table 3*.

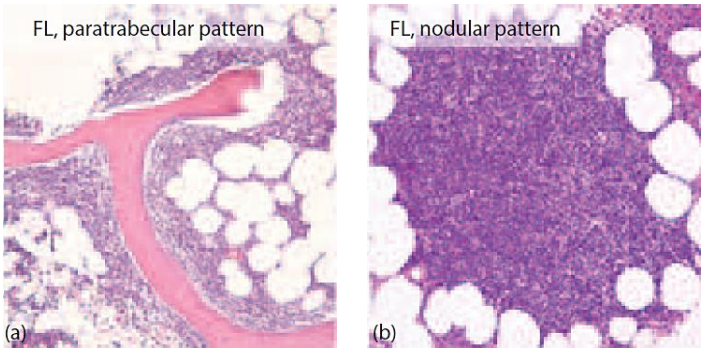

Figure 8. FL patterns of bone marrow involvement. From (20)

Table 3. Characteristics of the histology of BMB. Adapted from (32)

ADVANTAGES	LIMITATIONS
Almost all tissue biopsy samples can be assessed for immunohistochemistry Differentiation of follicular lymphoma from follicular hyperplasia	Tumour cells cannot be quantified Turnaround time is longer Decalcification can compromise some further molecular studies

The comparison between FL staging tests can be seen in the *Annex II (table 7)*.

3.7.3. PRE-TREATMENT TESTS

Some drugs can affect some organs functions, so before the treatment we must evaluate the function of these organs according to the prescribed treatment and its **toxicity**:

- Creatinine clearance.
- Electrocardiogram, cardiac ultrasound.
- Pulmonary function.
- Reproductive counselling in young patients.

3.8. CLINICAL STAGING

When a patient is diagnosed with follicular lymphoma, we have to stage it. **Ann-Arbor** classification (*Annex III. Table 8*) was made to stage HL, but it is also used to stage LNH, although it is less precise. At the diagnoses, approximately 75% of patients with FL have stages III or IV, and 25% have stages I or II (34).

3.9. PROGNOSIS

3.9.1. PROGNOSIS FACTORS

FLIPI

Follicular Lymphoma International Prognostic Index (FLIPI) is a prognostic index with 5 factors (10):

- ≥60 years.
- Stage III-IV.
- <12 g/dl.
- >4 lymph node regions.
- ↑LDH.

Each criteria counts 1 point, and finally we can separate 3 risk groups for overall survival:

- Low risk: 0-1 points.
- Medium risk: 2 points.
- High risk: 3-5 points.

FLIPI 2

FLIPI was revised and follicular Lymphoma International Prognostic Index 2 (FLIPI 2) was created with data from patients with a recent diagnosis of FL and already treated with chemotherapy schemes that included rituximab (12,18).

This system includes the following criteria:

- >60 years.
- Bone marrow involvement.
- <12 g/dl.
- Maximal diameter of lymph node >6 cm.
- ↑β_2-microglobulin.

As in FLIPI, each criteria counts 1 point, and finally we can separate 3 risk groups for overall survival:

- Low risk: 0 points.
- Medium risk: 1-2 points.
- High risk: 3-5 points.

GELF

The Groupe d'Étude des Lymphomes Folliculaires (GELF) considers patients with low tumour mass those patients that do not present any of the following criteria (18):

- Node or extranodal tumour mass ≥7 cm of diameter.
- Involvement of at least 3 lymph node regions, each with ≥3 cm of diameter.
- Presence of any B-symptoms.
- Splenomegaly.
- Compressive syndrome.
- Pleural effusion or ascites.
- Peripheral blood involvement (leukemization) (>5 x 10^9/l tumour cells).
- Peripheral cytopenias (absolute neutrophil count <1 x 10^9/l tumour cells and/or platelets <100 x 10^9/l tumour cells).

These criteria are usually used in clinical practise.

BNLI

British National Lymphoma Investigation (BNLI) considers that patients with LF and some of the following clinical data must be considered high tumour mass patients (18):

- Presence of any B-symptoms or itching.

- Rapidly progressive disease in the past 3 months.
- Involvement of a vital organ.
- Bone marrow involvement that produces cytopenias.
- Localized bone disease.
- Kidney infiltration.
- Gross liver involvement.

3.9.2. SURVIVAL

With the introduction of rituximab in the treatment of FL, the overall survival of patients with FL has increased. The median survival rate is of 8-10 years, although most patients relapse after treatment. In many cases, the FL can be transformed to an aggressive lymphoma, which can be the terminal phase of the disease (35).

3.10. TRANSFORMATION

Patients with FL have the risk of histologic transformation (HT) to an aggressive lymphoma. It commonly transforms to a diffuse large B-cell lymphoma, and less commonly to Burkitt lymphoma or other types of aggressive lymphomas. Its transformation occurs at a frequency of about 40% of all FL patients. It can cause emergence of extranodal lesions and the exacerbation of other symptoms, and it is a factor that contributes to therapy resistance, and it conditions poor prognosis.

Some factors that have been associated with increased risk to involve histologic transformation are an age below 65 years at presentation and initial involvement of extranodal sites other than bone marrow (36).

3.11. TREATMENT

Follicular lymphoma **treatment** depends on the stage of the tumour based on Ann-Arbor classification (19,37):

3.11.1 I-II FOLLICULAR LYMPHOMA

Treatment options in patients with a **FL in I-II stages** are the following:

- Watch-and-wait: it can be the best choice in selected patients, for example in those patients with a limited life expectancy or with difficulties to tolerate the treatment.
- Rituximab: it can be used in the same patients mentioned in the previous option.
- Radiotherapy (RT): this treatment option is generally the treatment of choice in patients with a FL in limited stage. Involved field radiotherapy (IFR) with 24Gy is usually the chosen option with a curative intent.
- Radiotherapy (RT) + chemotherapy (ChT) +/- rituximab: treatment based on RT in addition to ChT plus rituximab or not has demonstrated an improvement of progression free survival (PFS) compared with RT alone, but this combination has no impact on patients' overall survival (OS). It is indicated in patients with a high tumour burden, and in those with adverse clinical prognostic features.

3.11.2. III-IV FOLLICULAR LYMPHOMA

INDUCTION

In patients with **FL in III-IV stages** there is no curative therapy, and they do not require immediate treatment unless:

- B-symptoms.
- Symptomatic node disease.
- Symptomatic extranodal disease.
- Vital organ compression.
- Cytopenias.

In patients with FL in those stages we have the following treatment choices:

- Watch-and-wait: treatment can be deferred without disadvantage for patients with FL in III-IV stages with no treatment indicated.
- Rituximab: we can choose rituximab to treat patients with mild symptoms.

- Immunotherapy-ChT: this combination consists of treating the patient with ChT, such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), CVP (cyclophosphamide, vincristine, and prednisone), or with bendamustine, in combination with immunotherapy with rituximab.

CONSOLIDATION

After the induction treatment, patients will do the consolidation or maintenance treatment:

- Rituximab: treatment with rituximab every 2 months for 2 years improves PFS but it has no impact on the OS of the patient.
- Radioimmunotherapy: it also prolongs PFS after the induction treatment, but it is worse than the anterior treatment choice.

3.11.3. RELAPSED DISEASE

When a disease relapse is suspected, it is recommended to do a new biopsy to exclude the transformation of the FL to an aggressive lymphoma.

INDUCTION

- Watch-and-wait: observation is accepted in asymptomatic patients with low tumour burden and follicular histology confirmed.
- Radiotherapy (RT): in localised symptomatic disease.
- Chemotherapy (ChT): there are multiple chemotherapy schemes that can be used, like bendamustine after CHOP, alkylating agents-based regimens…
- Rituximab: it may be added if the initial treatment included it and the disease has relapsed >6-12 month after the remission. It can be also used in monotherapy in symptomatic cases with low tumour burden.
- Lenalidomide-rituximab: this treatment must be considered in patients with short remissions after chemotherapy.
- Idelalisib: it is a phosphoinositide 3-kinase (PI3K) inhibitor and it can be useful in double-refractory FL.

CONSOLIDATION

- Rituximab: for the consolidation, patients can be treated with rituximab every 3 month for up to 2 years. Nevertheless, it has not been investigated in patients who received antibody maintenance in first-line therapy.
- Chemotherapy (ChT) + autologous stem cell transplantation (ASCT): it should be considered in patients with brief first remissions (<2-3 months) after a treatment containing rituximab.

A scheme of possible treatment options of FL is shown in *Annex IV (figure 12)*.

4. JUSTIFICATION

Follicular lymphoma (FL) is the second most prevalent non-Hodgkin's lymphoma (NHL). It is considered an indolent tumour and its clinical presentation is heterogeneous: in most cases it has a slow progression with patterns of recurrences and remissions.

Treatment options for the patient are different depending on the stage of the lymphoma, and most patients with this tumour are diagnosed in stage IV of Ann-Arbor. For this reason, it is so relevant to have a well-established protocol of staging tests including the study of the bone marrow (BM).

Unfortunately, the use of multiparametric flow cytometry (FC) of bone marrow aspirate (BMA) in the staging and monitoring of follicular lymphoma is not validated or standardized among the centres: it is widely used as a complement to the histology of bone marrow biopsy (BMB).

There are controversial studies on its clinical utility compared to histology, that is the Gold Standard, although the techniques used are very disparate, and most of them are of low sensitivity with respect to those currently in use. There are virtually no multiparametric FC studies (10 fluorescences) that assess the usefulness of this technique in follicular lymphoma despite being used routinely for over 5 years.

The aim of this study is to compare the validity of flow cytometry to assess if this staging test should be included in the protocol together with histology to achieve a better characterization of bone marrow infiltration of follicular lymphomas.

In conclusion, due to FL's relatively high incidence, it is worth having good diagnostic and staging tests in order to offer the best treatment strategies to the patients, who will most likely have to live with the disease.

5. HYPOTHESIS

- The detection capacity of follicular lymphoma cells in bone marrow aspirate by a 10-colour flow cytometry panel is similar to the detection capacity of bone marrow histology in the staging of patients with FL.

6. OBJECTIVE

- To compare the capacity of a systematized analysis of 10-colour flow cytometry (FC) panel compared to histology (Gold Standard) to detect bone marrow involvement in patients with follicular lymphoma diagnosed in the province of Girona between 2017-2021.

7. SUBJECTS AND METHODS

7.1. STUDY DESIGN

This study is designed as a transversal and retrospective study which compares the detection of FL bone marrow infiltration by the histology of bone marrow biopsy and by flow cytometry of bone marrow aspirate.

Staging tests of patients with FL diagnosed in Girona between 2017-2021 will be recovered and re-analysed. Hospital Universitari Dr. Josep Trueta (Girona), Hospital de Palamós (Baix Empordà) and Fundació Hospital d'Olot i Comarcal de la Garrotxa (Garrotxa) will participate in the study. Information between the investigators will not be shared to avoid contamination of results (*figure 9*).

After this study, it would be useful to do a validation study including different patient cohorts with similar panels with the same analysis strategy. Bone marrow biopsy samples and flow cytometry files of patients of 3 other hospitals could be available: Hospital Universitari Germans Trias i Pujol (Badalona), Hospital Universitari de Bellvitge (l'Hospitalet de Llobregat) and Hospital Universitari Mútua Terrassa (Terrassa).

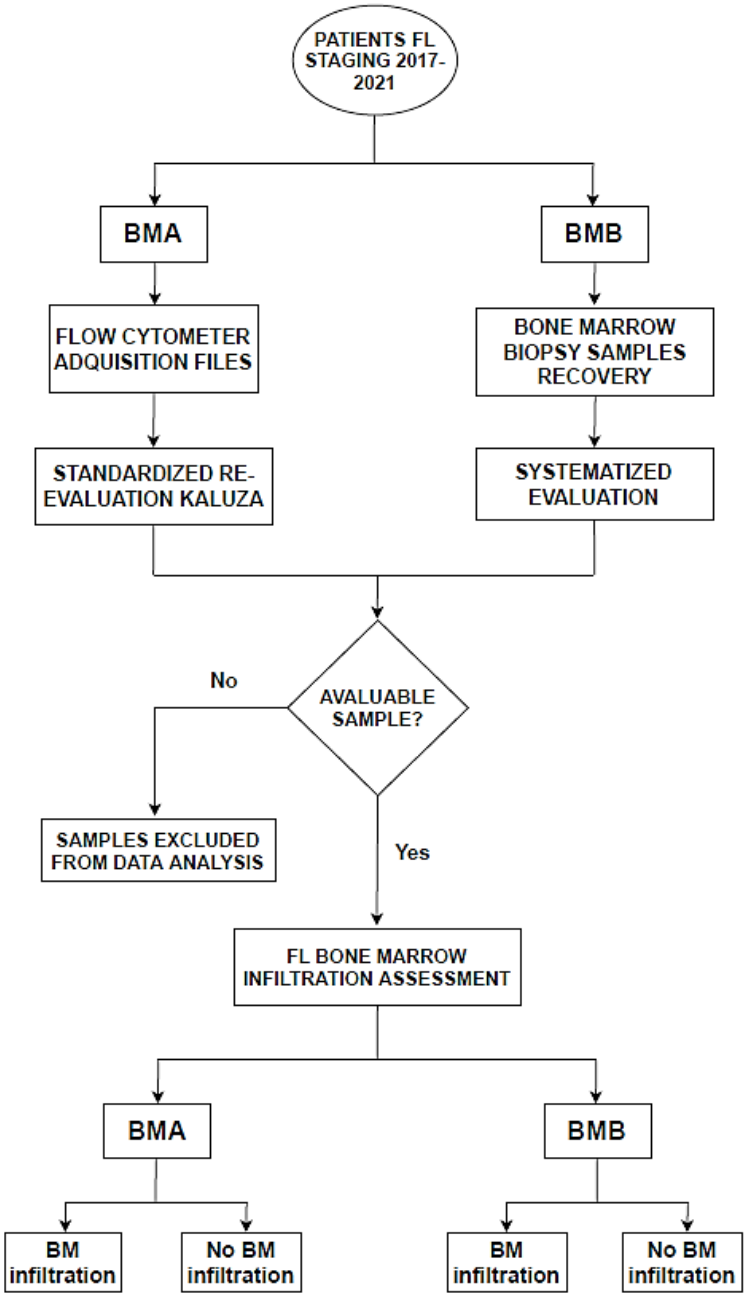

Figure 9. Study scheme

7.2. STUDY POPULATION

The eligible population includes patients diagnosed with follicular lymphoma in the 2017-2021 period in the province of Girona who have available files of BMA and available samples of BMB.

7.2.1. INCLUSION CRITERIA

- Patients with FL with multiparametric FC study of BMA samples available and with BMB recoverable samples.

7.2.2. EXCLUSION CRITERIA

- Patients with no BMA and/or BMB samples available.
- Patients with concomitant lymphoma at diagnosis.

7.3. SAMPLE SIZE

A global study of follicular lymphoma in Girona established an incidence of 3,45 cases/100.000 person-year. If we consider that the population of the province of Girona is 800.000, and our study lasts 5 years, we will have approximately $3{,}45 \cdot 8 \cdot 5 =$ **138** patients diagnosed with FL during this period.

We calculated the necessary theoretical sample size with the online software GRANMO. With the bibliography, we can consider that the sensitivity of the histology of BMB is 0,99, and that the sensitivity of the FC of BMA is 0,92. Accepting an alpha risk of 0,05% and a beta risk of 0,2%, and expecting a 5% of loses, we need a theorical sample of **136** subjects for the study to be statistically significant.

7.4. SAMPLE SELECTION

Samples of all patients diagnosed with FL in the province of Girona during the 5-year period 2017-2021 and who have signed the Informed Consent will be recovered and re-analysed.

7.5. VARIABLES

As it is a cross-sectional study, there are not either dependent or independent variables. Instead of that, we can define a main variable and covariables that influence it.

7.5.1. MAIN VARIABLE

The **main variable** is the capacity of flow cytometry to detect bone marrow infiltration of FL. An evaluation sheet of flow cytometry and histology will be delivered to the investigators (*Annexes V and VI)* to have a consensus analysis.

7.5.2. COVARIABLES

PATIENT

- Age: this variable will be measured as the number of years old.
- Chromosomic sex: this variable will be measured as a dichotomous qualitative variable (XX/XY).
- Leukocytes: this variable will be measured as $x10^9$/L leukocytes.
- Haemoglobin: this variable will be measured as g/L of haemoglobin.
- Platelets: this variable will be measured as $x10^9$/L platelets.
- LDH: this variable will be measured as UI/L.
- Beta-2-microglobulin: this variable will be measured as mg/L.

LYMPHOMA

- Histology grade: it is an ordinal qualitative variable, and the possible values are I, II, IIIA and IIIB.
- Lymph node involvement: the number of lymph nodes involved, their diameter (mm), and if it/they are supradiaphragmatic and/or infradiaphragmatic (S/I) will be recorded.

- PET-CT result: it will be considered positive or negative for tumour detection (+/-).

SAMPLES

Histology – BMB

- Date: the date of the sample obtention will be recorded.
- Site of puncture: if the samples have been obtained from the anterior iliac crest or from the posterior iliac crest (A/P) will be recorded. It will be also necessary to record if it is from the left iliac crest or from the right one, and also if the sample has been obtained bilaterally (L/R/B).
- Size of cylinder: it will be measured with mm.
- Trabecular spaces: we must record the number of trabecular spaces observed in the bone marrow sample.
- Infiltration: the variable will be measured answering yes or no.
- Pattern of infiltration: it can be diffuse, nodular, or others.

Multiparametric FC – BMA

- Date: the date of the sample obtention will be recorded.
- Site of puncture: if the samples have been obtained from the anterior iliac crest or from the posterior iliac crest (A/P) will be recorded. It will be also necessary to record if it is from the left iliac crest or from the right one, and also if the sample has been obtained bilaterally (L/R/B).
- Panel of Ab used: it is a qualitative variable. The order of monoclonal antibodies will be registered.
- Number of leukocytes: it will be measured with leukocytes/micL.
- Number of leukocytes CD45+ events acquired: it will be measured as a discrete quantitative variable.
- Number of B-cell CD19+ events acquired: it will be measured as a discrete quantitative variable.
- Relation pathological cells/bone marrow leukocytes: it will be measured in %.
- Relation pathological cells/B-cell CD19+: it will be measured in %.
- Detection of clonal B cells with FL phenotype: the variable will be measured answering yes or no.

7.6. MEASURE INSTRUMENTS

7.6.1. BMA FCM

- Beckman Coulter Navios: it can analyse up to 12 parameters; it has 10 colours and 3 lasers.
- Monoclonal antibodies: a panel of monoclonal antibodies conjugated with fluorochromes is used to study the complete immunophenotype of each sample. In our study multiparametric FC samples were processed in a standard protocol wash-stain-lyse, with a panel of 12 antibodies: (anti-)CD8-κ-FITC, (anti-)CD4-λ-PE, (anti-)CD19-ECD, (anti-)CD79b-PC5.5, (anti-)CD10-PC7, (anti-)CD5-APC, (anti-)CD38-APC-AF700, (anti-)CD3-APC-AF750, (anti-)CD20-PB, (anti-)CD45-KrO.
- Kaluza Software – Standardized Analysis Template: this software is designed in order to simply and efficiently analyse multicolour data.
- Revision by Flow Cytometrist: the cytometrist from the Hospital Universitari Dr. Josep Trueta (Girona) will fill the evaluation sheet (*Annex V. Table 9*).

7.6.2. BMB HISTOLOGY

A systematized review of Haematoxylin/Eosin slides and immunohistochemical slides (at least CD20 and CD3) if available of BMB of diagnose study will be done by the central pathologist of the study in Hospital Universitari Dr. Josep Trueta (Girona), Hospital de Palamós (Baix Empordà) and Fundació Hospital d'Olot i Comarcal de la Garrotxa (Garrotxa). It will be necessary an optical microscopy up to 400x-600x. Pathologists will fill the evaluation sheet (*Annex VI*).

7.7. DATA COLLECTION

As it is a retrospective study, most part of data collection will consist of recovering information already collected.

- Review of clinical records: needed patient variables will be recorded of clinical records. We will record patients' age in the diagnosis, the chromosomic sex, and some analytics dates, like haemoglobin, leukocytes, platelets, LDH and beta-2-

microglobulin in the diagnosis. Participants will have signed an Informed Consent allowing us to access the clinical records.

- Systematized analysis on Kaluza of Beckman Coulter Navios Files (.FCS): Kaluza Analysis Software is designed to analyse multi-colour data simply, efficiently, and quickly. Flow cytometry files of participants achieved in diagnosis will be recovered and a cytometrist will re-analyse them. An evaluation sheet will be filled (*Annex V. Table 9*).
- Recovery and analysing of bone marrow biopsy samples: samples stay at the 3 Hospitals participating in the study. 3 histology investigators will re-analyse all samples and they will fill the evaluation sheets (*Annex VI*).
- REDCap: it is a secure web application for building and managing online surveys and databases. It can be used to collect virtually any type of data, so it will be useful for the investigators to share the results of the re-analysis of the samples with the main coordinators of the study.

8. STATISTICAL ANALYSIS

8.1. DESCRIPTIVE ANALYSIS

Depending on the variable type we will use different statistical analysis. For **quantitative** variables, such as patient's age, number of leukocytes, haemoglobin, number of platelets, LDH, beta-2-microglobulin, size of BMB cylinders and number of trabecular spaces in BMB samples, we will calculate arithmetic mean and standard deviation if the variable has a normal distribution, and median and interquartile range if the variable does not have a normal distribution.

Qualitative variables, such as the site of puncture, the pattern of infiltration and the presence or not of clonal B-cells with FL phenotype, will be expressed with frequencies and percentages.

All study variables will be recorded to describe clinical characteristics of the cohort to be able to assess if the % of infiltration is the one expected.

Non-adequate samples will not be considered in the analysis interpretation, but reasons which make the samples unanalysable should be studied.

8.2. COMPARATIVE ANALYSIS

The **validity** of a test indicates the degree to which the investigator can get the assurance that the study conclusions are error free or accurate. It can be divided in:

- Internal validity: it is the extent to which the observed results represent the truth in the population that we are studying. It depends on the sensitivity and the specificity of the test.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{false negative}}$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{false positive}}$$

- External validity: it is the extent to which the observed results can be applied outside the context of the study. It depends on the positive predictive value (PPV) and the negative predictive value (NPV).

$$\text{Positive predictive value} = \frac{\text{True positive}}{\text{True positive} + \text{false positive}}$$

$$\text{Negative predictive value} = \frac{\text{True negative}}{\text{True negative} + \text{false negative}}$$

The results of the FC will be recorded in a contingency table (*table 4*) considering the result of the test and the correlation with the presence or not of bone marrow infiltration observed by histology. Finally, the validity of the flow cytometry will be calculated and compared with the Gold Standard test. Result will be considered statistically significant when p value is <0.05, with a confidence interval of 95% (95% CI).

Table 4. Study results of FC

	Affected BM	**Non-affected BM**
Positive FC	True positive (TP)	False positive (FP)
Negatives FC	False negative (FN)	True negative (TN)

After re-analysing all samples, there can be 4 different situations: both test positives, only flow cytometry positive, only histology positive, and both test negatives.

Cases in which we have concluded there are not bone marrow infiltration by histology, but flow cytometry has concluded the opposite situation should be discussed. Instead of being a false positive of the analysis with FC, it could be a false negative of the histology analysis. This is the main point in our study: it could be demonstrated that FC should be added as a staging test jointly with histology.

9. ETHICAL AND LEGAL CONSIDERATIONS

This protocol will be revised by the "*Comité d'Ètica d'Investigació Clínica*" (**CEIC**) of the Hospital Universitari Dr. Josep Trueta, the coordinating hospital of the study.

This study is designed in accordance with "*The World Medical Association Declaration of **Helsinki** of Ethical Principles for Medical Research Involving Human Subjects (2013)*" to guarantee human rights and ethical considerations.

All medical ethical principles are respected in this study. The objective of this study is to ensure an adequate staging of the disease, a fact that could affect patients' quality of life and their survival, so the study is based on the principle of **beneficence**. If the study is significant and flow cytometry is added to the staging protocol, it does not suppose harm to the patients, respecting **non maleficence** principle. This study has not discrimination, following **justice** principle.

To respect patients' **autonomy**, according to the "*Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica*", patients included in the study will be informed of the protocol with an **information document** where there will be explained the study (*Annex VII*).

The investigator/s will ensure that participants have understood all the information and will solve any doubt. Then patients will sign an **informed consent** authorizing the access to clinical records and samples used in the diagnosis (*Annex VIII*).

The study requires the reassessment of samples used in the diagnosis, so we will take into consideration the "*Ley 14/2007, de 3 de julio, de Investigación **biomédica***".

Personal dates of the participants will be **confidential**, according to the "*Reglamento (UE) 2016/679 del Parlamento Europeo y del Consejo, de 27 de abril de 2016, relativo a la protección de las personas físicas en lo que respecta al tratamiento de datos personales y a la libre circulación de estos datos*", and the "*Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales*".

Due this study is retrospective, and dates are already recorded, there is no risk or inconvenience for the participants.

Finally, investigators will declare they have no **conflicts** of interest.

10. STUDY STRENGTHS AND LIMITATIONS

10.1. STRENGTHS

- Time and cost: as it is a retrospective study, samples are already collected, so the study takes less time and it has less cost.
- Sample selection: it is a retrospective study, so there is no loss of patients because samples are already collected. There can be only a loss due to non-adequate samples.

10.2. LIMITATIONS

- Retrospective study: it would be interesting to follow patients with FL with flow cytometry and histology to analyse the predictive value of both tests in minimal residual disease and patients' survival, and it can not be done with a retrospective study design. In addition, some necessary information from the medical history of the participants can be missing.
- Sample size: the number of samples available could be considered small, but the final goal of this project is to define the bases to do a multicentric study with the participation of other Catalonia hospitals, having a bigger number of samples.
- Bone marrow biopsy sample bias: some bone marrow biopsy samples may not be available or may be inadequate, so there can be a loss of samples. Reasons which make samples unanalysable must be studied.
- Specific flow cytometer: samples have been analysed by Beckman Coulter Navios Cytometer; we would have to consider if the protocol is applicable to hospitals with other 10-color flow instruments.
- Uniprovincial study: as the study analyses samples of patients with FL of Girona population, a multicentric study including some other hospitals of Catalonia should be done.
- Subjective interpretation of samples: the interpretation of the results of flow cytometry has a point of subjectivity, as well as the interpretation of the histology, which could mean a bias in our study. The systematic approach with preset Kaluza templates minimizes subjectivity in flow cytometry.

11. WORK PLAN AND CHRONOGRAM

This study will last about 18 months, from August 2021 to January 2023, divided in 6 principal phases (*table 5)*. Finally, a multicentric study could be done including some other Catalonia hospitals.

- **Phase 0: protocol elaboration** (August 2021 – November 2021)

  During this phase the study will be planned. After bibliographic research, the protocol will be elaborated, containing clear hypotheses, objectives, variables, and methodology.

- **Phase 1: ethical evaluation** (December 2021)

  This protocol must be evaluated and approved for the "*Comité d'Ètica d'Investigació Clínica*" (CEIC) of the Hospital Universitari Dr. Josep Trueta.

- **Phase 2: information to participants and Informed Consent obtaining** (January 2022)

  Participants will be contacted. The information sheet will be given to them, and they will have time to read it and to solve all doubts. Informed Consent will be signed by those participants who want to participate in the study.

- **Phase 3: samples recovery** (February 2022 – March 2022)

  Patient's samples (BMA and BMB) will be recovered: bone marrow aspirates files are saved at Hospital Universitari Dr. Josep Trueta, the reference hospital of flow cytometry; bone marrow biopsy samples are in the reference hospitals, which are Hospital Universitari Dr. Josep Trueta (Girona), Hospital de Palamós (Baix Empordà) and Fundació Hospital d'Olot i Comarcal de la Garrotxa (Garrotxa).

- **Phase 4: samples reassessment** (April 2022 – July 2022)

  Bone marrow samples will be evaluated by the professional investigators. Histology will be studied for 3 investigators, and flow cytometry files will be interpreted by a

professional of Hospital Universitari Dr. Josep Trueta. The results will be reflected in the guide sheets (*Annexes V and VI*).

- **Phase 5: data analysis** (August 2022 – October 2022)

  The statistical analysis will be performed by a specialized statistician. Results will be presented in tables and conclusions will be drawn by the investigators.

- **Phase 6: publication of the results** (November 2022 – January 2023)

  The study will be recorded in an article, and it will be edited and published.

Table 5. Chronogram

STAGES	**2021**					**2022**												**2023**
	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J
PHASE 0: protocol elaboration																		
PHASE 1: ethical evaluation																		
PHASE 2: information to participants and Informed Consent obtaining																		
PHASE 3: samples recovery																		
PHASE 4: samples reassessment																		
PHASE 5: data analysis																		
PHASE 6: publication of the results																		

12. BUDGET

The research team participating in this study is already employed by the participating Hospitals. Pathologists will receive 10€ per analysed case.

A statistician will be contracted to do the data analysis, which will take approximately 50 hours.

Necessary material resources are still available in the Hospitals participating in the study.

Finally, we consider publication fees will cost about 2.000€.

The approximate final cost of the project will be of 5.130€ (*table 6*).

Table 6. Budget

	UNIT	COST PER UNIT	SUBTOTAL
HUMAN RESOURCES			
1 investigator analysing flow cytometry files	FC investigator will not receive any financial compensation for the study.		
3 investigators analysing BMB samples	138 cases	10 €/analysed case	**1.380 €**
1 statistician	50 hours	35 €/hour	**1.750 €**
MATERIAL RESOURCES			
Flow cytometer	Hospitals that participate in the study have the necessary material resources.		
Microscopies			
Informatic software			
PUBLICATION FEES			
Publication fees			**2.000 €**
TOTAL BUDGET			**5.130 €**

13. FEASIBILITY

Our study will recover samples from 2017 to 2021. The study is designed as retrospective study of a 5-year period with the participation of Hospital Universitari Dr. Josep Trueta (Girona), Hospital de Palamós (Baix Empordà) and Fundació Hospital d'Olot i Comarcal de la Garrotxa (Garrotxa). Each of these hospitals have professional haematologists and necessary material resources (flow cytometer in Hospital Universitari Dr. Josep Trueta and microscopies in Hospital Universitari Dr. Josep Trueta, Hospital de Palamós and Fundació Hospital d'Olot i Comarcal de la Garrotxa), so no additional resources are needed to analyse the available samples.

In Catalonia Hospitals, bone marrow biopsy samples are not removed until after 10 years of diagnosis, so samples are still available. Flow cytometry files are saved too, so they can be re-analysed.

A consensus meeting among the 3 involved pathologist and specific instructions for the systematic review of samples (2 hours) will be given for a professional previously to analyse the available samples in order to obtain histology results as standardized as possible.

After carrying out this study, an aim would be to repeat it but with the participation of 3 other Catalonia Hospitals: Hospital Universitari Germans Trias i Pujol (Badalona), Hospital Universitari de Bellvitge (l'Hospitalet de Llobregat) and Hospital Universitari Mútua Terrassa (Terrassa), which use the same cytometer than Hospital Universitari Dr. Josep Trueta. This way the sample would be bigger, and results would be more representative.

14. CLINICAL AND HEALTHCARE IMPACT

Follicular lymphoma is the second most prevalent non-Hodgkin's lymphoma (NHL), and its incidence in the province of Girona is of 3,45 cases/100.000 person-year. Therefore, refine its staging techniques is necessary to offer these patients adequate treatment options.

If the results of the study are relevant and show flow cytometry of bone marrow aspirate brings advantages over only analysing the histology of bone marrow biopsy in the staging of follicular lymphoma, it should be considered to include flow cytometry in the protocol to stage follicular lymphomas.

Having in consideration that having a bone marrow infiltration means that the follicular lymphoma is in stage IV of Ann-Arbor, achieving a better staging would mean being able to offer better treatment options to these patients, and consequently to improve their quality of life and their survival.

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16. ANNEXES

16.1. ANNEX I: B-cells normal maturation and FL in FC

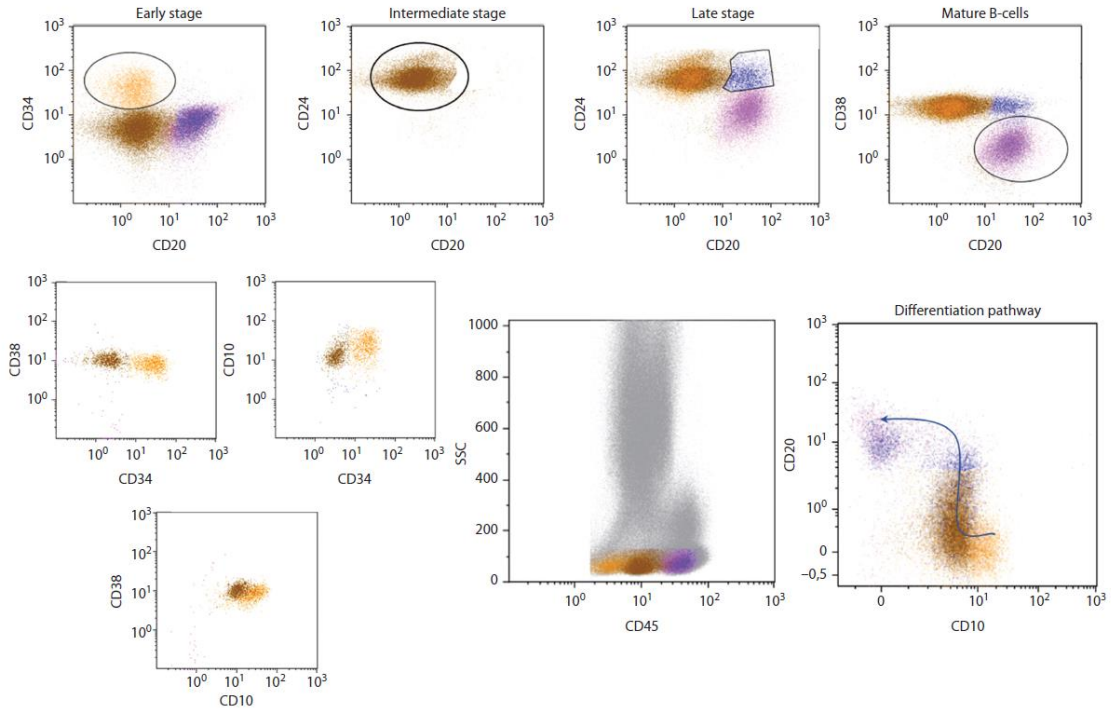


Figure 10. CD during B-cells maturation. From (14)

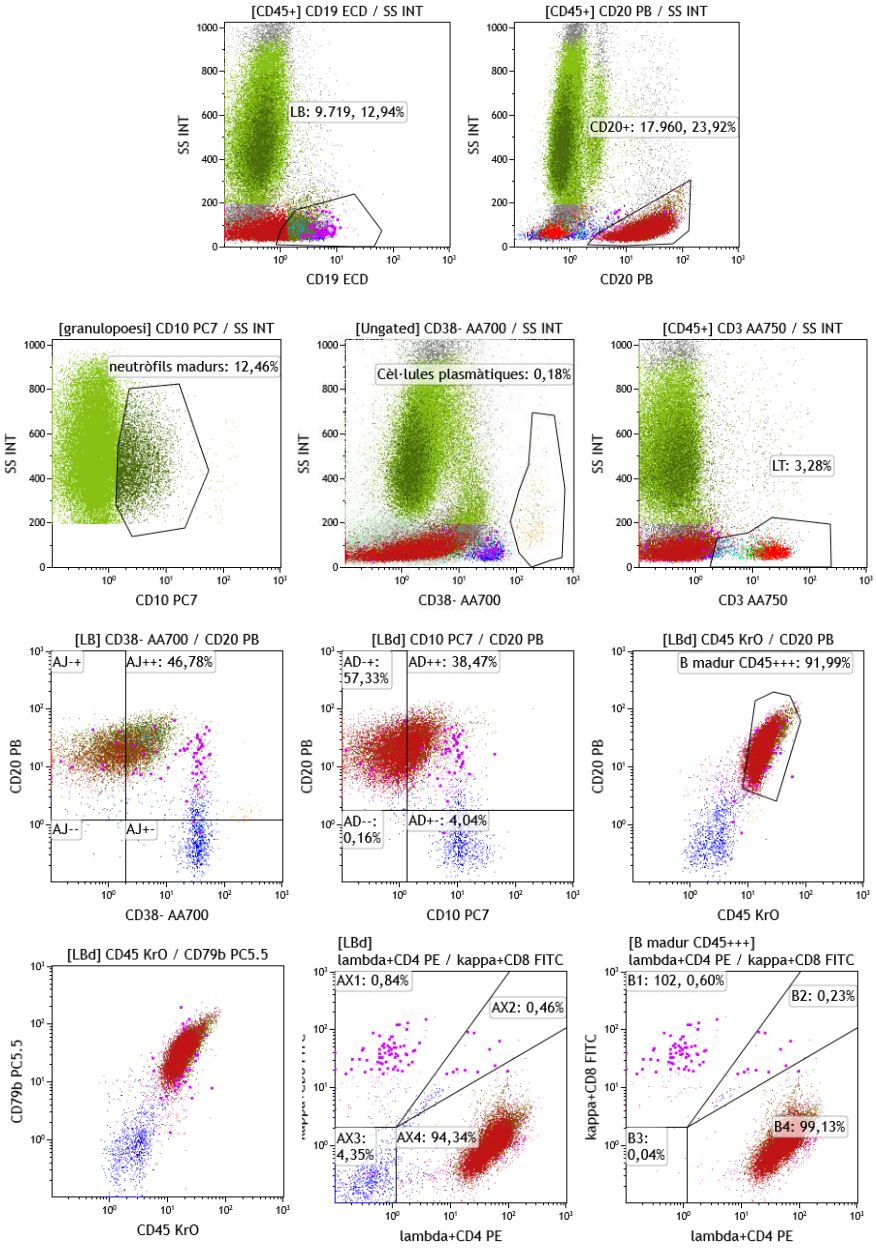


Figure 11. Flow cytometry of a bone marrow aspirate from a patient with follicular lymphoma. From Hospital Universitari Dr. Josep Trueta

16.2. ANNEX II: Comparison between available FL staging tests

Table 7. Comparison between available FL staging tests. Adapted from (32)

	PET-CT	**Histology**	**FC**
Information obtained	Nodal and/or extranodal involvement	Cellularity, architecture, fibrosis, focal lesions, bone structure	Morphology, cytochemistry, immunophenotyping…
Quantitative	No	No	Yes
Availability of antibodies	No	Limited	Abundant
Morphology and tissue architecture	No	Preserved	Lost
Sensibility	Low	Moderate-low	Very high
Specificity	Moderate-low	High	High-moderate
Time for routine examination report	Medium	Medium (7 days)	Low (24 hours)
Limitations	Little lesions with low glycolytic index False positives in infections	Investigator's subjectivity and experience Multiple staining of same cells can not be done simultaneously	It can not see space distribution Non standardization

16.3. ANNEX III: Ann-Arbor stages

Table 8. Ann-Arbor stages. Adapted from (5)

Stages	
I	Involvement of 1 lymph node (I) or 1 extralymphatic organ or site (IE)
II	Involvement of ≥2 lymph nodes on same side of diaphragm (II), or localized extralymphatic organ or site and ≥1 involved lymph node on same side of diaphragm (IIE)
III	Involvement of lymph nodes on both sides of diaphragm (III) or same side with localized involvement of extralymphatic site (IIIE), spleen (IIIS), or both (IIIS + E)
IV	Diffuse or disseminated involvement of extralymphatic organ or tissues with or without lymph node enlargement
Modifiers	
A	Absence of B-symptoms
B	Temperature >38º, night sweats, and weight loss of greater than 10% of body weight in the 6 months preceding admission are defined as systemic symptoms
X	Bulky disease (greater than 1/3 widening of the mediastinum or >10 cm diameter of nodal mass)

16.4. ANNEX IV: Possible treatment options of FL

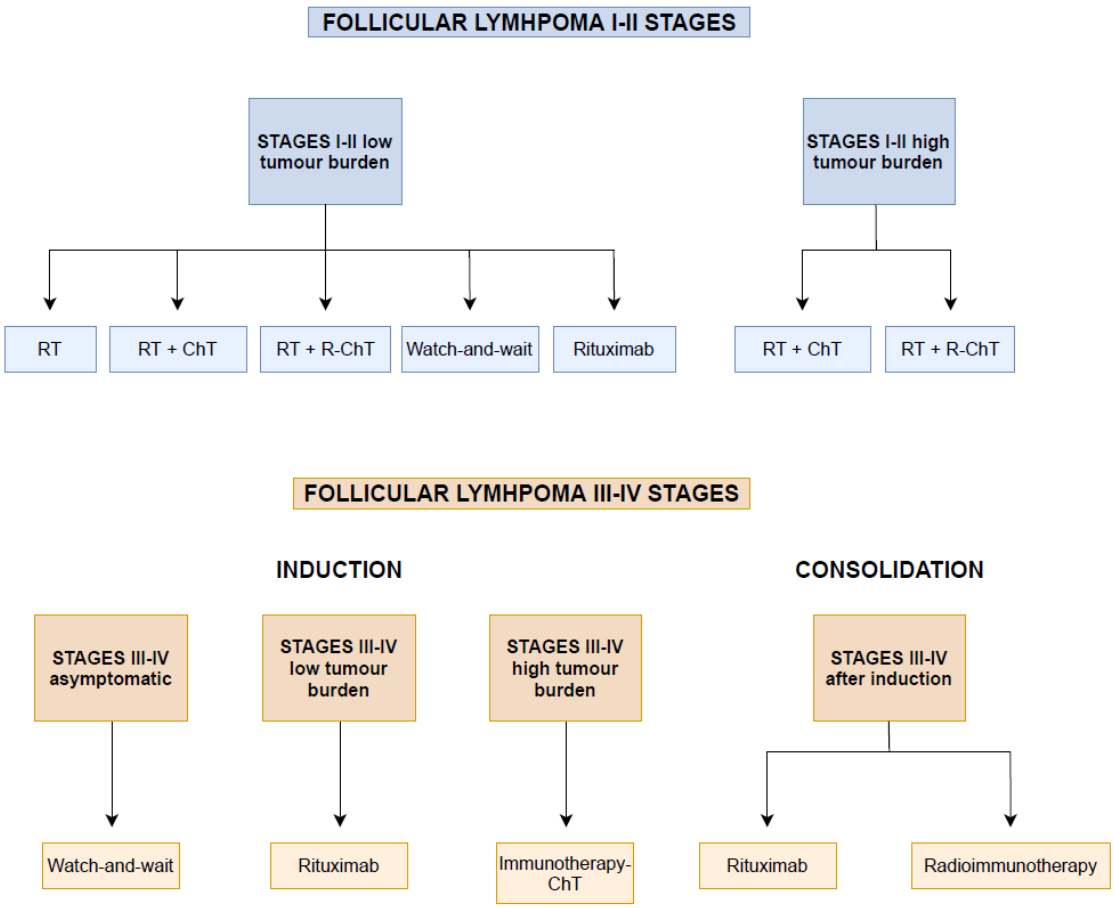


Figure 12. Possible treatment options of FL. RT: radiotherapy; ChT: chemotherapy; R: rituximab

16.5. ANNEX V: Checklist flow cytometry evaluation

1. **Sample characteristics**

1.1. Site of puncture: anterior/posterior (A/P); left, right, bilateral (L/R/B)

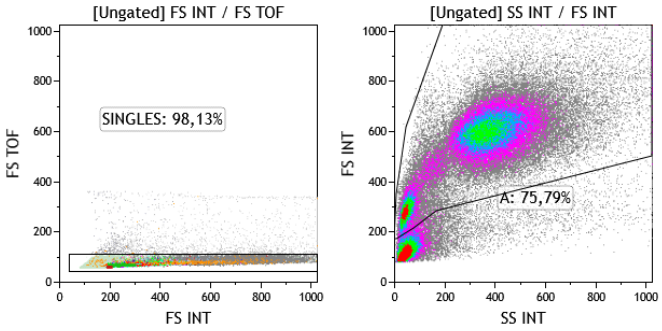
1.2. Date of puncture

1.3. Panel of Ab used

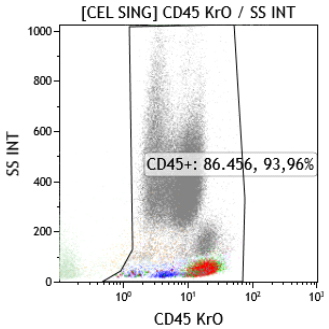
2. **Optimal sample**: yes/no

2.1. Leukocyte's evaluation

- Removal of doublets, removal of debris and dead cells

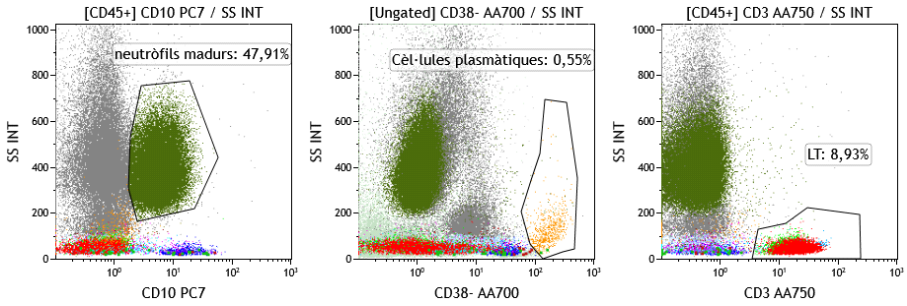


- Leukocytes (CD45+) identification



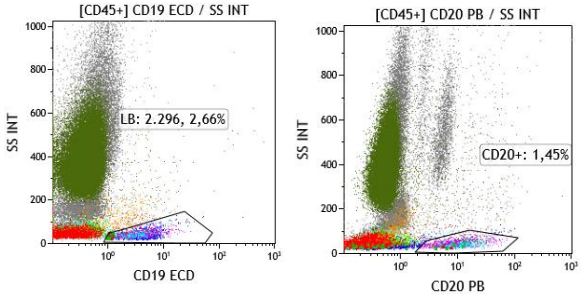
2.2. Assessment of contamination of the sample: inadequate if:

- Absence of plasma cells (<0,1%)
- Mature neutrophils/total granulopoiesis (>85%)
- Proportional increase in T-lymphocytes



3. Sample evaluation

3.1. B-cell selection: it must be done by a Boolean selection (CD19 and CD20) in order to be able to assess all the immature or pathological CD19+/CD20- B-cells and the pathological cells that could lose CD19 (frequent finding in the FL)



3.2. Mature b-cell selection and detection of FL suggestive aberrations and verification of clonality in suspicious populations

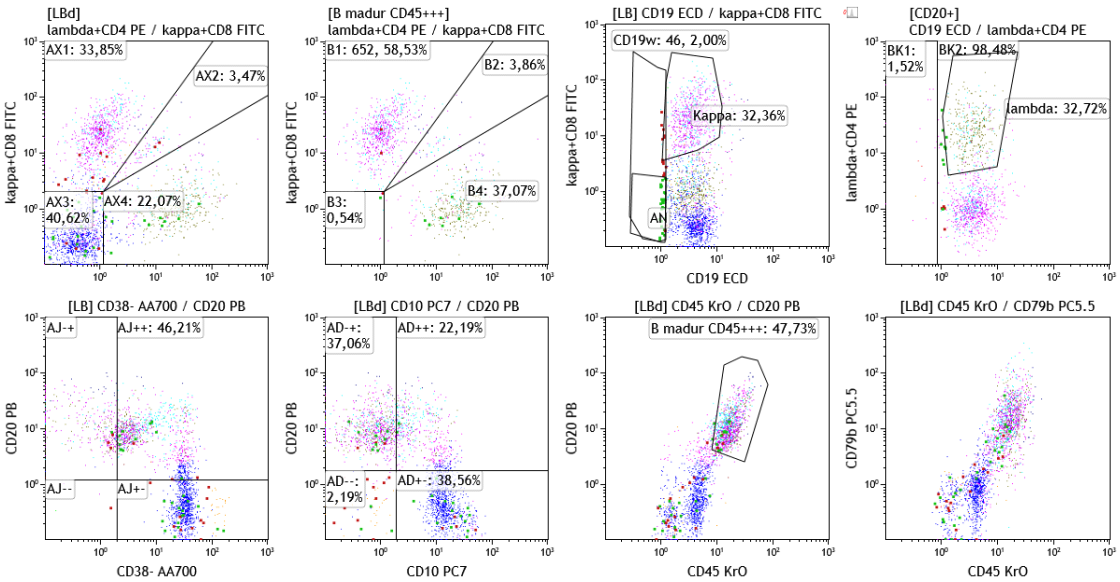


Table 9. Checklist flow cytometry evaluation

	%/mature b-cells	Intensity (dim, +, ++, +++)	Comment
Kappa			
Lambda			
CD19			
CD20			
CD79b			
CD10			
CD38			
CD44			
BCL2			

Conclusion	% pathologic B-cells/leukocytes	___%
	% pathologic B-cells/B-cells CD19+	___%
	Infiltration	Yes/no

16.6. ANNEX VI: Checklist bone marrow biopsy evaluation

Adapted from (38)

1. **Sample characteristics**
   - **1.1. Site of puncture**: anterior/posterior (A/P); left, right, bilateral (L/R/B)
   - **1.2. Date of puncture**

2. **Optimal sample**: yes/no
   - **2.1. Cylinder size**: ….. cm
   - **2.2. Cylinder integrity**: complete, fragmented
   - **2.3. Number of trabecular spaces**

3. **Sample evaluation**
   - **3.1. Global hematopoietic cellularity**: normal, increased, decreased
   - **3.2. Lymphocytes**:
     - Lymphocytosis: yes, no
     - Nodules/aggregates: yes, no
     - Distribution: central, paratrabecular, plate, diffuse, interstitial, intrasinusoidal, mixed, others
     - Cellularity: typical, atypical; monomorph, polymorph; high mitotic activity, low mitotic activity
     - Non-Hodgkin
       - Small: scarce, abundant; regular nucleus, irregular nucleus
       - Medium: scarce, abundant
       - Large: scarce, abundant
       - Cellular type
       - Immunophenotype: B, T, no B no T
       - Annexed cells: histiocyte, epithelioid, other
     - Lymphocytes: scarce, abundant
     - Plasma: scarce, abundant
     - Neutrophiles/eosinophils: scarce, abundant
     - Histiocytes/epithelioid: scarce, abundant
   - **3.3. Valued techniques**
     - **Usual:**
       - Haematoxylin-eosin: yes, no
     - **Specials:**
       - Immunohistochemistry: yes (CD3, CD20, others)/no

Conclusion	**Infiltration**	Yes/no
	Pattern	Diffuse/nodular /others

16.7. ANNEX VII: Information sheet

Hospital Universitari de Girona
Doctor Josep Trueta

ICO
Institut Català d'Oncologia

FULL D'INFORMACIÓ AL PACIENT

VALIDACIÓ D'UN PANELL DE CITOMETRIA DE FLUX DE 10 COLORS COMPARAT AMB LA BIÒPSIA DE MEDUL·LA ÒSSIA PER AVALUAR LA INFILTRACIÓ DE LA MEDUL·LA ÒSSIA EN EL LIMFOMA FOL·LICULAR

INVESTIGADORS PRINCIPALS: Dra. Natàlia Lloveras Guelque i estudiant Mar Segundo Felip.

TÍTOL DE L'ESTUDI: Validació d'un panell de citometria de flux de 10 colors comparat amb la biòpsia de medul·la òssia per avaluar la infiltració de la medul·la òssia en el limfoma fol·licular.

CENTRES: participen en l'estudi l'Hospital Universitari Dr. Josep Trueta (Girona), l'Hospital de Palamós (Baix Empordà) i la Fundació Hospital d'Olot i Comarcal de la Garrotxa (Garrotxa).

INTRODUCCIÓ: ens dirigim a vostè per informar-lo sobre un estudi al que està convidat a participar. Aquest full informatiu pretén fer-li arribar tota la informació necessària perquè vostè pugui decidir si vol o no participar en l'estudi. Llegeixi atentament la informació proporcionada i consulti en cas de tenir qualsevol dubte.

PARTICIPACIÓ VOLUNTÀRIA: la seva participació en aquest estudi és de caràcter totalment voluntari. Si decideix no participar-hi, les seves dades i mostres no es tindran en compte en cap moment i no li suposarà absolutament cap perjudici en un futur.

DESCRIPCIÓ DE L'ESTUDI:

La finalitat de l'estudi proposat és avaluar la citometria de flux, una prova que possiblement hauria de ser inclosa en les proves estàndards d'estadiatge en pacients diagnosticats de limfoma fol·licular, i que actualment no consta en el protocol.

Aquesta modificació en l'estadiatge podria contribuir a estadiar amb més precisió els pacients diagnosticats de limfoma fol·licular i, per tant, oferir-los les opcions de tractament adequades.

Per poder realitzar l'estudi, es pretén re-analitzar els fitxers de l'anàlisi de les mostres d'aspirat de medul·la òssia i les mostres de biòpsia de medul·la òssia que se li van extreure en el moment del diagnòstic del limfoma. També seria necessari accedir a la seva història clínica per la obtenció d'algunes dades rellevants en l'estudi.

CONFIDENCIALITAT: les dades dels participants seran confidencials, segons el "*Reglamento (UE) 2016/679 del Parlamento Europeo y del Consejo, de 27 de abril de 2016, relativo a la protección de las personas físicas en lo que respecta al tratamiento de datos personales y a la libre circulación de estos datos*", i la "*Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales*". La informació serà emmagatzemada en una base de dades anonimitzada. En cas d'acceptar la participació en l'estudi, en qualsevol moment vostè pot sol·licitar l'esborrament de les seves dades als investigadors.

16.8. ANNEX VIII: Informed Consent

Hospital Universitari de Girona
Doctor Josep Trueta

ICO
Institut Català d'Oncologia

CONSENTIMENT INFORMAT

VALIDACIÓ D'UN PANELL DE CITOMETRIA DE FLUX DE 10 COLORS COMPARAT AMB LA BIÒPSIA DE MEDUL·LA ÒSSIA PER AVALUAR LA INFILTRACIÓ DE LA MEDUL·LA ÒSSIA EN EL LIMFOMA FOL·LICULAR

INVESTIGADORS PRINCIPALS: Dra. Natàlia Lloveras Guelque i estudiant Mar Segundo Felip.

TÍTOL DE L'ESTUDI: Validació d'un panell de citometria de flux de 10 colors comparat amb la biòpsia de medul·la òssia per avaluar la infiltració de la medul·la òssia en el limfoma fol·licular.

Jo, ...,

DECLARO QUE:

- He llegit el full d'informació al pacient
- He rebut suficient informació sobre les característiques de l'estudi
- He pogut resoldre tots els meus dubtes sobre l'estudi
- He entès que la participació en l'estudi és voluntària
- He entès que em puc retirar de l'estudi en qualsevol moment

Accepto l'obtenció de les mostres diagnòstiques: SÍ ☐ NO ☐

Accepto l'obtenció de dades de la història clínica: SÍ ☐ NO ☐

Signatura de l'investigador

Signatura del participant

Data: ___ /___ /_____

Data: ___ /___ /_____