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GENETIC ABNORMALITIES AS DIAGNOSTIC AND PROGNOSTIC MARKERS IN B CELL LYMPHOMAS: ROLE OF NEW MOLECULAR TECHNOLOGIES IN PERSONALIZED MEDICINE FOR EXTRANODAL DIFFUSE LARGE B CELL LYMPHOMA (EN-DLBCL) AND FOLLICULAR LYMPHOMA (FL)

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INTRODUCTION

B cell lymphomas (BCL) are a group of heterogeneous hematological malignancies assumed to originate at different stages of lymphocyte development through different molecular genetic aberrations. Thus, different subtypes resemble lymphocytes at distinct differentiation stages and show peculiar clinic, morphologic, immunophenotypic and genetic features. Schematically, mature (non-lymphoblastic) BCL are proposed to be derived from pre-germinal center (GC), GC or post-GC B cells. In detail, mantle cell lymphoma (MCL) is considered a pre-GC neoplasm, follicular lymphoma (FL), Burkitt lymphoma (BL) and GC-type diffuse large B cell lymphoma (GCB-DLBCL) are of GC origin, whereas activated-type diffuse large B cell lymphoma (ABC-DLBCL), primary mediastinal B cell lymphoma (PMBL), marginal zone lymphoma (MZL), lymphoplasmacytic lymphoma (LPL), chronic lymphocytic leukemia (CLL) and classical Hodgkin lymphoma (cHL) appear to be derived from post GC B cells (figure 1).

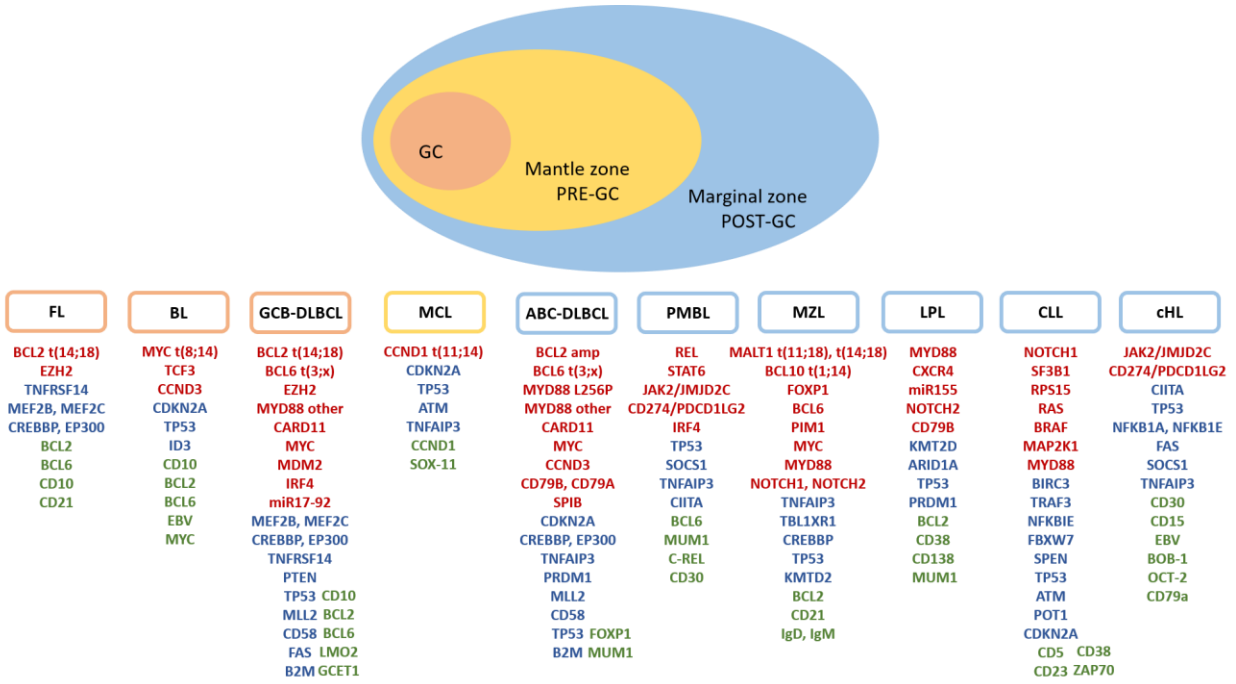

Figure 1. Origin of BCL with a list of the main molecular (red=gain of function and blue=loss of function) and immunohistochemical (green) biomarkers characteristic of each entity (modified from *Sun 2016*).

Novel insights into the origin and biology of BCL have been achieved with the advance of new technologies, and a considerable number of molecular and immunohistochemical biomarkers that are related to alterations at the genetic, epigenetic, and protein level as well as the tumor microenvironment have become available nowadays (Shaffer 2012). They can be useful both at a speculative level to clarify details regarding the mechanisms underlying lymphomagenesis and for

practical purposes in the diagnostic workup, to predict the outcome and properly treat patients with personalized targeted therapies. For example, figure 1 summarizes the most important gain of function (red) and loss of function (blue) of molecular biomarkers involved in the various subtypes of BCL, as well as immunohistochemical markers (green) routinely used by pathologists in the differential diagnosis among different entities. Despite a plethora of candidate biomarkers with potential clinical value have been suggested, it is crucial to understand when and how they can be integrated into the clinical setting, translating experimental results from bench to bedside, with the aim of improving patients' care.

Among BCL, DLBCL and FL are the most common types of non-Hodgkin lymphomas worldwide (WHO 2017) and still represent a challenge for both researchers and clinicians.

Diffuse large B cell lymphoma (DLBCL)

According to the latest revision of the WHO classification of tumours of the hematopoietic and lymphoid tissues, DLBCL is defined as diffuse proliferation of neoplastic B cells with a nuclear size greater than or equal to that of a histiocyte nucleus, or more than twice the size of a small lymphocyte (WHO 2017) (figure 2).

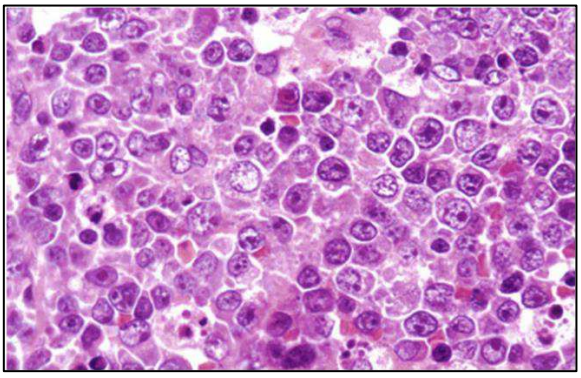

Figure 2. DLBCL (Hematoxylin & Eosin X600).

DLBCL represents the most common histological subtype of non-Hodgkin lymphoma (NHL), accounting for about 30% of adult NHL worldwide (WHO 2017). The median age of onset is 64 years, but any age can be affected and there is a slight male predominance (male-to-female ratio of 1.2:1) (WHO 2017).

Although DLBCL is an aggressive lymphoma with a median survival of less than one year if left untreated, it is a potentially curable disease. Since the 1970s, the CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) regimen has represented the standard therapy, with 50% of complete remissions (CR) and 30–40 % of long survivors (Rovira 2015). Since the early 2000's, the addition of rituximab, the first approved anti-CD20 monoclonal antibody, to the chemotherapy has improved overall survival by approximately 20% (Feugier 2005, Sehn 2005, Bachy 2015).

Overall, the disease exhibits a striking heterogeneity in terms of clinical presentation and outcome, morphology, immunophenotype and gene expression profiles. Some well-defined entities exhibiting peculiar clinico-biological characteristics have been recognized and classified as specific variants in the WHO classification (WHO 2017). In this scenario, the term DLBCL, Not Otherwise Specified (NOS) is used to collectively define a group of biologically and clinically heterogeneous cases not conforming to the defined subtypes and entities (figure 3).

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
EBV$^+$ DLBCL, NOS*
*EBV$^+$ mucocutaneous ulcer**
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B–cell lymphoma
Intravascular large B–cell lymphoma
ALK$^+$ large B–cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
*HHV8$^+$ DLBCL, NOS**

Figure 3. DLBCL subclassification according to the WHO classification of tumours of the hematopoietic and lymphoid tissues (from *Swerdlow 2016*).

* Changes from the 2008 WHO classification

Microarray studies have uncovered distinct molecular signatures in DLBCL that have unique biology and natural history (Alizadeh 2000, Rosenwald 2002, Monti 2005). They are characterized by distinct gene expression profiles either characteristic of normal GC B-cells or of activated blood memory B-cells. It is now well established that the cell of origin (COO) represents one of the major

sources of diversity in DLBCL, being associated with different molecular alterations and clinical evolution (Bea 2005, Tagawa 2005). In detail, patients with GCB-DLBCL have more favorable outcomes than those with ABC DLBCL when treated with standard R-CHOP immunochemotherapy (Alizadeh 2000, Rosenwald 2002). The original method used to define these entities performed GEP using microarrays on RNA extracted from frozen tissue (Lenz 2008). As the gold standard GEP methods are not readily accessible, being based on the availability of fresh frozen samples and microarray technology, immunohistochemistry (IHC)-based algorithm applicable in every laboratory to formalin fixed paraffin embedded (FFPE) tissues have been developed as a surrogate for GEP data (Hans 2004, Muris 2006, Natkunam 2008, Choi 2009, Nyman 2009). Among these classifiers, the most widely used is the Hans' algorithm (Hans 2004), which is based on the combination of three markers: CD10, BCL6 and MUM1 (figure 4).

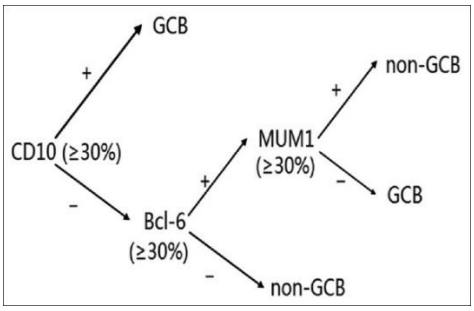

Figure 4. Hans' algorithm to determine the COO based on sequential evaluation of three immunohistochemical markers.

However, it is evident that information obtained from complex gene-expression signatures on large numbers of genes cannot be reproduced using a very small number of antigens. Moreover, its intrinsic subjectivity and variability in scoring, lowers the reliability of such IHC-based methodology (Gutierrez-Garcia 2011, Gleeson 2015). More recently, a robust and highly accurate molecular assay for COO distinction using GEP techniques applicable to FFPE samples has been developed (Scott 2014). The nCounter platform of NanoString Technologies (Seattle, WA, USA) is useful for the direct multiplex measurement of gene expression using FFPE samples, and numerous clinical research studies with this platform have been performed. Scott and coworkers have developed a 20-gene version of a NanoString code set for a COO typing assay of DLBCL named Lymph2Cx (Scott 2014). Fifteen genes, along with 5 housekeeping genes, were selected among 93 genes, based on their ability to accurately replicate the COO model originally proposed by Lenz (Lenz 2008). This assay brings to fruition the potential to use gene expression-based COO assignment in standard practice.

However, as the latest WHO update requires the identification of the COO and GEP is still not a routine clinical test, the use of IHC algorithms is considered acceptable (WHO 2017).

Moreover, about 40% of DLBCL arise in non-lymphoid organs and are referred to as primary extranodal (EN) DLBCLs (Moller 2004, Vannata 2015, Vitolo 2016,). Some of them present distinct biology and clinical behavior and have been segregated out in the WHO classification (WHO 2017). These include primary DLBCL of the central nervous system (CNS), primary cutaneous DLBCL, leg type, intravascular large B-cell lymphoma, primary mediastinal (thymic) large B-cell lymphoma and lymphomatoid granulomatosis. However, DLBCL can originate in almost every organ. Although several studies have been published, each addressing DLBCL arising in a different body site, the literature on primary EN lymphomas as a group is limited to a few papers.

Finally, in the latest update of the WHO classification (WHO 2017), a new category named high-grade B-cell lymphoma (HGBL) has been introduced. This entity includes high grade lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements and cases of diffuse aggressive B-cell lymphoma with high grade features (such as intermediately sized blastoid cells, starry sky pattern, expression of CD10, variable BCL2, high proliferative fraction) that lack "double-hit" genetic features and fall under the category of HGBL, NOS. The so called "double/triple-hit" (DH/TH) configuration of the former is strictly defined by the presence of rearrangements and breakpoints at the sites of both *MYC* and *BCL2* and/or *BCL6*, as detected by cytogenetic techniques. *MYC* and *BCL2* rearrangements may result in an overexpression of the related proteins, however, even if identification of the more common immunohistochemically defined double-expressor (DE) lymphomas is of interest, it is important to keep in mind that they only partially overlap with true DH/TH lymphomas. In detail, coexpression of BCL2 (≥50% of tumor cells) and MYC (≥40% of tumor cells) seems to be associated with worse prognosis, independent of other clinical risk factors in patients treated with standard immuno-chemotherapy. For this reason, clinical trials have also been developed for DE cases (Green 2012, Johnson 2012, Clark Schneider 2016). Nonetheless, they do not seem to be as aggressive as DH/TH HGBL, and there is less agreement on the clinical utility of their recognition (Swerdlow 2014). At the morphological level, HGBL-DH/TH may include, among others, all cases of otherwise typical DLBCL carrying a proven DH/TH (figure 5). Accordingly, it should be advisable to routinely perform FISH analysis in all DLBCL. If resources preclude this broad approach, as most DH/TH cases show a GCB phenotype and express MYC at immunohistochemistry, a limiting testing to this group represents an acceptable, despite inferior, alternative (Friedberg 2017, Sesques 2017).

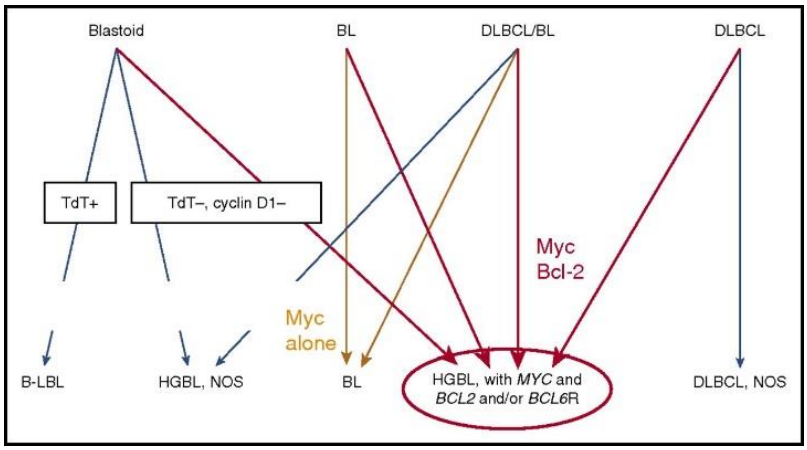

Figure 5. *HGBL-DH/TH categories according to WHO 2017 (from Friedberg 2017).*

In this scenario, a few months ago, the Italian Group of Haematopathology (GIE) proposed a practical workup for the diagnosis of aggressive mature B cell lymphomas. Based on a rational stepwise approach, the application of such algorithm allows the selection of cases deserving molecular analysis, with the aim of optimizing the use of costly and time-consuming techniques, and of assuring the optimal management of any patient (Di Napoli 2019).

Primary EN DLBCL

Due to the tendency of DLBCL to disseminate in both nodal and EN locations, the definition of a DLBCL as either primary nodal or EN has been a controversial issue, especially in advanced stages at presentation. Three alternative ways of defining EN lymphomas emerged when Krol and coworkers reviewed the available literature on the subject (Krol 2003) (figure 6). The same authors used data from a population-based NHL registry to illustrate the selection bias that is introduced when a strict definition of primary EN NHL, that excludes cases with disseminated disease, is used (Krol 2003).

Definition 1	
Primary nodal NHL	Presentation in lymph node, Waldeyer's ring, spleen or bone marrow
	Lymph node involvement clinically dominant; at most one extranodal organ involved (usually bone marrow)
Primary extranodal NHL	Presentation in other organs; no or only minor lymph node involvement
Extensive involvement	Presentation in both extranodal and nodal sites (often other side of diaphragm)
Definition 2	
Primary nodal NHL	As in definition 1, but also including patients with extensive involvement
Primary extranodal NHL	As in definition 1
Definition 3	
Primary nodal NHL	As in definition 1, but also including patients with extensive involvement, and patients with disseminated disease presenting in an extranodal site
Primary extranodal NHL	Presentation in an extranodal site with or without regional lymph node involvement

Figure 6. *Alternative ways of defining primary nodal and EN DLBCL*, as reviewed in *Krol 2003.*

Currently, it is accepted to operationally define as EN those lymphomas presenting as clinically dominant EN masses, with no, or only "minor", nodal involvement at presentation (Zucca 1997, Vannata 2015). Another controversial issue is whether to consider peculiar body sites, namely the Waldeyer's ring, the spleen and the bone marrow, as either nodal or EN. More commonly, cases arising in the Waldeyer's ring and in the spleen are considered as primary nodal, whereas bone marrow is considered an EN location (López-Guillermo 2005, Kim 2011).

The gastrointestinal (GI) tract is the most common EN site of presentation in DLBCL, with 10-15% of all the DLBCL cases and 30-40% of all the EN cases, followed by the head and neck (H&N) district (Castillo 2014, Vannata 2015).

Globally speaking, patients with EN disease present more frequently with early stage disease than those with nodal DLBCL (Møller 2004, Lal 2008, Castillo 2014). A potential explanation for this finding is that involvement of EN sites could be detected earlier based on signs and symptoms associated with the neoplastic mass effect. Results pertaining to other clinical characteristics, such as performance status and LDH levels are conflicting (Moller 2004, López-Guillermo 2005, Lal 2008). More interestingly, specific EN sites present distinct clinical and prognostic features. For example, lymphomas arising in the Waldeyer's ring and in the digestive tract have been associated to early-stage disease, no bone marrow infiltration, normal serum LDH, low- to low/intermediate-risk international prognostic index (IPI), and better outcome compared to DLBCL of other sites, though the outcome largely depends on other factors (López-Guillermo 2005, Lal 2008, de Leval 2012, Oh 2013, Wang 2016). In contrast, other authors, found GI, together with pulmonary, hepatic and pancreatic DLBCL to carry a significantly worse prognosis (Castillo 2014), even at multivariate analysis. Finally, lymphomas arising at immune-privileged sites, namely the testes and the CNS, are

traditionally considered aggressive diseases requiring prompt diagnosis and specific therapeutic approaches (Vitolo 2008, Phillips 2014, Deng 2016, Grommes 2019).

As far as the COO and the corresponding B cell differentiation markers are concerned, it has been hypothesized that DLBCL of primary nodal and EN origin might differ immunophenotypically. Data collected so far are often contradictory and difficult to compare, as most published works are based on small retrospective and heterogenous cohorts of patients. Moreover, although several studies have been published, each addressing DLBCL originating in a different EN body site, the literature on primary EN lymphomas as a group is limited to a few papers. The Korean group of Kim and coworkers didn't find any significant difference in the frequencies of GCB and ABC subtypes or in CD10, BCL6 and MUM1 expression when analyzing a series of consecutive de-novo DLBCL arising in nodal and EN sites (Kim 2011). Only BCL2 expression differed between the two groups, being significantly less frequent in primary EN cases (Kim 2011). In contrast, other authors found BCL6 expression to be more frequent in EN DLBCL, as a group, thain in nodal DLBCL (López-Guillermo 2005). More commonly, specific EN sites were related to peculiar immunophenotypes. In detail, it has been reported that gastric DLBCL usually show a non-GCB profile, suggesting a relationship with marginal zone lymphoma, whereas intestinal DLBCL commonly belong to the GCB subset (Connor 2007, Mitchell 2008). GC origin has been attributed to cervico-cephalic cases as well, especially when including DLBCL arising in the Waldeyer's ring (López-Guillermo 2005, de Leval 2012, Wang 2016). On the other hand, DLBCL of the breast, of the testis and of the central nervous system have been associated to a non-GCB phenotype (Yoshida 2005, Al Abbadi 2006, Booman 2008, Gill 2014, Magnoli 2015, Li 2017). Overall, it seems that peculiar immunophenotypes are not related to the mere nodal or EN origin, but rather to the specific site of presentation (Wang 2016).

Peculiar biologic and molecular characteristics have been claimed for EN DLBCL, suggesting that these lymphomas might originate through different genetic pathways and even represent distinct pathological entities (Raghoebier 1991, Houldsworth 1996, Kramer 1998, Al-Humood 2011). Using conventional comparative genomic hybridization (CGH), some authors documented significantly distinct chromosomal aberrations between nodal and EN DLBCL (Al-Humood 2011), which appeared to be genuinely related to the site and not to the ABC/GCB subclassification, as assessed by Choi's algorithm (Choi 2009). Moreover, it has been reported that *BCL2/IGH* rearrangement is more frequent in nodal DLBCL (Kramer 1998). By contrast, *BCL6*, *MYC* and *REL* molecular cytogenetic aberrations have been associated with an EN site of origin (Houldsworth 1996, Muramatsu 1996, Kramer 1998, Rao 1998). However, these single gene alterations have been found in relatively small and heterogeneous series and not widely confirmed (Houldsworth 2004, López-

Guillermo 2005). Once again, it seems not the generic study of DLBCL as either nodal or EN to be more informative, but rather the analysis of the characteristics of each specific site.

Follicular lymphoma (FL)

FL is defined as a neoplasm composed of GC B cells (typically both centrocytes and centroblasts) with at least a focal follicular growth pattern (WHO 2017) (figure 7).

As it has been demonstrated that the clinical aggressiveness of the lymphoma increases with the number of centroblasts, FL grading is based on the average number of large transformed cells in 10-20 randomly selected neoplastic follicles at X400 high-power field (HPF) examination. A case with up to 5 centroblasts/HPF is grade 1; 6 to 15 centroblasts is grade 2; and greater than 15 centroblasts is grade 3 (Mann 1982). Grades 1 and 2 do not significantly differ in terms of clinical behavior and are now considered together as low grade. Grade 3 is further subdivided into 3A, with admixed centrocytes still present, and 3B, with solid sheets of centroblasts. Finally, diffuse areas composed solely or predominantly by centroblasts in FL of any grade have to be reported as DLBCL with FL (WHO 2017).

The growth pattern can be predominantly follicular (>75% follicular), combined follicular and diffuse (25–75% follicular), or predominantly diffuse (<25% follicular). Diffuse areas are more common in grade 3 FL and predictive for worse prognosis (WHO 2017).

Among clinical parameters, Ann Arbor stage and the Follicular Lymphoma International Prognostic Index (FLIPI) and FLIPI2 are well-known prognostic factors for patients with FL (Solal-Celigny 2004, Federico 2009).

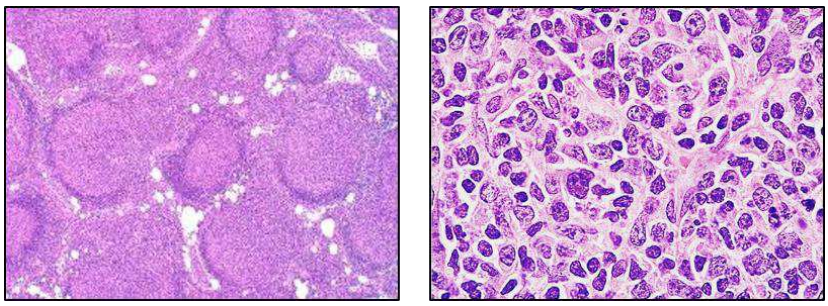

Figure 7. *Left: FL with follicular growth pattern. Back to back neoplastic follicles with attenuated mantle zones lack cell polarization and tingible body macrophages* (Hematoxylin & Eosin X100). Right: focus on centrocytes (small cleaved cells) and centroblasts (large noncleaved cells) (Hematoxylin & Eosin X600).

FL predominantly affects adults, with a median age of 55 to 59 years. It represents the most frequent form of NHL in Western countries and the second most common lymphoma worldwide (after DLBCL), accounting for 20% of all NHL (The Non-Hodgkin's Lymphoma Classification Project 1997).

Despite it follows a chronic indolent clinical course, FL remains incurable with standard therapies, being characterized by response to initial treatment with frequent relapses and shorter duration responses to salvage therapy. Management of FL has traditionally been based on a watch-and-wait approach or chemotherapy. The introduction of the anti-CD20 monoclonal antibody rituximab has favorably impacted on prognosis, with median overall survival (OS) exceeding 12 years (Kahl 2016).

In the last few years, it has become clear that FL represents a biologically complex and heterogenous disease (Magnoli 2019). Such aspect has been at least partly acknowledged by the inclusion of four clinicopathological variants of FL in the latest WHO update, namely in situ follicular neoplasm, duodenal-type FL, testicular FL, ad diffuse FL. In addition, separate entities, such as pediatric-type FL, large B-cell lymphoma with IRF4 rearrangement, and primary cutaneous follicle center lymphoma have been recognized (WHO 2017). However, besides these well-defined forms, other aspects of inter- and intra-tumor heterogeneity are evident at morphologic, immunophenotypic, genetic, and clinical levels. If correctly recognized and interpreted, these differences might represent the starting point for a tailored treatment. Particularly, the analysis of the genetic profile of tumor cells highlights important relationships between specific genetic lesions and tumor initiation, progression, and transformation.

Green and coworkers have proposed an elegant multistep model to explain FL tumorigenesis in which founder mutations turn a non-malignant B cell clone into a premalignant tumor cell population, stable enough to acquire one or more secondary driver mutations, leading to an early malignant clone. Finally, tertiary mutations may either act as passenger or accelerator mutations, the latter providing a selective advantage to a progressed malignant subclone (Green 2013). In this context, the translocation (14;18)(q32;q21) is considered the genetic hallmark of FL and is reported with a prevalence of 85–90% in most published literature (WHO 2017). Subsequent aberrations of chromatin modifiers, genes involved in B-cell development, JAK-STAT and NF-κB signaling, as well as interactions with tumor microenvironment play a major role in the genesis, progression, and transformation of FL (Okosun 2014) (figure 8).

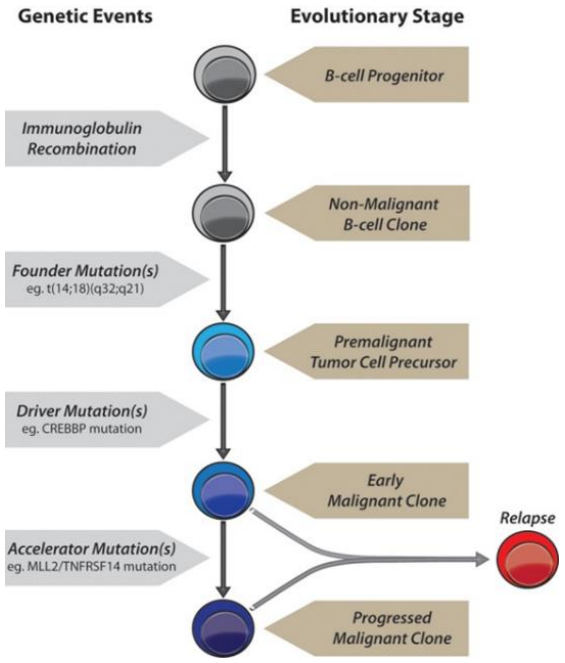

Figure 8. *Genetic evolution of FL from early development to progression and transformation (from Green 2013).*

The t(14;18) seems to occur in bone marrow immature pre-B cells due to erroneous V(D)J recombination, resulting in constitutive expression of the BCL2 antiapoptotic protein. BCL2 overexpressing B cells can survive irrespective of their B cell receptor (BCR) affinity, with the risk of acquiring additional genetic aberrations and a fully malignant phenotype (Sungalee 2014). Intriguingly, it has been demonstrated that both naïve and antigen-experienced t(14;18)+ B lymphocytes can be detected at low levels in the peripheral blood of up to 70% of healthy adults, most of which will never develop overt FL (Schüler 2009, Roulland 2006). Thus, the IGH-BCL2 translocation has been considered necessary, although not sufficient to promote lymphomagenesis by itself.

BCL2 negative FL

As previously stated, the translocation (14;18)(q32;q21) is considered the genetic hallmark of FL (WHO 2017). However, some authors have observed a proportion of FL lacking t(14;18) as high as 50% in their series, suggesting the existence of marked geographical differences and alternative mechanisms of genetic deregulation in BCL2- cases. In detail, reported detection rates are significantly lower in Far East and, to a less extent, European studies, compared to United States (US) series (Pezzella 1990, Albinger-Hegyi 2002, Biagi 2002, Payne 2011). It has been suggested that

these discrepancies may be technical rather than real, due to the use of methods unable to detect rearrangements involving *BCL2* sequences outside of the major breakpoint region (MBR) and minor cluster region (mcr) (Aster 2002, Guo 2005). However, most false-negative results have been eliminated using more sensitive FISH methods, which are able to detect variant rearrangements that are missed by G-banding cytogenetics and PCR-based methods (Chang 2013). Some authors have pointed out that some cytogenetic changes may be missed by FISH analyses as well, such as translocations between *BCL2* gene and unusual partner or cryptic translocations not detected by commercially available probes. However, this represents a rare occurrence (Godon 2003, Bentley 2005). In a meta-analysis, Biagi and Seymour observed that the relative lower incidence of FL registered in Eastern populations seems not to depend on a lower frequency of BCL2 rearrangements in healthy individuals, but rather on distinct molecular pathways operating in different geographical areas (Biagi 2002). On the other hand, some Asian researchers noted that the proportion of circulating lymphocytes carrying the t(14;18) in healthy subjects was much less prevalent than in the US, suggesting that ethnic disparity begins at a very early stage of disease development (Yakusawa 2001, Wu 2016). These findings do not exclude the original hypothesis that Western and Asian FLs represent heterogeneous entities, with different molecular pathogenesis. These discrepancies seem to be due more to lifestyle and environmental exposures rather than to ethnic background, as suggested by epidemiological data derived from Asian emigrants to the US and their descendants (Herrinton 1996, Wu 2019). Among others, high caloric intake, cigarette smoke and chemical agents such as pesticides, solvents, hair dyes, arsenic and compounds, asbestos, diesel fuel, nitrate, nitrite, or nitrosamine, have been called into question (Biagi 2002, Pan 2005, Richardson 2008, Zhang 2008, Cocco 2010), but the precise nature of such putative factors is far from being elucidated.

It is well-established that nodal grade 1–3A FLs without t(14;18) are morphologically indistinguishable from their translocated counterpart, but they are variably characterized by weak or loss of CD10 expression, increased Ki-67 labeling, higher MUM1 and granzyme B immunoreactivity and occasional CD23 positivity in lymphoma cells (Leich 2009). Moreover, this subset revealed a characteristic miRNA expression profile indicating a late GC B cell phenotype. Accordingly, GEP analyses documented an enrichment of GC B cell associated signatures in t(14;18)+ FL, whereas ABC-like, NFkB, proliferation, and bystander cell signatures were enriched in negative cases (Leich 2011). Taken together, these findings demonstrate distinct molecular features between the two subsets, however, they do not shed light on the molecular pathogenesis of t(14;18)-negative FL. In this regard, it is important to remember that BCL2 protein overexpression has been recorded in a subset of FL

lacking t(14;18) (Falini 2002, Horsman 2003). Many of them showed extra copies of chromosome 18, which may implicate an increased dosage effect, and alternative mechanisms may operate in the remaining unamplified cases (Horsman 2003).

In an attempt to identify pro-survival signals alternative to BCL2 overexpression, increased levels of some proteins such as Bcl-XL and activation of Akt/Bad pathway have been preferentially described in t(14;18)-negative FL (Ambrosini 1997, Zha 2004), despite no convincing deregulation of anti-apoptotic proteins alternative to BCL2 has been demonstrated so far.

BCL6 gene encodes a transcriptional repressor whose oncogenic effect is well-recognized (Albagli-curiel 2003, Saito 2007). 3q27/BCL6 rearrangement has been variously reported as a transforming and proliferating stimulus alternative to the classic BCL2 deregulation in high grade FL (Katzenberger 2004, Guo 2005) or in low grade disease (Marafioti 2013). Others again have challenged its putative role as a crucial pathogenetic factor in BCL2-negative FL (Karube 2008, Leich 2009).

Katzenberger and coworkers identified a distinctive subtype of t(14;18)-negative FL, characterized by a predominantly diffuse growth pattern, localized involvement of inguinal lymph nodes and 1p36 deletion (Katzenberger 2009). Aberrations of this chromosomal region have been reported in BCL2- morphologically classical FL with a predominantly follicular growth pattern, but it should be noted that they represent one of the most common alteration in classical *BCL2*+ FL too (Kridel 2012, Launay 2012).

Finally, an important player in clonal heterogeneity of FL is represented by activation-induced cytidine deaminase (AID), a gene whose product is required in the highly specialized GC microenvironment for both somatic hypermutation (SHM) and class switch recombination (CSR) to generate high affinity antibodies (Muramatsu 2000). In a small series of 2 and 3A FLs, Gagyi and coworkers found no differences in terms of ongoing SHM of the IGVH genes, aberrant SHM and AID expression between cases without *BCL2* gene rearrangement and protein expression and lymphomas carrying the t(14;18). The authors hypothesize that, besides different molecular alterations at the starting point of lymphomagenesis, BCL2-positive and BCL2-negative FL represent the same entity sharing several molecular pathways, as in both cases the immunoglobulin receptor complex provides additional signals required for malignant transformation (Gagyi 2008).

Fluorescent in situ hybridization (FISH)

FISH is a powerful technique firstly introduced in the early 1980's to detect and localize specific DNA sequences on chromosomes using fluorescent-labelled DNA probes (Langer-Safer PR 1982). These probes are single-stranded DNA sequences, previously marked with fluorochromes, that are complementary to target regions, genes or genetic sequences of interest.

FISH can be used both as a stand-alone technique on archived material, as it does not require cells in division (interphasic FISH), and in conjunction with chromosome banding to aid karyotype characterization. Interphasic FISH can be applied to different types of tumor nuclei, including imprinted nuclei, nuclei obtained from conventional cytogenetic procedures, frozen nuclei, paraffin-embedded nuclei, and nuclei extracted from paraffin-embedded sections. As such, it may allow the simultaneous assessment of chromosomal aberrations, cellular phenotype and tissue morphology. Despite its high sensitivity and versatility, FISH is an indirect cytogenetic method and needs accurate controls to have adequate specificity.

For the detection of chromosomal translocations, a variety of FISH probe strategies are available, each with its own limitations and benefits (Tibiletti 2007). Dual-color single-fusion probes are useful in detecting high percentages of cells harboring a specific chromosomal translocation. The DNA probe hybridization targets are located on one side of each of the two genetic breakpoints (generally one probe labeled in red and one in green). A nucleus lacking the translocation will exhibit a two-red, two-green signal pattern, whereas in a positive cell a yellow fusion in addition to single red and green signals corresponding to the normal alleles will be observed (figure 9A). Dual-color break-apart probes are designed on the opposite sites of a known genetic breakpoint which has multiple translocation partners. Each probe is labelled in a different color (generally red and green). In interphases of normal cells, two yellow fusion signals will appear, whereas following a translocation one separate green signal and one separate red signal, together with a preserved fusion signal will be observed (figure 9b). Finally, dual-color dual-fusion strategy is useful in detecting low numbers of nuclei harboring a simple balanced translocation. Two differently labelled probes (generally red and green) are designed to span the breakpoint of the genes involved in the translocation. Two fusion signals (derivative chromosomes), in addition to one green and one red signal (normal chromosomes), will be detected when a specific translocation involving both genes is present (figure 9c).

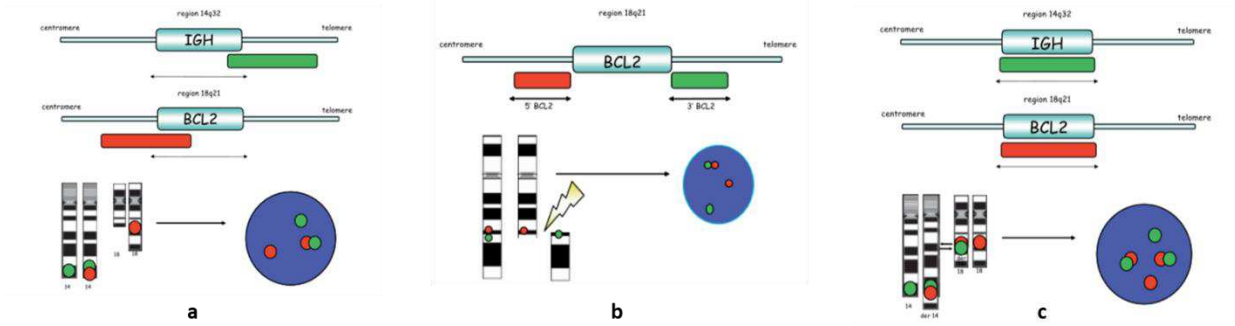

Figure 9. *Probes strategies. Dual-color single-fusion (a); dual-color break-apart (b); dual-color dual-fusion (c). See text for explanations* (from *Tibiletti 2007*).

Commercial probes are currently available to detect chromosomal abnormalities relevant to the pathogenesis of lymphoproliferative disorders. For instance, the translocation (14;18)(q32;q21) is considered the genetic hallmark of FL, as previously stated. Moreover, about 20% to 30% of DLBCLs carry a t(14;18), these cases being predominantly centroblastic in morphology and belonging to the GCB subgroup (Copie-Bergman 2009, Tibiletti 2009, Akyurek 2012, Visco 2013). FISH analysis is clearly superior to PCR-based assays in investigating *BCL2* translocations, due to breakpoints heterogeneity (Espinet 2008). Rearrangements of the 3q27 locus, where *BCL6* maps, have been described in approximately 1/3 of DLBCL (Copie-Bergman 2009, Tibiletti 2009, Akyurek 2012) and have also been detected in FL (Díaz-Alderete 2008). *MYC* chromosomal aberrations have been observed in up to 15% of DLBCL, usually associated with complex karyotypes and poor outcome (Copie-Bergman 2009, Tibiletti 2009, Akyurek 2012, Visco 2013). Finally, the aforementioned DH/TH lymphomas are defined by the presence of rearrangements and breakpoints at the sites of both *MYC* and *BCL2* and/or *BCL6*, as detected by cytogenetic techniques.

The WHO classification of tumors of hematopoietic and lymphoid tissues emphasizes the importance of assessing chromosome abnormalities for accurate diagnosis, appropriate treatment and monitoring response to therapy. In the era of personalized medicine, patients are categorized into several groups based on their tumor characteristics. FISH represents a pivotal tool in identifying biomarkers that may predict the natural history of the disease and allow the administration of more precise therapy for different patients.

AIMS

A plethora of immunohistochemical and molecular biomarkers have been identified and can be used nowadays both at a speculative level to clarify details regarding lymphomagenesis and for practical purposes in the diagnostic workup, to predict the outcome and properly treat patients with personalized target therapies. However, it is crucial to understand when and how they can be integrated into the clinical setting, translating experimental results from bench to bedside, with the aim of improving patients' care.

We decided to investigate the role of some of these biomarkers in two subtypes of NHL which still represent a challenge for both researchers and clinicians.

The aim of the first part of my project was to evaluate the possible existence of differential immunophenotypic and genotypic profiles in DLBCLs arising at different primary EN sites. In addition, survival analyses were performed in order to identify possibly significant prognostic variables.

We next moved to FLs, with the aim of aim of testing the incidence of *BCL2*-negative cases in a series of Italian patients from the Insubric region. Moreover, we evaluated the clinico-pathological features and investigated alternative genetic aberrations of this subset.

MATERIALS & METHODS

Primary EN DLBCL

Case selection

One hundred and seventeen FFPE EN DLBCLs from 117 different patients were retrospectively collected from 6 institutes (Department of Pathology of the Ospedale di Circolo-University of Insubria, Varese, Italy; Surgical Pathology Division of the Ospedale A. Manzoni, Lecco, Italy; Department of Pathology of the Ospedale Valduce, Como, Italy; the Department of Pathology of the IRCCS MultiMedica, Milan, Italy; the Cantonal Institute of Pathology, Locarno, Switzerland; Centre de Pathologie, Strasbourg, France). The inclusion criterion was the diagnosis of DLBCL in an EN site, with no or only minor lymph node involvement at presentation (Zucca 1997, Vannata 2015). We excluded from further analyses cases with incomplete clinical data or scarce material, insufficient to undergo basic morphological, immunohistochemical and genetic characterization. As in case of a mediastinal mass most of the times we receive for the diagnosis small biopsy samples which do not satisfy such quantitative requirement, primary mediastinal LBCL are not present in our series. Moreover, cases primarily arising in the tonsils, in the spleen, in the bone marrow and in the thymus were excluded. Using these criteria, a total of 106 EN DLBCLs were included in our study.

Primary sites were as follows: 44 gastrointestinal, 21 testicular, 13 central nervous system (CNS), 9 head and neck (H&N), 8 cutaneous and 11 DLBCLs arising at miscellaneous sites (Table 1). A subset of data regarding 18 out of the 21 testicular cases were previously included in two papers, recently published by our group (Bernasconi 2014, Magnoli 2015).

SITE	NUMBER OF CASES
Stomach	34 (32,1%)
Testis	21 (19,8%)
Central Nervous System	13 (12,3%)
Head and Neck	9 (8,5%)
Cutis	8 (7,6%)
Small Bowel	6 (5,7%)
Large Bowel	4 (3,8%)
Bone	3 (2,8%)
Breast	2 (1,9%)
Thyroid Gland	2 (1,9%)
Adrenal Gland	1 (0,9%)
Pleura	1 (0,9%)
Retroperitoneum	1 (0,9%)
Urinary Bladder	1 (0,9%)
TOTAL	106 (100%)

Table 1. *106 EN-DLBCL detailed by site of origin.*

All cases were histologically reviewed by two pathologists (FM and SU), and the morphological diagnosis of DLBCL was confirmed according to the criteria of the WHO classification of tumors of the hematopoietic and lymphoid tissue (WHO 2017).

Clinical and follow-up data were obtained by consulting the files of the Oncology departments at the different Institutions. In detail, for all the patients we collected data regarding age at diagnosis, sex, the lymphoma site of origin, Ann Arbor stage, International Prognostic Index (IPI), therapy protocols, response to treatment and overall survival (OS), defined as the time from diagnosis to death from any cause. Moreover, survival results were compared to those we previously observed in a series of 71 primary nodal DLBCL (Uccella 2008).

The study has been performed in compliance with the Helsinki Declaration and with policies approved by the Local Boards of Ethics.

Immunohistochemical analysis

Immunohistochemistry was performed on 3-µm-thick sections using the antibodies listed in table 2, either with an automated immunostainer (Benchmark XT; Ventana Medical Systems, Tucson, AZ) or manually.

Staining results were evaluated semiquantitatively. In detail, a cutoff value of 30% of immunoreactive tumor cells was used to consider a case positive for CD10, BCL6 and MUM1, and to classify cases as GC or non-GC subtypes, as originally proposed by Hans and coworkers (Hans 2004).

BCL2 and MYC expression were interpreted as positive if ≥50% and ≥40% of all lymphoma cells were immunoreactive, respectively. On the base of these results, we assigned each patient a double expressor score (DES) that ranged from 0 to 2, giving one point for each of the two markers expressed at or above the selected cutoff values.

ANTIBODY	M (CLONE)/P	DILUITION	MANUFACTURER
CD3	M (2GV6)	Ready to use	Ventana, Tucson, AZ
CD10	M (SP67)	Ready to use	Ventana, Tucson, AZ
CD20	M (L26)	Ready to use	Ventana, Tucson, AZ
BCL2	M (124)	Ready to use	Ventana, Tucson, AZ
BCL6	M (GI191E/A8)	Ready to use	Ventana, Tucson, AZ
Cyclin D1	M (SP4)	Ready to use	Ventana, Tucson, AZ
Ki-67	M (MIB1)	1:100	DakoCytomation, Carpinteria, CA
MUM1	MUM1p	1:50	DakoCytomation, Carpinteria, CA
MYC	M (Y69)	Ready to use	Ventana, Tucson, AZ

Table 2. *List of the antibodies used in the immunohistochemical analyses. M: monoclonal; P: polyclonal.*

FISH analysis

In a subset of 58 cases including 15 testicular, 11 CNS, 14 gastrointestinal, 6 head and neck, 5 cutaneous and 7 DLBCLs arising at miscellaneous EN sites, interphasic FISH was performed on sections used for conventional histologic examination (3–4 µm). Two slides respectively obtained before and after the one used for FISH analysis were hematoxylin-eosin stained to confirm the presence of an adequate neoplastic cellularity. Section thickness was established considering the cytoarchitectural organization and overlapping of nuclei in DLBCL. In our lab experience, 3 to 4 µm represents a good compromise between loss of evaluable spots due to nuclei truncation and overlapping.

Probes for split-signal FISH targeting *BCL2*, *BCL6*, *MYC*, *BCL10*, *CCND1*, and *MALT1* genes were provided by Dakocytomation Denmark A/S (Copenhagen, Denmark) and the FISH experiments were carried out as described elsewhere (Tibiletti 2004, Tibiletti 2009). Besides BCL2, BCL6 and MYC, whose importance in the genetic landscape of DLBCLs is well established, we speculatively decided to study BCL10, CCND1 and MALT1 genes similarly to what we did in a series of nodal DLBCL previously published by our group, due to their role in lymphomagenesis (Uccella 2008). Briefly, the analysis was performed using direct viewing on a standard fluorescence microscope (Leica DMRA) at 100x magnification. The presence of rearranged alleles was defined when the distance between red and green spots exceeded three times the fusion signal diameter.

Each FISH experiment was analyzed blindly by two independent operators. In each case, more than 100 nuclei on PE sections were examined from at least 5 to 8 areas with well-preserved cellular and nuclear morphology. Only experiments with 100% hybridization efficiency were considered. The threshold values for the presence of specific chromosome rearrangements, trisomies and polysomies of the hybridized chromosome regions were evaluated for each probe on a panel of 10 PE sections of hyperplastic lymph nodes showing normal karyotypes by conventional cytogenetics. Reactive nodes were preferred to tonsils because the architectural organization of their nuclei is more similar to the one observed in DLBCL. The cutoff points were set as the mean value plus 3 standard deviations of nuclei showing split signals or 3, 4, and more than 4 spots. The standard deviation was calculated assuming a binomial distribution of the spots. Resulting cutoffs were 1,3%, 18,3%, 1,7% and 0,0% for chromosomal rearrangements, trisomies, tetrasomies and polysomies, respectively.

BCL2 negative FL

Case selection

Eighty-three consecutive nodal FLs diagnosed in our Pathology Department between 2013 and 2016 were retrospectively collected. After excluding 7 cases with scarce material, a total of 76 FLs were further characterized in our study.

All cases were histologically reviewed by two pathologists (FM and SU), and the morphological diagnosis of FL was confirmed according to the criteria of the WHO classification of tumors of the hematopoietic and lymphoid tissue (WHO 2017).

Clinical and follow-up data were obtained by consulting the files of the Oncology department at our Institution. In detail, for all the patients we collected data regarding age at diagnosis, sex, the lymphoma site of origin, Ann Arbor stage, Follicular Lymphoma International Prognostic Index (FLIPI) and OS. Epidemiologic data on birthplace and migration flows were obtained from the registry offices at the municipalities of residence.

The study has been performed in compliance with the Helsinki Declaration and with policies approved by the Local Boards of Ethics.

Immunohistochemical analysis

Immunohistochemistry was performed on 3-µm-thick sections using the antibodies listed in table 3, either with an automated immunostainer (Benchmark XT; Ventana Medical Systems, Tucson, AZ) or manually.

ANTIBODY	M (CLONE)/P	DILUITION	MANUFACTURER
CD3	M (2GV6)	Ready to use	Ventana, Tucson, AZ
CD10	M (SP67)	Ready to use	Ventana, Tucson, AZ
CD20	M (L26)	Ready to use	Ventana, Tucson, AZ
BCL2	M (124)	Ready to use	Ventana, Tucson, AZ
BCL6	M (GI191E/A8)	Ready to use	Ventana, Tucson, AZ
CD21	M (EP3093)	Ready to use	Ventana, Tucson, AZ
CD23	M (SP23)	Ready to use	Ventana, Tucson, AZ
Ki-67	M (MIB1)	1:100	DakoCytomation, Carpinteria, CA
MUM1	MUM1p	1:50	DakoCytomation, Carpinteria, CA

Table 3. *List of the antibodies used in the immunohistochemical analyses. M: monoclonal; P: polyclonal.*

FISH analysis

Interphasic FISH was performed on FFPE sections used for conventional histologic examination (3–4 µm), as described elsewhere (Tibiletti 2004, Tibiletti 2009).

Commercial break apart probes targeting *BCL2*, *IGH*, *BCL6* and *MYC* were provided by ZytoVision GmbH (Bremerhaven, Germany). Moreover, a subset of these cases was also investigated with an alternative BCL2 break apart probe provided by Vysis (Abbott Laboratories, Abbott Park, IL, Usa) at the Cantonal Institute of Pathology, Locarno, Switzerland.

In a subset of 32 cases, FISH experiments were performed both on FFPE sections and on nuclei obtained from chromosome preparation of fresh lymphoma samples.

Dual-color break-apart probes are designed on the opposite sites of a known genetic breakpoint which has multiple translocation partners. Each probe is labelled in a different color (red and green). In interphases of normal cells, two yellow fusion signals will appear, whereas following a translocation one separate green signal and one separate red signal, together with a preserved fusion signal will be observed (figure 9b, Introduction).

Karyotype reconstruction analysis was performed in 11 FLs using direct chromosome preparations, as previously described by our group (Tibiletti 1996). A minimum of 10 metaphases were analyzed for each case and chromosome abnormalities were defined according to the most recent International System for Chromosome Nomenclature (ISCN) recommendations (ISCN 2016). When different tumor cell populations were identified, the chromosome complement of each population was described.

Statistical analysis

Associations in 2-way tables were tested for statistical significance using either the X^2 test or Fisher exact test (2-tail), as appropriate.

OS was defined as the time from the date of diagnosis until death from any cause or until the last date of follow-up. Patient survival was evaluated using the Kaplan-Meier method and statistically tested with the log-rank test.

All analyses were performed using GraphPad Prism V5.0 software. A p value ≤0.05 was considered statistically significant.

RESULTS

Primary EN DLBCL

Clinico-pathological results

The summary of the clinico-pathological results is reported in table 4. The median age at diagnosis was 71 years (range 28-94 years). Sixty-two patients were males and 44 females.

Most lymphomas in our series were confined to one side of the diaphragm and were classified as Ann Arbor stage I and II (59/83, 71.1%); in the remaining 24 cases (28.9%) more advanced disease was classified as stage III and IV. An International Prognostic Index (IPI) lower than 3 was observed in most cases (57/80, 71.25%), whereas high-intermediate and high scores were attributed to the remaining 23 patients (28.75%).

Most patients received R-CHOP immunochemotherapy, alone or in combination with surgery (77/87, 88.5%), whereas only 10 were treated with either involved field radiotherapy or surgical resection alone. First line therapy achieved a complete remission in the majority of patients (71/80, 88.75%). During the follow-up period, 22 of them (27.5%) experienced a relapsing disease, the highest rate of relapse being observed in intestinal DLBCLs (40%).

Follow-up time ranged from 1 and 122 months. Survival data, available in 96 cases, showed that 45 (46.9%) patients were dead, while 51 (53.1%) were still alive at last follow up, with a median overall survival of 47.5 months from the first diagnosis. In detail, 40 out of 96 patients died of disease and 5 died of other cause.

Patients' characteristics	Whole series (n=106)	Stomach (n=34)	Intestine (n=10)	H&N (n=9)	CNS (n=13)	Testis (n=21)	Skin (n=8)	Other sites (n=11)
Age at diagnosis								
- Median age	71	69	75	82	66	69	75.5	66
- Range	28-94	28-87	47-82	54-88	31-80	34-94	35-85	36-87
Sex								
- Male	62	18	7	4	5	21	4	3
- Female	44	16	3	5	8	0	4	8
Stage								
- I-II	59	23	6	3	2	12	7	6
- III-IV	24	9	1	3	3	4	0	4
- Not known	23	2	3	3	8	5	1	1
IPI								
- Low (0/1)	41	20	2	1	2	6	6	4
- Low-intermediate (2)	16	7	1	2	2	2	1	1

- High-intermediate (3)	17	4	3	2	1	2	0	5
- High (4)	6	1	0	0	0	5	0	0
- Not known	26	2	4	4	8	6	1	1
Therapy								
- Surgery only	5	0	1	0	0	4	0	0
- CHT* only	47	28	1	4	2	0	5	7
- Surgery + CHT*	30	5	5	1	2	12	1	4
- RT#	5	1	1	1	1	0	1	0
- Not known	19	0	2	3	8	5	1	0
Response to first line therapy								
- Complete remission	71	30	6	5	4	11	6	10
- Partial remission	2	1	0	0	0	0	1	0
- Progression	7	3	1	0	1	0	0	1
- Lost	26	0	3	4	8	10	1	0
Follow-up:								
- Alive without disease	47	20	3	3	1	8	5	8
- Alive with disease	4	2	1	0	0	0	1	0
- Dead of disease	40	9	4	4	10	10	0	3
- Dead of other cause	5	2	0	0	0	1	0	0
- Not known	10	1	2	2	2	2	2	0
Median OS (months)	47.5	57.5	25	27	19	21	63	72

Table 4. Clinico-pathological features of 106 DLBCLs. CHT: chemotherapy; RT: radiotherapy.

*R-CHOP or R-CHOP- like regimens; Methotrexate for CNS DLBCL; #independently from other therapy

Morphology, immunohistochemistry, COO and DES

All cases showed a diffuse proliferation of large or medium-sized B lymphocytes, with a nuclear size greater than or equal to that of a histiocyte nucleus, or more than twice the size of a small lymphocyte. They were diagnosed as DLBCL, according to the typical histological and immunophenotypic features based on the WHO classification of Tumors of Haematopoietic and Lymphoid Tissue (WHO 2017), most of them belonging to the centroblastic variant (figure 10).

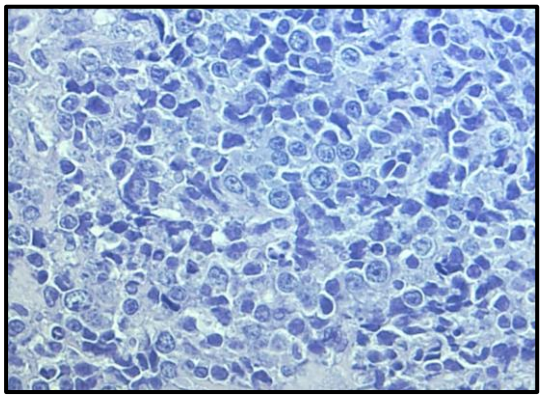

Figure 10. DLBCL, centroblastic cytologic variant. The cells have vesicular chromatin and often membrane-bound nucleoli (Giemsa stain, X400).

Immunohistochemical results are detailed in table 5. Considering the antibodies included in Hans' algorithm, CD10, BCL6 and MUM1 positivity was found in 20 (18.9%), 83 (78.3%) and 72 (67.9%) out of 106 cases, respectively. According to these results, we classified 41 (38.7%) DLBCLs as GC type and 65 (61.3%) as non-GC type and observed a significant predominance of the GC type in gastrointestinal tumors compared to other sites (p=0.008), whereas the non-GC type was more frequent in the immunological sanctuaries (CNS and testis) (p=0.0026). Most GC type lymphomas had a DES score of 0; conversely, most non-GC DLBCLs had an immunohistochemical score of 1 or 2 (p=0.018). BCL2 expression was observed in half of our cases (54/106, 50.9%), with significantly different distribution according to the site of origin. In detail, all cervico-cephalic DLBCLs, three quarters of the testicular DLBCLs and half of the intestinal DLBCLs were BCL2 immunoreactive, whereas CNS, gastric and cutaneous cases showed BCL2 positivity in a lower percentage of cases (p=0.003). MYC expression was evaluable in a subset of 99 cases, 18 (18.2%) of which turned out to be positive. The statistical analysis demonstrated that lymphomas arising in the head and neck district had the highest expression of MYC protein compared to both the whole series and the other sites singularly (p<0.05). Ninety-nine of our 106 DLBCL were assessable for the DES index, as defined in the *Material and Methods* section. When grouping our cases according to the site of origin, we found a statistically significant difference in the distribution of the scores. Namely, DES 0 prevailed in gastrointestinal DLBCLs and DES 1 in lymphomas arising at immunological sanctuaries (CNS and testis), whereas most cases arising in the head and neck were attributed a DES 2 (p=0.02).

Site (n)	CD10 (%)	BCL6 (%)	MUM1 (%)	HANS		BCL2 (%)	MYC (%)	DES (%)		
				GC (%)	NON GC (%)			0	1	2
CNS (13)	2 (15.4%)	11 (84.6%)	11 (84.6%)	3 (23.1%)	10 (76.9%)	4 (30.7%)	1 (7.7%)	9 (69.2%)	3 (23.1%)	1 (7.7%)
Testis (21)	2 (9.5%)	15 (71.4%)	20 (95.2%)	3 (14.3%)	18 (85.7%)	16 (76.2%)	3 (15%)	4 (20%)	14 (70%)	2 (10%)
Stomach (34)	4 (11.7%)	24 (70.6%)	16 (47%)	17 (50%)	17 (50%)	12 (35.3%)	3 (10.7%)	17 (60.7%)	8 (28.6%)	3 (10.7%)
Intestine (10)	6 (60%)	9 (90%)	6 (60%)	7 (70%)	3 (30%)	5 (50%)	2 (20%)	4 (40%)	5 (50%)	1 (10%)
Skin (8)	3 (37.5%)	8 (100%)	6 (75%)	4 (50%)	4 (50%)	3 (37.5%)	2 (25%)	4 (50%)	3 (37.5%)	1 (12.5%)
H&N (9)	0 (0%)	5 (55.5%)	9 (100%)	0 (0%)	9 (100%)	9 (100%)	6 (66.7%)	0 (0%)	3 (33.3%)	6 (66.7%)
Other sites (11)	3 (27.3%)	11 (100%)	4 (36.4%)	7 (63.6%)	4 (36.4%)	5 (45.5%)	1 (9.1%)	5 (45.5%)	6 (54.5%)	0 (0%)
TOTAL (106)	**20 (18.8%)**	**83 (78%)**	**72 (67.9%)**	**41 (38.7%)**	**65 (61%)**	**54 (50.9%)**	**18 (18.2%)**	**43 (43.4%)**	**42 (42.4%)**	**14 (14.2)**

Table 5. Immunohistochemical analysis.

FISH results

Molecular cytogenetic results are detailed in table 6. Overall, *BCL2*, *BCL6*, *MYC*, *CCND1*, *BCL10* and *MALT1* gene rearrangements, including translocations and inversions, were identified in 34 (58.6%) of the 58 cases.

According to the nomenclature previously proposed by our group (Tibiletti 2009), in half of the cases (17/34), gene rearrangements were present as major clones (more than 5% of rearranged cells) whereas in 13 out of 34 cases minor rearranged clones were identified (1.3-5% of rearranged cells). In the remaining 4 cases (11.8%), coexistence of major and minor clones was observed.

BCL6 was the most commonly rearranged gene (39.5%), followed by *MYC* (20.9%), *BCL2* (14%), *CCND1* (11.6%), *BCL10* (9.3%) and *MALT1* (4.7%).

The genes more involved in minor clones were *BCL6* (6 cases) and *MYC* (4 cases).

Multiple rearrangements were detected in 7 cases (20.6%): *BCL6* and *CCND1* (4 cases); *BCL6* and *BCL10* (1 case); *BCL6*, *BCL10* and *MALT1* (1 case); *BCL2*, *MALT1* and *MYC* (1 case). In most of DLBCLs with multiple rearrangements (4/7, 57.1%), one major and one or more minor populations were observed in the same sample.

Considering the specific EN sites of origin, intestinal lymphomas showed the highest rate of gene rearrangements (89%), followed by H&N (67%), gastric (60%), testicular (60%), cutaneous (40%) and CNS (27%) DLBCLs. *BCL6* was the most commonly rearranged gene in all sites, with exception of the GI tract and the skin, where *MYC* and *BCL2* alterations prevailed, respectively. In DLBCLs arising in immune-privileged sites, i.e. CNS and testicular cases, we observed a higher frequency of minor rearranged clones than in other sites, with a trend towards statistical significance (p = 0,07).

Interphasic FISH analysis with probes of different chromosome regions also provided data for chromosome assessment of DLBCL. Polysomies were observed for all investigated regions and, assuming that the presence of polysomy for more than 3 chromosomal regions suggests a polyploid chromosome assessment, 16 out of 58 (27.6%) EN DLBCLs resulted to be polyploid, whereas 42 (72.4%) were classified as diploid. Polyploidy confirmed by FISH with centromeric probes coexisted with chromosome rearrangements in 10/16 cases. Interestingly, gene amplification was observed in 3 DLBCLs arising in the GI tract and involved *MYC* alone in 2 cases and both *MYC* and *MALT1* in one case. Two out of 3 *MYC* amplified cases also revealed a concomitant *MYC* rearrangement (figure 11).

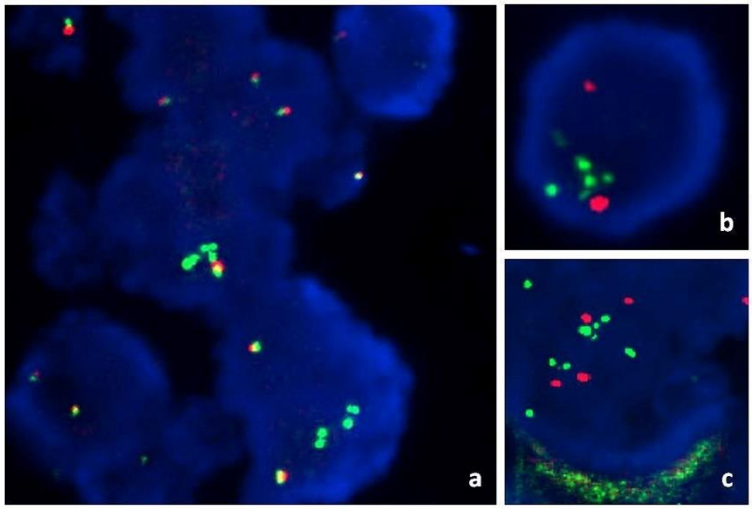

Figure 11. FISH analysis of case #38 using *MYC* break-apart probe (a) and dual color *MYC* (green)/chromosome 8 centromere (red) probe (b and c). These analyses demonstrated: a) *MYC* rearrangement and concomitant 5' *MYC* region deletion, b and c) Amplification of *MYC* region (green signals) compared to chromosome 8 centromere (red signals).

Case n°	*Site*	*FISH analysis*			*Immunohistochemical analysis*						
		Translocations	**Poliploidy**	**Amplifications**	***Bcl-2 (50%)***	***Myc [%]***	***DES***	***CD10 (30%)***	***Bcl-6 (30%)***	***MUM1 (30%)***	***HANS***
1	CNS	NO	YES	NO	-	25%	0	+	+	+	GC
2	CNS	NO	NO	NO	-	-	0	+	+	+	GC
3	CNS	NO	NO	NO	-	20%	0	-	+	+	nonGC
4	CNS	BCL6 (sc)	NO	NO	+	30%	1	-	-	+	nonGC
5	CNS	NO	NO	NO	-	10%	0	-	+	+	nonGC
6	CNS	NO	YES	NO	-	30%	0	-	+	+	nonGC
7	CNS	NO	NO	NO	+	60%	2	-	+	+	nonGC
8	CNS	NO	NO	NO	+	-	1	-	+	+	nonGC
9	CNS	NO	NO	NO	-	-	0	-	-	-	nonGC
10	CNS	BCL6, CCND1 (sc)	NO	NO	-	5%	0	-	+	+	nonGC
11	CNS	BCL10 (sc)	NO	NO	-	30%	0	-	+	+	nonGC
12	Testis	BCL6 (sc), BCL10, MALT1 (sc)	YES	NO	+	30%	1	-	+	+	nonGC
13	Testis	BCL6 (sc)	NO	NO	+	15%	1	-	+	+	nonGC
14	Testis	MYC (sc)	YES	NO	+	20%	1	-	+	+	nonGC
15	Testis	NO	NO	NO	+	-	1	-	+	+	nonGC
16	Testis	NO	NO	NO	-	20%	0	-	-	+	nonGC
17	Testis	BCL6	NO	NO	+	30%	1	-	+	+	nonGC
18	Testis	BCL6	YES	NO	+	-	1	-	-	+	nonGC
19	Testis	BCL6 (sc)	YES	NO	+	-	1	-	+	+	nonGC
20	Testis	NO	YES	NO	+	-	1	-	-	+	nonGC
21	Testis	BCL2 (sc)	NO	NO	+	40%	2	+	+	+	GC
22	Testis	NO	NO	NO	+	-	1	-	+	+	nonGC

23	Testis	BCL6	NO	NO	-	-	0	+	+	+	GC
24	Testis	NO	NO	NO	+	60%	2	-	-	+	nonGC
25	Testis	NO	NO	NO	+	-	1	-	-	+	nonGC
26	Testis	BCL2, MALT (sc), MYC (sc)	YES	NO	+	5%	1	-	+	+	nonGC
27	Stomach	MYC	NO	NO	+	60%	2	+	-	+	GC
28	Stomach	BCL6, BCL10 (sc)	NO	NO	+	-	1	-	+	+	nonGC
29	Stomach	NO	NO	NO	+	10%	1	-	-	+	nonGC
30	Stomach	BCL6, CCND1	YES	MYC	-	-	0	-	+	+	nonGC
31	Stomach	NO	NO	NO	+	-	1	-	+	+	nonGC
32	Small bowel	BCL10 (sc)	YES	NO	+	-	1	+	-	-	GC
33	Small bowel	MYC (sc)	NO	NO	-	80%	1	+	+	+	GC
34	Small bowel	MYC	NO	MYC	+	80%	2	+	+	+	GC
35	Small bowel	BCL2	NO	NO	+	10%	1	+	+	-	GC
36	Small bowel	BCL6, CCND1 (inv)	NO	NO	-	-	0	-	+	+	nonGC
37	Colon	BCL6	NO	NO	-	-	0	-	+	-	GC
38	Colon	MYC	NO	MYC, MALT	-	-	0	+	+	+	GC
39	Colon	MYC (sc)	NO	NO	+	30%	1	-	+	+	nonGC
40	Colon	NO	NO	NO	-	20%	0	+	+	-	GC
41	Skin	NO	YES	NO	-	40%	1	-	+	-	GC
42	Skin	BCL2	NO	NO	-	-	0	+	+	-	GC
43	Skin	NO	NO	NO	-	7%	0	-	+	+	nonGC
44	Skin	NO	NO	NO	+	-	1	+	+	+	GC
45	Skin	BCL2 (sc)	YES	NO	-	-	0	+	+	+	GC
46	H&N	NO	YES	NO	+	70%	2	-	-	+	nonGC
47	H&N	CCND1 (sc)	YES	NO	+	40%	2	-	-	+	nonGC
48	H&N	BCL6	NO	NO	+	30%	1	-	+	+	nonGC
49	H&N	MYC	NO	NO	+	40%	2	-	+	+	nonGC

50	H&N	NO	YES	NO	+	50%	2	-	+	+	nonGC
51	H&N	BCL6	NO	NO	+	-	1	-	+	+	nonGC
52	Adrenal gland	BCL6	NO	NO	+	-	1	-	+	+	nonGC
53	Bone	BCL2	NO	NO	+	25%	1	+	+	-	GC
54	Mammary gland	BCL6 (sc)	YES	NO	+	-	1	-	+	-	GC
55	Retroperitoneum	NO	NO	NO	-	-	0	-	+	-	GC
56	Thyroid gland	MYC	NO	NO	-	70%	1	+	+	-	GC
57	Thyroid gland	BCL6 (sc), CCND1 (sc)	NO	NO	+	-	1	-	+	+	nonGC
58	Urinary bladder	NO	NO	NO	+	-	1	-	+	+	nonGC

Table 6. FISH analyses as compared with immunohistochemical results.
SC: small clone.

Comparison between immunophenotypic profile and chromosomal rearrangements

In the cytogenetically investigated subset of EN DLBCLs, the immunohistochemical algorithm identified 38 DLBCLs with the non-GC phenotype and 20 with the GC phenotype. Overall, the number of gene rearrangements detected in the two groups was not statistically different (p = 0.27). Multiple rearrangements occurred in a significantly higher proportion of non-GC DLBCLs (p = 0.03). *BCL6* rearrangements were significantly more frequent in non-GC than in GC lymphomas (p = 0.01). Conversely, *BCL2* rearrangements occurred in a higher portion of the GC cases, with a trend towards statistical significance (p = 0.06). No significant differences were found regarding *CCND1*, *MALT1*, *BCL10* and *MYC*, even though *CCND1* and *MALT1* alterations were exclusive of non-GC cases (5 and 2 cases, respectively). All four cases displaying the coexistence of major and minor clones belonged to the non-GC group.

As far as BCL2 was concerned, 34 (60.7%) out of 56 DLBCLs showed immunohistochemical positivity for this protein. This finding was not systematically related to the presence of *BCL2* rearrangements, as in the majority of cases (32 out of 56, 57.14%) we observed discordant results between immunohistochemistry and FISH analysis (namely, 30 DLBCLs were BCL2-immunoreactive without showing concurrent *BCL2* gene rearrangement and, vice-versa, 2 cases didn't stain with anti-BCL2 antibody but were positive by FISH). Among the 24 concordant cases, 20 turned out to be negative both for immunohistochemistry and FISH, whereas 4 were positive for both analyses. On the other hand, MYC immunohistochemical expression was well related with cytogenetic data (p = 0.02). In detail, 43 out of 54 cases (79,63%) were concordant (38 "double negative" and 5 "double positive); 7 were positive for immunohistochemistry but not at FISH analysis and 4 resulted cytogenetically rearranged without overexpression of MYC protein.

Concurrent translocation of both *BCL2* and *MYC* genes was present in a single DLBCL of the testis (double hit lymphoma), which also harbored *MALT1* rearrangement. Thirteen lymphomas showed rearrangement of either *BCL2* or *MYC*, whereas most patients (41/55, 74.5%) had no cytogenetic abnormality in the two genes. Immunohistochemical analysis of MYC and BCL2 expression expanded the proportion of DLBCLs with identifiable double hit biology from 2% (patients with a cytogenetically defined DHL) to 15% (patients with an immunohistochemically defined double expressor lymphoma).

Correlation with clinical outcome

Survival data were available for 103 patients and the median follow-up time was 37 months. Among clinical parameters, older age (>71 years), high IPI score (≥3) and high Ann Arbor stage (III-IV) were significantly related to unfavorable outcome ($p = 0.04$, $p < 0.0001$ and $p = 0.0003$, respectively).

The whole series of EN DLBCLs did not show significantly different overall survival, when compared to the previously published nodal series (Uccella 2008). However, when the cases were subdivided according to the site of origin, significant differences emerged. In fact, we found that cutaneous and gastrointestinal DLBCLs had a better overall survival than nodal DLBCL, whereas cases involving immunological sanctuaries (CNS and testis) and the cervico-cephalic district demonstrated a worse outcome ($p=0.0009$) (figure 12).

Among immunohistochemical markers, the expression of BCL2 and MUM1 carried a poor prognostic value ($p = 0.0390$ and $p = 0.0035$, respectively). MYC immunoreactive cases showed a trend to shorter OS. Univariate analysis showed patients in the DES 0 group to have better OS than patients in the DES 1 or 2 group. Interestingly, when applying the DES to patients with non-GC DLBCL or with low IPI scores (0 to 2), patients in the DES 2 group had inferior OS compared with patients in the DES 0/1 group. Conversely, DES was not prognostic in patients with GC DLBCL or high IPI score. GC subtype, as defined according to Hans' algorithm, was significantly associated with longer OS compared with non-GC cases ($p = 0.018$) (figure 12).

At univariate analysis, the presence of one or more gene rearrangements was not associated with survival, as well as the presence of polyploid clones. The presence of rearrangements involving single genes (*BCL2*, *BCL6*, *MYC*, *CCND1*, *BCL10* and *MALT1*) had no prognostic significance when considering the whole series. Only *MYC* translocation was associated with worse OS in gastrointestinal lymphomas ($p = 0.008$). The presence of either *MYC* or *BCL2* rearrangement significantly predicted worse outcome in gastrointestinal DLBCLs ($p = 0.02$) but did not carry any prognostic value in the whole series (figure 12).

Finally, multivariate analysis of the significant parameters revealed that the variables independently influencing OS were IPI score, the primary site of origin and MUM1 expression.

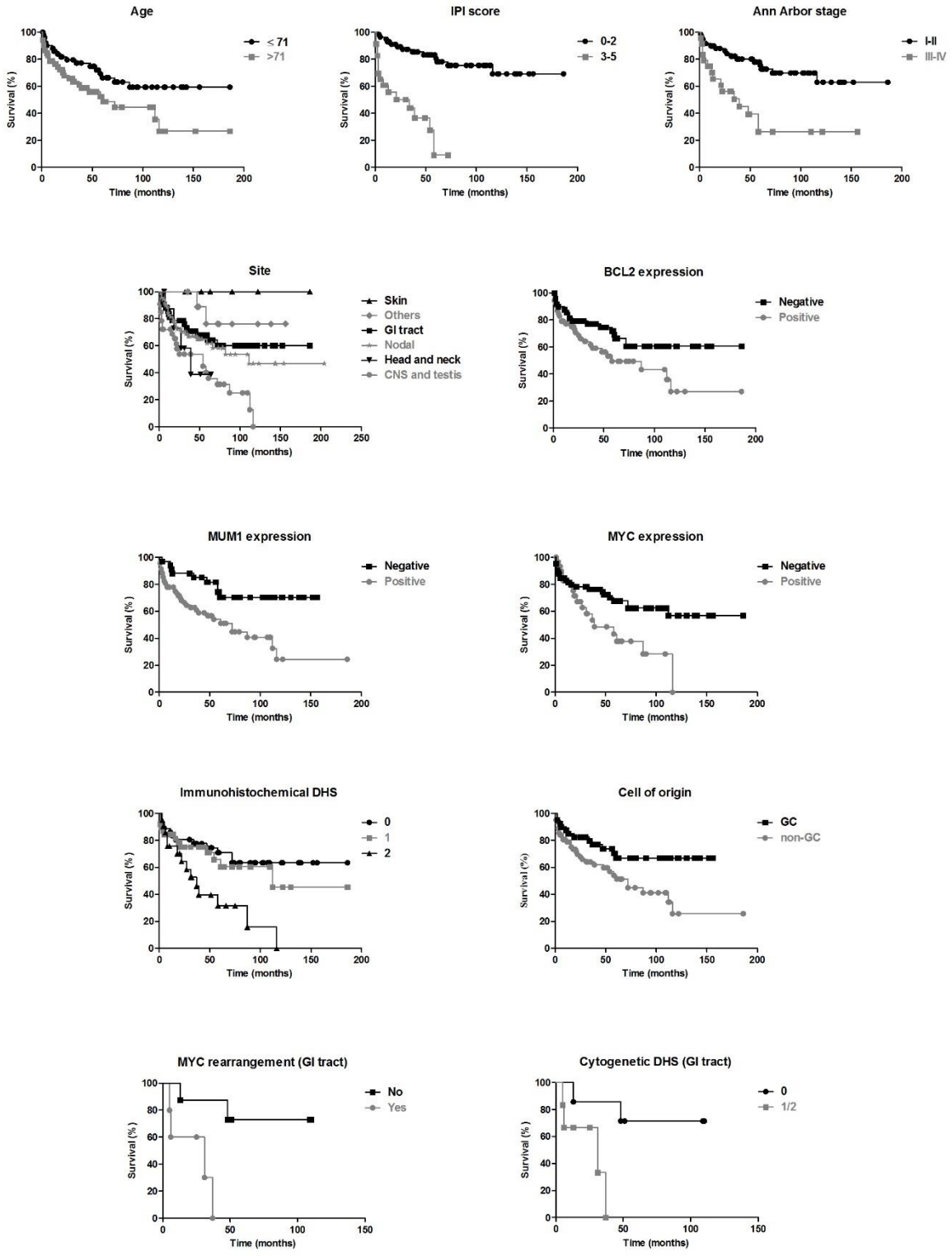

Figure 12. Overall survival according to clinical parameters, immunohistochemical and molecular cytogenetic results, and the site of origin.

BCL2 negative FL

Clinico-pathological results

The summary of the clinico-pathological results is reported in table 7. The mean age at diagnosis was 63.5 years (range 30-87 years). Thirty-two patients were males and 44 females.

Most lymphomas in our series presented at advanced Ann Arbor stages III and IV (46/61, 75.4%); in the remaining 15 cases (24.6%) the disease was confined to one side of the diaphragm and classified as stage I and II. Low FLIPI was observed in half cases (31/61, 50.8%), whereas intermediate and high scores were attributed to the remaining 16 (26.2%) and 14 (23%) patients, respectively.

	Grade 1/2 (51)	***Grade 3A (21)***	***Grade 3B (4)***	***Total (76)***
Age at diagnosis				
mean value	64.2	60	70.3	63.5
range	44-87	30-87	60-81	30-87
Gender				
Male	21	9	2	32
Female	30	12	2	44
Stage				
I-II	10	4	1	15
III-IV	30	15	1	46
not known	11	2	2	15
FLIPI				
low	21	10	0	31
intermediate	12	4	0	16
high	7	5	2	14
not known	11	2	2	15

Table 7. Clinico-pathological results of 76 FL.

As far as geographical data are concerned, despite the great variability in birthplaces, analysis of migration flows from the early Seventies documented that most (70 out of 76, 92%) of our patients were either born in or moved early in their life to the Insubric region. This area is located on the southern side of the European Alps, between Lombardy (Italy) and Canton Ticino (Switzerland), and it is bordered by Lake Maggiore, Lake of Como and Lake of Lugano (figure 13).

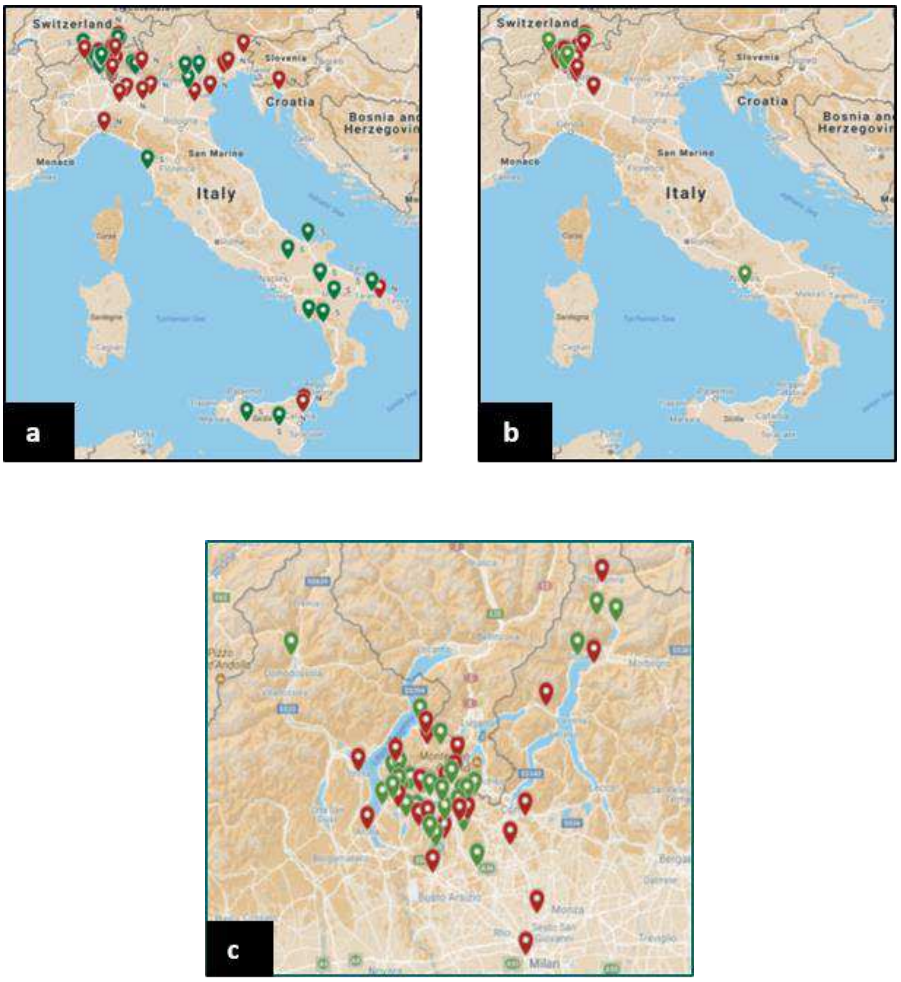

Figure 13. Birthplace (a) and residency at diagnosis (b) of patients in our series, with focus on the Insubric region were most of them were born or moved early (c). Red tags represent cases without *BCL2* translocation; green tags represent *BCL2+* cases. Maps created with Google Maps.

Morphological and immunohistochemical results

All cases in our series had morphological and immunophenotypic features typical for FL, including growth pattern, admixture of centroblasts and centrocytes within neoplastic follicles, and germinal center phenotype. In detail, most of them (62/75, 82.3%) showed a follicular architecture, whereas the remaining were either follicular and diffuse (8/75, 10.7%) or predominantly diffuse (5/75, 7%) (table 8). In one case we couldn't properly define the architecture of the neoplastic proliferation due to the scarcity of biopsy material.

PATTERN	PROPORTION of FOLLICULAR (%)	NUMBER of CASES
Follicular	>75	62 (81.6%)
Follicular and diffuse	25-75	8 (10.6%)
Predominantly diffuse	<25	6 (7.9%)

Table 8. Distribution of pattern of growth in our series of FL.

Tumor grade defined according to the method of Mann and Berard (Mann 1982) was assessed for all FL cases, as well (table 9). Overall, in our cohort, low-grade (grade 1-2) FL represented approximately 2/3 of cases, and grade 3A-3B FL the remaining 1/3.

GRADE	DEFINITION	NUMBER of CASES
Grade 1-2 (low grade)	0-15 centroblasts/HPF	51 (67.1%)
Grade 1	0-5 centroblasts/HPF	19 (25%)
Grade 2	6-15 centroblasts/HPF	32 (42.1%)
Grade 3	>15 centroblasts/HPF	25 (32.9%)
Grade 3A	centrocytes still present	21 (27.6%)
Grade 3B	solid sheets of centroblasts	4 (5.3%)

Table 9. Grading of FL in our series, according to WHO classification. HPF: high power field of 0.159 mm^2 (40X objective).

All cases expressed nuclear BCL6. The other germinal center associated protein, CD10, was detected in 68/76 FLs (89.5%). CD10-negative cases were equally distributed between low and high grade FLs, and all of them were positive for BCL2 immunostaining.

Overall, BCL2 protein was expressed in 66/76 cases (86.8%). The proportion of BCL2 positive lymphoma cells ranged from 20% to 100%. In detail, the frequency distribution showed in only 2 cases a value <40%, whereas in most FLs 53/76 (69.7%) ≥80% of neoplastic cells stained with the anti-BCL2 antibody. The cytoplasmic staining intensity of the tumor cells was compared with that of the admixed reactive T lymphocytes on the same slide and positive cases were classified into a 3-tiered score as follows: in 39/66 FLs (59.1%) tumoral B cells stained similar to reactive T cells and they were categorized as 3+; 10/66 FLs (15.1%) with weak immunostaining were classified ad 1+; 17/66 (25.8%) with intermediate intensity of BCL2 staining formed the 2+ subgroup (figure 14).

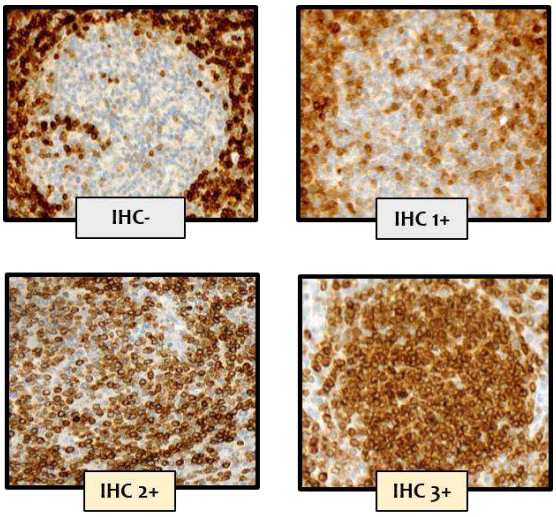

Figure 14. Evaluation of BCL2 intensity of staining with the anti-BCL2 (clone 124) antibody.

A strong positive linear dependence between the proportion of positive cells and the intensity of immunostaining was observed (r = 0,75). In contrast, no relationship between FL grade and either intensity of BCL2 staining or percentage of positive lymphoma cells was found.

Finally, as far as proliferation index is concerned, Ki-67 labeling index ranged from 6% to 70% (median value = 20%) and was <50% in all but three cases. Pearson linear correlation between Ki-67 and other variables, namely histological grade and percentage of BCL2 positive cells was poor (r = 0.4 and r = -0.2, respectively). Intriguingly, among pathological parameters, low proliferation index as assessed by Ki-67 rather than WHO-defined grade was related to BCL2 immunohistochemical expression (p = 0.02).

Molecular cytogenetic results

FISH on FFPE sections

FFPE sections used for conventional histologic examination were available for all cases. Overall, BCL2 rearrangements were detected in approximately half of FLs in our series (39/76, 51.3%), independently from histological grade (table 10 and table 16). This observation was confirmed in a subset of 29 FLs enriched in *BCL2*-negative cases, which were investigated with an alternative *BCL2* break apart probe provided by Vysis (Abbott Laboratories, Abbott Park, IL, Usa) at the Cantonal Institute of Pathology, Locarno, Switzerland (table 16).

GRADE	*BCL2* rearranged	*BCL2* not rearranged	TOTAL	*BCL2* polysomic	*BCL2* non polysomic	TOTAL
Grade 1-2	28 (55%)	23 (45%)	51	13 (25%)	38 (75%)	51
Grade 3A	11 (52%)	10 (48%)	21	14 (67%)	7 (33%)	21
Grade 3B	1 (25%)	3 (75%)	4	2 (50%)	2 (50%)	4
TOTAL	40	36	76	29	47	76

Table 10. Comparison among histological grade, *BCL2* rearrangements and polysomies.

Even if most 3B FLs did not show *BCL2* rearrangement, the sample size was too small to reach statistical significance. Polysomic cases were significantly prevalent among high grade FLs (p = 0.002) (table 10).

To further characterize *BCL2* translocations and identify its partner, we used a commercial break apart probe targeting *IGH*. In most cases (66/76, 87%) these two molecular cytogenetic aberrations either coexisted or were both absent in the same sample, corroborating, although indirectly, either the presence or the absence of t(14;18). The ten discordant cases behaved as follows: 6 were *IGH+/BCL2-*, 3 were *IGH+* in a percentage of neoplastic cells much higher than those carrying *BCL2* rearrangement and 1 was *IGH+* in a proportion of lymphoma cells much lower than those positive for *BCL2* translocation (table 11). Such discordant cases were further investigated with break apart probes targeting *BCL6* and *MYC*, since these genes are frequently involved in the pathogenesis and evolution of lymphoproliferative B cells disorders as *IGH* translocation partners. However, in only one of the 10 discordant cases we observed coexistence of *IGH* and *BCL6* rearrangements, suggesting the presence of a translocation involving these two genes.

Case	R-*BCL2*	R-*IGH*	R-*BCL6*	R-*MYC*	
4	-	50%	-	-	6 *IGH+/BCL2-*
26	-	80%	-	-	
52	-	100%	-	-	
68	-	40%	-	-	
71	-	100%	60%	-	
76	-	100%	-	-	
15	36%	80%	-	-	3 *IGH+>>BCL2+*
17	20%	85%	-	-	
62	25%	100%	-	-	
65	70%	35%	-	-	1 *IGH+<<BCL2+*

Table 11. Detail of 10 *BCL2/IGH* discordant cases, which were further analyzed for *BCL6* and *MYC* translocations.

FISH on fresh frozen lymphoma samples

In a subset of 32 cases, FISH experiments were replied on nuclei obtained from chromosome preparation of fresh lymphoma samples (table 16). Results were in agreement with those obtained on FFPE nuclei, with the exception of 3 cases. As detailed in table 12, these three discordant cases were *BCL2*-negative when analysis was performed on FFPE archived material, whereas turned out to be *BCL2*+ when the corresponding FL separated nuclei were investigated. However, in all 3 cases we were able to identify the possible reason why FISH analysis on FFPE sections did not identify *BCL2* rearrangement. Indeed, cases 33 and 68 showed only a minor percentage of translocated lymphoma nuclei on frozen samples. Moreover, in case 76, a "cryptic" *BCL2* translocation characterized by an atypical breakpoint difficult to be identified on FFPE samples, was found.

Case	R-*BCL2 FFPE*	R-*BCL2 fresh*	
33	-	28%	SMALL CLONES
68	-	14%	SMALL CLONES
76	-	60%	ATYPICAL BCL2 BREAKPOINT

Table 12. Comparison between FISH experiments on FFPE and fresh frozen nuclei, with focus on discordant cases.

Karyotype analysis

We could perform karyotype reconstruction in a subset of 11 cases (figure 15 and table 13).

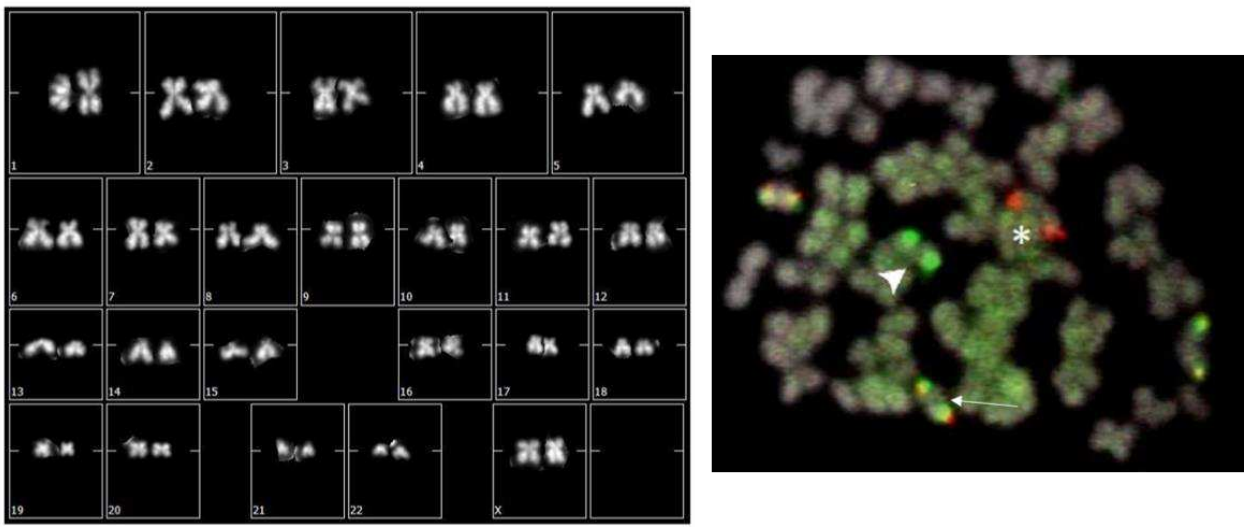

Figure 15. Patient N. 47: XX, t(14;18)(q34;q21) + der(18) t(14;18)(q34;q21). Karyotype reconstruction (left) and FISH analysis (right). The arrow indicates the normal copy of chromosome 18. The asterisk and the arrowhead show the derivative chromosomes.

Case	*R-BCL2 FFPE (%)*	*R-BCL2 fresh (%)*	*Karyotype*
13	0%	0%	46,XX[4]/46,XX,+der(1q)t(1p;10)[7]
17	20%	40%	46,XY
18	90%	95%	46,X,+3,t(14;18)(q21;q32)
20	60%	80%	46,XY,t(6;22)(p25;q12.1),t(14;18)(q32;q21)
21	55%	N.A.	47,XX,del(6)(q24qter),t(14;18)(q32;q21),+mar1[8]
39	55%	66%	46,XX,t(14:18)(q34;q21)[22] Nuc ish(BCL6X2)[100],(MYCX3)[71/100],(BCL2X2)(5'BCL2 sep3'BCL2X1)[66/100]
45	100%	N.A.	47,XY,t(14;18)(q34;q21),+mar1
47	100%	100%	46 XX,t(14;18)(q34;q21)[3]/47 XX,t(14;18)(q34;q21),+ der(18) t(14;18)(q34;q21)[9]/47 XX,t(1;2)(p34;p21),t(14;18)(q34;q21)+ der(18)t(14;18)(q34;q21)[9]
60	0%	0%	46,XX,t(3;6)(q27.3;p22)[4]/46,XX,t(3;6)(q27.3,p22),+14[4]. Ish t(3;6)(5'BCL6-,3'BCL6+; 5'BCL6+, 3'BCL6-). Nuc ish(BCL6X2)(5'BCL6 SEP 3'BCL6X1[33/150], (BCL2,MYCX2)[150]
64	80%	71%	46,XX[10]/46,XX,t(11,14)?del(11)(q11q22)[13]/49XXX, t(2;18)(p11;q21),+4,del(5)(q?),der(17)t(1;17)(q22-q25;q25), +der(18)t(2;18)(p11;q21)[23]
73	0%	0%	46,XY,del(3)(q27.3)(5'BCL6X1,3'BCL6X2)(5'BCL6, 3'BCL6X1)[5/8],nuc ish(BCL2X2)[100]

Table 13. *BCL2* rearrangements assessed on FFPE and fresh frozen nuclei and karyotype reconstruction in a subset of 11 cases. N.A. = not available

Conventional cytogenetic analysis allowed us to gain a deeper insight into the pathogenesis of *BCL2*-negative cases. In detail, in case 13 we detected the unbalanced translocation der(1q)t(1p;10), involving the 1p region where *TNFRSF14* gene is located. Moreover, in two FLs (case 60 and 73), *BCL6* locus involvement at 3q27.3 was observed. Finally, case 64 deserves a particular mention, because it corresponds to a patient in which two synchronous lymphoproliferative B cell disorders were diagnosed, namely FL and in situ mantle cell neoplasia. In this case, karyotype reconstruction allowed us to identify t(11;14) between *CCND1* and *IGH* genes, which is characteristic of *MCL*, together with t(2;18)(p11;q21) unbalanced translocation, involving *BCL2* and an alternative partner, different from *IGH*, represented by the *IGK* gene. The analysis of such an unusual case became the object of a distinct paper published by our group (Vivian 2019, submitted) (figure 16).

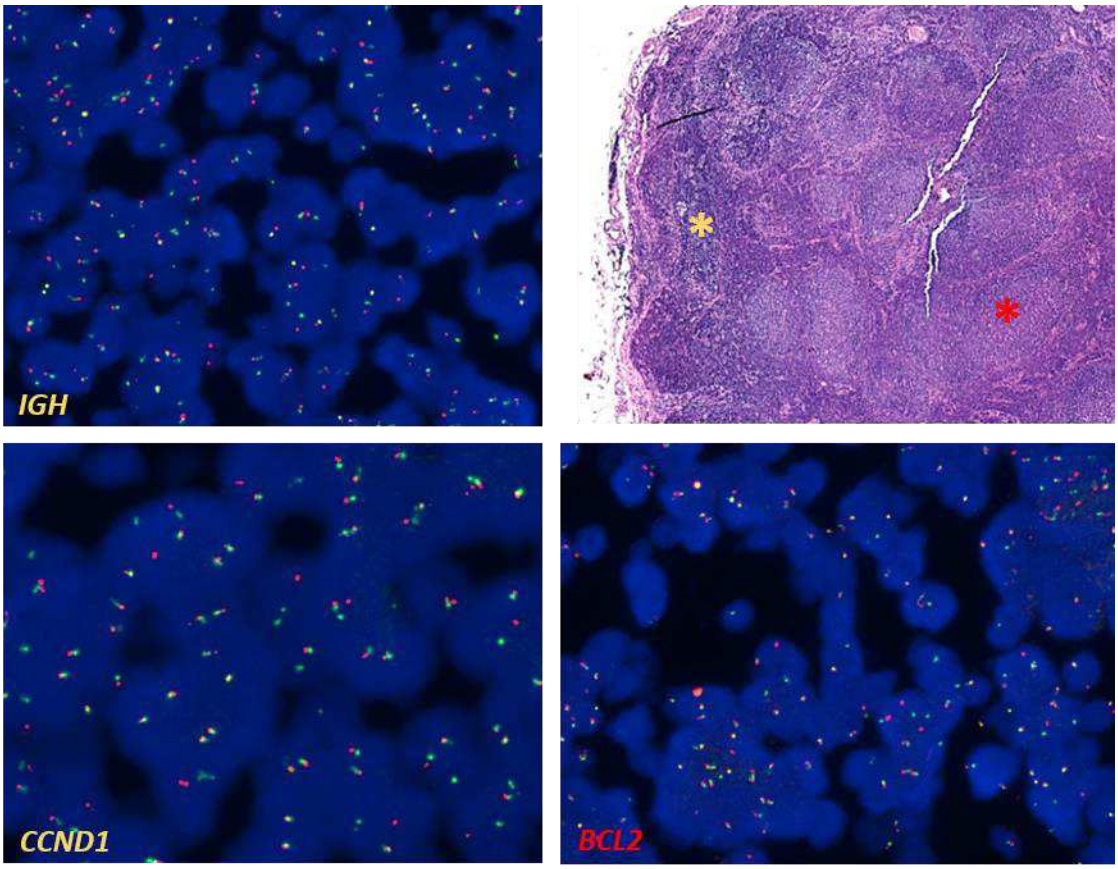

Figure 16. Patient 64. Hematoxylin and eosin showing the coexistence of FL (red asterisk) and ISMCN (yellow asterisk) in the same lymph node (X40). The corresponding FISH analyses in the two components show *BCL2* and *IGH* together *CCND1* rearrangements, respectively (X100).

Comparison between immunohistochemical and molecular cytogenetic results

As *BCL2* gene rearrangement results in overexpression of the corresponding oncogene, we next analyzed the relationship between immunohistochemical and molecular cytogenetic data. Overall, 47/76 (61.8%) cases showed concordant results (p = 0.02), as detailed in table 14.

	IHC-	IHC+	total
FISH-	8	27	35
FISH+	2	39	41
total	10	66	76

47 CONCORDANT CASES

Table 14. Comparison between FISH analysis of *BCL2* rearrangement and BCL2 immunohistochemical expression.

Among the remaining 29 discordant cases (38,2%), only 2 were negative at immunohistochemistry in presence of *BCL2* rearrangement as documented by FISH analysis, whereas most of them (27/29, 93.1%) turned out to express BCL2 protein without the corresponding gene translocation. Interestingly, if we consider FLs with weak BCL2 immunostaing (i.e. 1+) the same way as negative cases, concordance between the two methods dramatically increases ($p < 0.0001$) (table 15).

	IHC-	IHC 1+	IHC 2+	IHC3+	total
FISH-	8	9	9	9	35
FISH+	2	1	8	30	41
total	10	10	17	39	76

55 CONCORDANT CASES

Table 15. Comparison between FISH analysis of *BCL2* rearrangement and BCL2 immunohistochemical expression when grouping IHC1+ together with IHC negative cases.

In detail, 9 out of 10 (90%) IHC1+ cases were concordantly negative at FISH analysis, suggesting that weak immunohistochemical expression of the BCL2 protein is not predictive of *BCL2* chromosomal rearrangement. On the other hand, approximately one half (9/17, 52.3%) of IHC2+ cases and one quarter (9/39, 23.1%) of IHC3+ cases were FISH-, suggesting that BCL2 protein overexpression can be due to alternative mechanisms, different from gene translocation.

Correlation with clinical data and outcome

We found both BCL2 immunohistochemical expression and rearrangement to be significantly prevalent in patients with high Ann Arbor stage disease ($p = 0.023$ and $p = 0.026$, respectively), whereas no correlation was observed between BCL2 status and FLIPI.

Survival data were available for 61 patients and the median follow-up time was 52 months (range 12-273). Overall, only 7 patients died (11.5%), whereas most of them (88.5%) were alive at latest follow-up. Among clinical parameters, older age (>70 years), was significantly related to unfavorable outcome ($p = 0.005$), whereas high FLIPI score showed a trend towards statistically significant poor prognosis ($p = 0.09$).

Case	**BCL2** *IHC*	*R-BCL2 FFPE (%)*	*R-BCL2 fresh (%)*
1	95%	80%	N.A.
2	80%	70%	N.A.
3	100%	70%	N.A.
4	50%	-	N.A.
5	80%	-	N.A.
6	90%	-	N.A.
7	100%	70%	92%
8	80%	-	N.A.
9	100%	70%	69%
10	-	-	N.A.
11	20%	-	N.A.
12	90%	-	-
13	50%	-	-
14	100%	100%	100%
15	100%	36%	N.A.
16	-	100%	N.A.
17	90%	20%	40%
18	75%	90%	95%
19	70%	-	-
20	100%	60%	80%
21	100%	55%	N.A.
22	100%	70%	N.A.
23	100%	80%	75%
24	100%	-	-
25	70%	-	-
26	100%	-	N.A.
27	100%	60%	24%
28	100%	100%	N.A.
29	-	-	-
30	-	-	-
31	90%	70%	50%
32	60%	-	N.A.
33	90%	-	28%
34	90%	80%	N-A-
35	30%	-	-
36	-	-	N.A.
37	100%	70%	N.A.
38	90%	-	N.A.
39	100%	55%	66%
40	-	-	N.A.
41	100%	-	N.A.
42	90%	-	N.A.
43	80%	85%	N.A.
44	100%	85%	50%
45	90%	100%	N.A.
46	pos	-	N.A.
47	100%	100%	100%
48	pos	90%	N.A.
49	100%	-	N.A.

50	40%	-	N.A.
51	90%	100%	N.A.
52	90%	-	N.A.
53	-	-	-
54	-	100%	N.A.
55	100%	100%	N.A.
56	90%	80%	N.A.
57	100%	90%	N.A.
58	70%	-	N.A.
59	80%	-	N.A.
60	70%	-	-
61	100%	70%	N.A.
62	100%	25%	N.A.
63	100%	100%	N.A.
64	100%	80%	71%
65	80%	70%	N.A.
66	-	-	-
67	-	-	N.A.
68	70%	-	14%
69	80%	50%	N.A.
70	pos	100%	N.A.
71	80%	-	N.A.
72	100%	70%	N.A.
73	100%	-	-
74	95%	80%	100%
75	80%	-	-
76	100%	-	60%

Table 16. Comparison between immunohistochemical and FISH results.
Cases also investigated at the Cantonal Institute of Pathology, Locarno, are highlighted in light blue.

DISCUSSION

Personalized medicine is defined by the National Institute of Health (NIH) as a form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat diseases. This term was used for the first time in the way it is meant today on The Oncologist twenty years ago (Langreth 1999). Since then a plethora of biomarkers have been investigated in all cancer fields, both to clarify details regarding the pathogenetic mechanisms underlying neoplastic transformation and for practical purposes. However, it is crucial to understand when and how they can be integrated into the clinical setting, translating experimental results from bench to bedside, with the aim of improving patients' care.

B cell lymphomas (BCLs) represent a wide group of neoplasms whose categorization continues to evolve in parallel with the knowledge of their molecular landscape and their presumed cell of origin. Particularly, DLBCL and FL are the most common types of NHL worldwide (WHO 2017) and still represent a challenge for both researchers and clinicians. The research work along the three years of this PhD program has been focused on the molecular genetic characterization of these two groups of lymphomas and the results have been integrated with morphological, immunohistochemical and available clinical data.

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DLBCL category encompasses a heterogeneous group of aggressive BCL and currently, many clinicopathologic variants and distinct subtypes are recognized, although they account for only a minority of all cases. The remaining ones are referred to as DLBCL, not otherwise specified (WHO 2017). In contrast to indolent lymphomas, the survival curve typically shows an initial downward slope followed by a plateau, indicating the potential curability of a significant proportion of patients who achieve remission. In DLBCL, GEP analyses have identified two main biologic subtypes - GCB and non-GCB (Alizadeh 2000). This information has become more and more clinically relevant, as drugs that are expected to benefit one subtype or the other have been developed, such as ibrutinib, a selective, irreversible inhibitor of Bruton's tyrosine kinase, that is typically expressed in non-GCB cases. Another point is represented by so called *double hit (DH)* lymphomas. These high-grade lymphomas are strictly defined by the presence of two genetic abnormalities, namely rearrangements involving both *MYC* and *BCL2* and/or BCL6, and it's important that they are appropriately identified as they seem not to respond well to the current standard treatment for

DLBCL, NOS. Moreover, studies focusing on DLBCLs arising in several EN locations have suggested the existence of peculiar site-related molecular, pathological and clinical characteristics, with therapeutic and prognostic consequences (Møller 2004, Al-Humood 2011, Raghoebier 1991, Kramer 1998, Lal 2008, Jang 2011). Although DLBCL arising in specific EN sites have been classified by WHO as separate entities, such as CNS and cutaneous DLBCL, the significance of the primary EN origin itself has been poorly addressed.

The aim of the first part of our project was to investigate the clinico-pathologic, immunophenotypical and cytogenetic features of a series of 106 EN-DLBCLs, and to compare the obtained data to the available survival information. As only a subset of 58 cases in our series were cytogenetically investigated for the detection of *MYC*, *BCL2* and *BCL6* chromosomal rearrangements, we have used the generic term "DLBCL" throughout the manuscript to address diffuse aggressive lymphomas with DLBCL morphology. In fact, even if we have not excluded the possibility of double/triple hit in all the series, it is also true that in the cytogenetically investigated subset we found only one double hit lymphoma out of 58 cases (1.7%). Thus, it is reasonable that the vast majority of our cases belong to the DLBCL, NOS category, and the results obtained in our series can be extended to this group of neoplasms with little statistical effect.

The analysis of our results highlighted several features that seemingly delineate a specific profile for EN DLBCL, either as a group, or with reference to specific primary sites. From a clinical point of view, patients in our series presented most frequently with low or low-intermediate IPI score and early stage disease, as previously observed in large population-based studies on EN DLBCL from both the United States and Asia (Castillo 2014, Lal 2008). By contrast, in a series of primary nodal DLBCLs previously published by our group, we found a prevalence of advanced-stage diseases (Uccella 2008). We can hypothesize that site related symptoms associated with the tumor mass effect may contribute to early detection of the disease in primary EN DLBCLs. The immunohistochemical study firstly focused on the application of the Hans' algorithm for the identification of the cell of origin. Considered as a group, the majority of our EN DLBCL belonged to the non-GC subtype, as it was recently observed by Wang and coworkers (Wang 2016). However, in other unselected series, there were no differences in the frequencies of GC and non-GC cases between primary nodal and EN DLBCLs (Kim 2011). Such conflicting results may be due to several causes. First, the definition itself of a lymphoma as primary nodal or EN is still a controversial issue, particularly in cases of disseminated disease or involvement of peculiar sites, such as Waldeyer's ring, spleen and bone marrow (Krol 2003). In our study, we excluded cases arising in the spleen, in the tonsils and in the thymus, restricting the definition of "extranodal" to "extralymphoid" sites.

Second, immunohistochemical algorithms alternative to the Hans' one have been used in different studies, and this may affect the accuracy in defining the cell of origin (López-Guillermo 2005, Al-Humood 2011). Third, the adoption of different inclusion criteria, which may privilege peculiar sites and exclude some others, has introduced an important selection bias. In our series there is enrichment in testicular and CNS cases, which are predominantly of non-GC type, and this fact may have influenced our results.

When we separately looked at the various EN sites we found significant differences among them. Among digestive cases, most of the intestinal DLBCLs belonged to the GC subype, whereas gastric DLBCL were equally distributed between GC and non-GC, supporting the hypothesis that gastric and intestinal DLBCLs may recognize different pathways of lymphomagenesis (Connor 2007, Mitchell 2008). On the other hand, the majority of DLBCLs arising in immunological sanctuaries (CNS and testes) displayed a non-GC phenotype, confirming previous findings (Magnoli 2015 Al-Abbadi 2006, Booman 2008, Li 2010, Gill 2014). Interestingly, all the cervico-cephalic lymphomas investigated in our study belonged to the non-GC group. It is worth noting that, in our series, this group included DLBCL of the nose, paranasal sinuses and pharynx, whereas other studies focusing on H&N lymphomas included patients diagnosed with primary DLBCLs of the Waldeyer's ring (i.e. palatine tonsil, lingual tonsil and nasopharynx), which were predominantly of GC subtype (Lopez-Guillermo 2005, de Leval 2012).

We next moved to the immunohistochemical analysis of BCL2 and MYC expression. Intriguingly, we found the highest proportion of double expressor (DE) DLBCL among lymphomas of the cervico-cephalic district. The majority of our DLBCLs in this site presented with low or low-intermediate-risk IPI score and early-stage disease, all of them were attributed a non-GC phenotype, and, as a group, they had a poor outcome, in agreement with the reported adverse prognostic value of concomitant expression of BCL2 and MYC (Kramer 1998, Sarkozy 2015, Sohn 2003, Swerdlow 2014).

Molecular cytogenetic analysis using FISH technique with break-apart probes allowed us to investigate abnormalities, including rearrangements and copy number alterations, of key genes in lymphomagenesis. Considering the whole series, *BCL6* was the most commonly rearranged gene, followed by *MYC* and *BCL2*. These results were different from those that we previously observed in a series of 74 primary nodal DLBCL (Tibiletti 2009), in which *BCL6* was the most frequently rearranged gene, as well, but *MYC* alterations were present in a minority of cases, whereas rearrangements of *BCL2* and *BCL10* were more frequent than in the present series of EN DLBCL. The involvement of *MYC*

alterations in lymphomagenesis is known to be related to an increased biological aggressiveness of the neoplasm. A recently published FISH study by Quesada et al (Quesada 2017) reported increased *MYC* copy number in more than 10% of de novo DLBCL, claiming a relationship with a worse patients' outcome. However, the authors did not provide details on whether the increased copy number was attributable to polysomy of chromosome 8, or to an amplification of the gene locus. In our series, we observed true amplification of *MYC* locus in only 3 gastrointestinal DLBCLs (5.2%). Interestingly, in two of these cases, *MYC* amplification was associated with *MYC* rearrangement and the patients had a dismal outcome, as they died of disease 1 and 5 months after the diagnosis, respectively. Whilst the negative prognostic impact of MYC rearrangements seems to be well established, the clinical implications of extra copies of the gene are less clear. In the largest reported series of DLBCLs with MYC amplification, the authors concluded that MYC increased copy number was not predictive for inferior survival (Landsburg 2016). Similarly, results from a European series suggested that the detection of extra copies of MYC was not related to worse prognosis in the absence of concurrent del(8p) (Testoni 2011). In line with these data, we can state that in our patients with MYC amplification, a dismal outcome was observed only in the presence of concurrent MYC rearrangement. However, according to other authors, the presence of both MYC translocations and gains is associated with a poorer outcome (Yoon 2008). Taken together, these data suggest that larger series of patients would need to be studied to determine the prognostic significance of MYC amplification. Only a single case in our series, arising in the testis, could be defined as DH, bearing concomitant *MYC* and *BCL2* rearrangements.

Interestingly, in this study, we identified 17 cases showing small rearranged clones and, in the majority of them, the small clones coexisted with major rearranged clones. This peculiar cytogenetic condition could suggest the predisposition to double strand breaks and, consequently, a possible prognostic effect. However, the clinical behavior of this condition is still unknown.

A significant heterogeneity in the type and frequency of gene rearrangements was observed among DLBCLs arising in different body sites, paralleling immunohistochemical results and further supporting the existence of site-specific pathogenetic mechanisms. In fact, *BCL6* was frequently rearranged in testicular DLBCL, whereas intestinal DLBCLs showed a preferential rearrangement of *MYC*. Strikingly, CNS DLBCL showed very few rearrangements of the investigated genes, with a major clone of cells with *BCL6* rearrangement in only one case. This suggests that other mechanisms of lymphomagenesis exist in this site, and further supports their separation from DLBCL-NOS. Indeed, several years ago, another FISH analysis of the genes classically involved in lymphomagenesis (*BCL2*, *BCL6* and *MYC*), in a series of 13 cerebral DLBCL, showed the presence of *BCL6* rearrangement in only

three cases, with no other gene abnormalities (Montesinos-Rongen 2002). The involvement of non-classical *BCL6* locus alterations has also been reported in CNS DLBCL by Schwindt and coworkers, who suggested that they may be explained by aberrant class switch recombination or somatic hypermutation, which supported the origin of CNS DLBC from late germinal center cells (Schwindt 2006). More recently, other studies evaluating large numbers of primary CNS DLBCLs have confirmed that *BCL2*, *BCL6* and *MYC* rearrangements are rarely seen in this entity (Nosrati 2019, Villa 2019). When we looked at the cell of origin, 38 out of the cytogenetically investigated cases belonged to the non-GCB group and were characterized by a high frequency of BCL6 rearrangements, together with a significant proportion of aberrations in multiple genes, supporting the concept of a genetically unstable and heterogeneous entity. By contrast, the 20 GC cases preferentially showed *BCL2* rearrangements. This and other genomic differences have been already described in DLBCLs and support the view that GC and non-GC lymphomas are biologically distinct entities with different pathogenetic pathways (Nedomova 2013, Pasqualucci 2015). However, in the case of EN DLBCL, if we consider the different distribution of GC and non-GC subtypes in the various primary EN sites, we could assume that the different genomic profiles observed between nodal and EN-DLBCLs may be influenced by the primary site of origin, rather than exclusively depending on the cell of origin, as suggested by others (Al-Humood 2011).

The survival analysis of our series had unavoidable limits due to different therapeutic approach to DLBCLs involving specific body sites. Nevertheless, it has still been performed, only considering OS, to provide a general overview on the outcome of the patients with EN DLBCLs. OS is the best-established primary efficacy end point to evaluate DLBCL therapies, however progression-free and disease-free survival represent other important clinical parameters when evaluating the natural history of the disease. These parameters were not included in our analysis, as they were available only for a limited number of patients. Thus, in our minds, our survival analysis does not provide robust clinical indications, but it can give clues to the relevance of some biologic mechanisms involved in lymphomagenesis. Further controlled studies are needed to confirm our findings. The Cox's regression model did not show any significant difference in patients' outcome between the whole series of EN-DLBCL and the nodal series previously investigated by our group (Uccella 2008). However, when considering the specific site of origin, a statistically significant difference in terms of OS emerged among DLBCLs arising in different organs and tissues. In detail, cutaneous lymphomas showed, as expected, the best prognosis, followed by DLBCLs arising in the GI tract and by nodal cases. Finally, a dismal outcome was observed in lymphomas affecting the head and neck district and the immune-privileged sites. At multivariate analysis, the primary site of origin was one of the

variables independently influencing OS, together with IPI score and MUM1 expression. In line with the observation of Hans and coworkers, MUM1 turned out to be an independent risk factor (Hans 2004). The prognostic value of the Hans' algorithm was evident only if applied to the whole series, but not when considering the various sites separately, possibly due to small sample size. Other immunohistochemical markers related to worse survival were BCL2 and MYC. Furthermore, we confirmed that the combination of increased MYC protein, implying accelerated proliferation, with the expression of anti-apoptotic BCL2 factor, is a predictor of dismal prognosis in DE lymphomas. Most retrospective studies suggest that DH and DE lymphomas carry a poor prognosis when treated with conventional therapeutic regimens, such as R-CHOP. However, there is a clear need for further prospective studies to clarify the true prognostic value of these parameters, as the role of the two genes has been recently challenged in a large randomized trial by the GELA/LYSA consortium (Copie-Bergman 2015). We previously demonstrated that the presence of at least one gene rearrangement was associated with a worse outcome in nodal DLBCLs (Tibiletti 2009). In contrast, cytogenetic abnormalities did not carry prognostic significance when considering the whole EN series. In fact, only *MYC* translocation was related to shorter OS, but only in the gastrointestinal subset. It is conceivable that in peculiar EN location, such as, for example, CNS lymphomas, which bear a significantly low proportion of abnormalities in the investigated genes, the drivers of lymphomagenesis and of the aggressive behavior are genomic alterations different from the genes rearrangements classically involved in nodal DLBCLs. In a series of testicular DLBCLs we observed that both the amounts of the T-cell infiltrate and its composition seem to be related to patients' outcome (Magnoli 2015). Thus, it is conceivable that the composition of the microenvironment is one of the factors playing a major role in affecting the biology of the tumor, particularly in immune sanctuaries, where lymphomatous B cells and reactive inflammatory cells may establish peculiar interactions between each other. In conclusion, our results seem to suggest that the primary site is related to the peculiar immunophenotypic and genetic features of EN DLBCLs and strongly influences the natural history of the disease and the patients' outcome. Hence, this study provides further support to the existence of site-related mechanisms of lymphomagenesis, as also proposed by others (Lopez-Guillermo 2005, Castillo 2014, Wang 2016, de Leval 2012, Olszewski 2014).

Our results outline that both immunohistochemical and FISH approaches are needed to improve the detection rate of aggressive EN-DLBCL including DE and DH cases. Studies on large and well characterized series of EN DLBCLs are required to shed light on the complex interaction between the intrinsic biomolecular features of these heterogeneous diseases and the surrounding macro-/microenvironment, in the need to develop more effective site specific therapeutic strategies.

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FL has long been considered a well defined disease, with straightforward diagnostic criteria, a clear genetic background and recognizable precursor condition. In the last few years, a deeper insight in the clinicopathological features of FL has unveiled that this disease is, in fact, composed of many different entities, which raised the need of personalized diagnostic and therapeutic approaches. Such heterogeneity has been at least partly acknowledged by the inclusion of four clinicopathological variants of FL in the latest update of the WHO classification (WHO 2017), namely in situ follicular neoplasm, duodenal-type FL, testicular FL, ad diffuse FL. In addition, separate entities, such as pediatric-type FL, large B-cell lymphoma with IRF4 rearrangement, and primary cutaneous follicle center lymphoma have been recognized. However, besides these well-defined entities, other aspects of the inter- and intra-tumor heterogeneity of FL are evident. In particular, the analysis of the genetic profile of tumor cells highlights important relationships between specific genetic lesions and tumor initiation, progression, and transformation. Since the discovery of *BCL2* gene by Tsujimoto et al (Tsujimoto 1984), the translocation (14;18)(q32;q21) has been considered the genetic hallmark of FL. In recent years, it has been recognized that FL lymphomagenesis is a multistep process, in which early lesions progress to overt disease through complex events of selection/counter-selection. Using an exome sequencing approach, Green and coworkers proposed an elegant genetic evolution model for FL tumorigenesis in which founder mutations turn a non-malignant B cell clone into a premalignant tumor cell population, stable enough to acquire one or more secondary driver mutations, leading to an early malignant clone. Finally, tertiary mutations may either act as passenger or accelerator mutations, the latter providing a selective advantage to a progressed malignant subclone (Green 2013). At the beginning of this spectrum, the earliest known oncogenic event is the t(14;18)(q32;q21), and it is reported with a prevalence of 85-90% in most of the published literature (WHO 2017). However, detection of t(14;18) is not required for the diagnosis of FL, even if it can be a useful tool, when present, in small biopsies and atypical lymphoid proliferations, in the context of equivocal morphologic and immunophenotypic findings. As a consequence, most centers do not perform routine evaluation of *BCL2* status, so that they do not know the exact incidence of t(14;18) in their series. Moreover, some authors have observed a proportion of *BCL2*-negative FLs as high as 50% in their series, and, in general, reported detection

rates are significantly lower in Far East and, to a less extent, European studies, compared to the US ones (Pezzella 1990, Biagi 2002, Pan 2012, Segel 1998).

Daily diagnostic activity in our pathology department seems to confirm the latter observations, challenging the paradigm of *BCL2* translocation as a necessary early event in FL pathogenesis and suggesting the existence of marked geographical differences in the pathogenesis and of alternative mechanisms of genetic deregulation in *BCL2*-negative cases. Starting from this practical observation, the aim of the second part of our project was to test the incidence of *BCL2*-negative FLs in a series of Italian patients from the Insubric region, evaluate its association with clinicopathological features and investigate alternative genetic aberrations in this subset.

Epidemiological features of our series were in line with published literature (WHO 2017), as our patients were adults with a median age in the sixth decade of life and females were slightly more common involved than males. Overall, *BCL2* rearrangements were detected in approximately half of our cases (39/76, 51.3%), in contrast with the 85-90% rate reported in most series (WHO 2017). It can be hypothesized that such discrepancy depends on the demographic characteristics of our patients, which were either born in or moved early in their life to the Insubric region. Indeed, NHL incidence widely vary across Italy, and our territory is among the areas with the highest recorded rates (AIRT working group 2006) (figure 17).

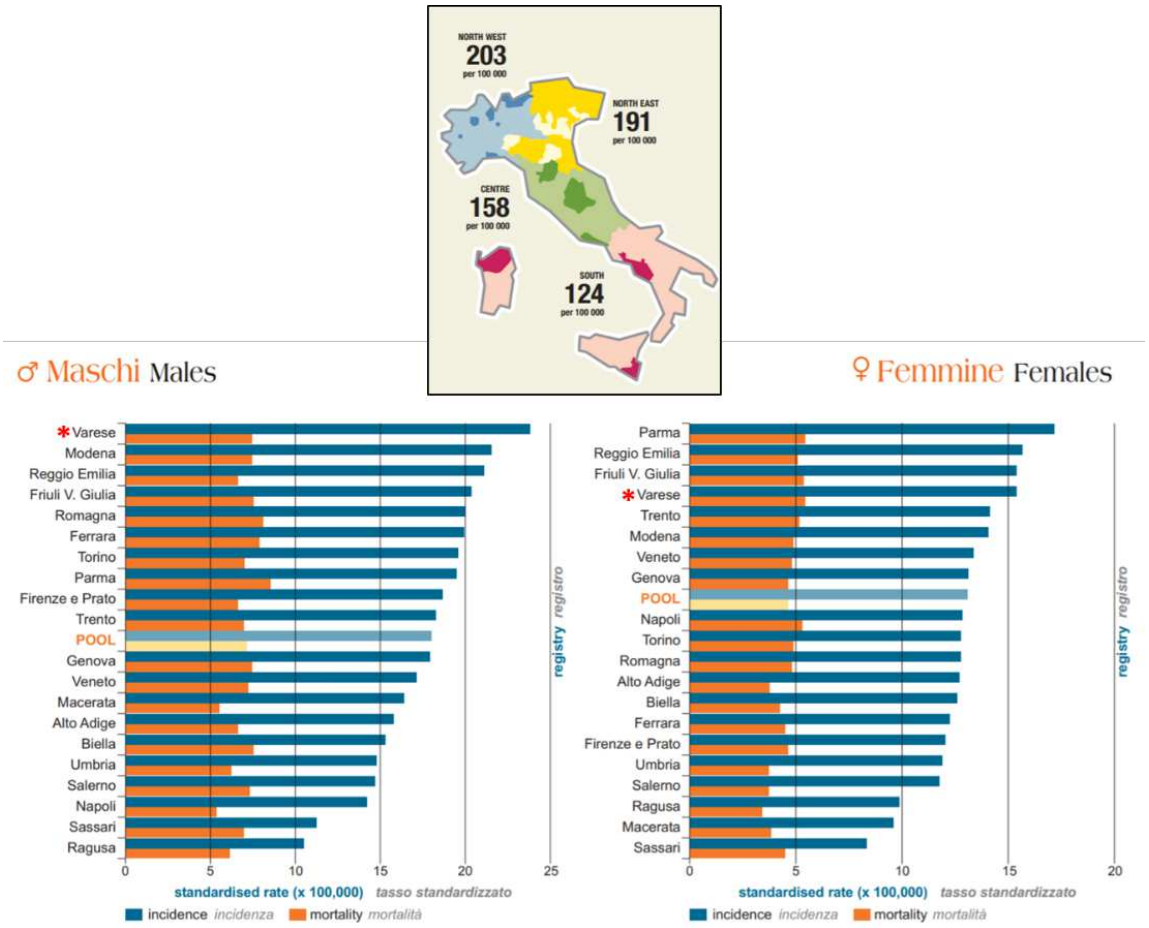

Figure 17. Non-Hodgkin lymphomas prevalence in Italy by macro-area (proportion per 100.000) and incidence by sex and micro-area.

It is conceivable that environmental factors may operate selectively in different geographical districts, favoring lymphomagenesis and resulting in FLs that are morphologically similar but molecularly distinct. This point has already been addressed to in previous studies, however, the authors concluded that the large variance in the detection frequencies of t(14;18) depended on methodological limitations (Albinger-Hegyi 2002, Aster 2002). These considerations, however, referred to the use of standard PCR conditions with major breakpoint region (MBR)/minor cluster region (mcr)-specific primer pairs, that indeed render false negative results when rearrangements involve *BCL2* sequences outside of the MBR and mcr. As a high fraction of rearrangements fall outside these regions (Akasaka 1998, Albinger-Hegyi 2002), the authors optimized long-distance PCR-protocols that offered some significant advantages in terms of sensitivity over the more commonly used standard methods. Interphasic FISH has a higher overall diagnostic sensitivity than PCR, because the FISH probes span almost all breakpoints. In addition, they do not require absolute sequence complementarity and are not as adversely impacted by poor quality of DNA specimen. Finally, FISH can also detect complex cytogenetic abnormalities and translocations involving the *IGH*

or *BCL2* genes. It is true that some cytogenetic changes may still be missed by FISH analysis, such as translocations between *BCL2* gene and unusual partner or cryptic translocations not detected by commercially available probes, however these represent rare occurrences (Godon 2003, Bentley 2005). We tested different commercially available probes for BCL2 translocation, obtaining overlapping results in terms of percentage of translocated cases lower than expected. Moreover, as we had also the opportunity of performing karyotype analyses on a consistent number of our cases, we demonstrated that chromosome abnormalities other that t(14;18) can be present in FL. As a whole, these observations tend to confirm that the variation in the incidence of t(14;18) across studies may be due to geographical factors rather than technical problems. The relative lower incidence of FL registered in Asian populations seems not to correspond to a lower frequency of BCL2 rearrangements in healthy individuals (Biagi 2002). Moreover, epidemiological data derived from Asian emigrants to the United States and their descendants seem to imply environmental rather than genetic influences (Herrinton 1996). For example, cigarette smoke and pesticide exposure have been called into question (Biagi 2002), but the precise nature of such putative factors is far from being elucidated.

After assessing the percentage of *BCL2*-rearranged cases, we systematically searched for translocations involving its classical partner, *IGH*. While all *BCL2*+ FLs showed concurrent *IGH* rearrangement, the opposite was not true, as we found 6 *IGH*+/*BCL2*- cases. Moreover, in 4 additional cases we observed discordant results, as rearrangements in both genes were present, but with quite different percentages of translocated cells. These 10 cases were tested with break-apart probes for *BCL6* and *MYC* genes, due to their pivotal role in the pathogenesis and evolution of lymphoproliferative disorders. As we found only *BCL6* translocation in a single case, further investigations are required to clarify the molecular genetic background of the remaining 9 discordant FLs.

In a subset of 32 cases, the availability of fresh lymphoma samples allowed us to confirm data obtained on FFPE material, but at the same time highlighted the limits of FISH analyses on archived material. Indeed, we observed three discordant cases which were *BCL2*-negative when analysis was performed on FFPE samples, whereas turned out to be *BCL2*+ when the corresponding fresh frozen nuclei were investigated. If it is true that the preservation of histological context in paraffin sections allows for the analysis of the topographical distribution of cytogenetically abnormal cells, FISH on isolated nuclei from FF tissue was able to detect either small neoplastic clones or an atypical *BCL2* breakpoint due to its major sensitivity.

Besides confirming FISH results, karyotype analyses proved that chromosome abnormalities other that t(14;18) could characterize FL. In detail, *BCL6* locus on 3q was involved in two cases. *BCL6* gene encodes a transcriptional repressor whose oncogenic effect is well-recognized (Albagli-Curiel 2003, Saito 2007). *BCL6* rearrangements have been variously reported as transforming and proliferating stimuli alternative to the classic *BCL2* deregulation in high grade FL (Guo 2005, Katzenberger 2004) or in low grade disease (Marafioti 2013). Others again have challenged its putative role as a crucial pathogenetic factor in *BCL2*-negative FL, suggesting that *BCL6* amplification/3q27 gain is associated with peculiar clinicopathologic characteristics, namely, high grade morphology, high BCL2 and MUM1 protein expression and frequent combination with *BCL2* gene amplification/18q21 gain (Karube 2008). Moreover, in one FL we observed an unbalanced translocation involving *TNFRSF14,* a member of the TNF-receptor superfamily with tumor suppressor function which encodes the herpes virus entry mediator (HVEM). Disruption of the HVEM-BTLA (B and T lymphocyte attenuator) axis has been documented during the early stages of GC lymphomagenesis (Boice 2016). Katzenberger and coworkers identified a distinctive subtype of t(14;18)-negative FL, characterized by a predominantly diffuse growth pattern, localized involvement of inguinal lymph nodes and 1p36 deletion (Katzenberger 2009). Aberrations of this chromosomal region have been reported in *BCL2*-morphologically classical FL with a predominantly follicular growth pattern, but it should be noted that they represent one of the most common alteration in classical *BCL2*+ FLs too (Launay 2012, Kridel 2012). Finally, we observed a translocation in which the fusion partner of *BCL2* was represented by the lambda light-chain gene (*IGL*). Variations in the t(14;18) can be classified into two categories: simple variants, involving chromosomes 18 and 2, or 22, in which the fusion partner of *BCL2* is a light-chain gene and complex variants, occurring among chromosomes 14, 18 and other chromosomes. Both represent rare events, reported as single cases or small series in the literature (Bentley 2005, Impera 2008). Taken together, these observations do not shed light on the pathogenesis of *BCL2*- FLs but suggest the existence of diverse mechanisms of genetic deregulation, which in many cases have not been elucidated, yet.

Another point of interest is represented by the correlation between molecular cytogenetic and immunohistochemical results. All FLs with weak or absent BCL2 staining were negative at FISH analysis, with the only exception of 2 cases. This result minimizes the need for testing additional antibodies in the work-up of suspected FLs that are negative with one immunostaining, as suggested by other authors (Xerri 2016). On the other hand, nearly half of cases with moderate to intense BCL2 staining did not show t(14;18). This phenomenon is well documented in the literature and attributed

to the existence of alternative mechanisms leading to protein overexpression, such as amplification of chromosome 18 which may implicate an increased dosage effect (Falini 2002, Horsman 2003).

In our study, *BCL2* immunohistochemical expression and intensity of staining, as well as the presence or absence of *IGH/BCL2* fusion were not significant prognostic indicators of OS. Conflicting results have been published on the prognostic significance of BCL2 status in FL, mostly based on small series of cases with relatively short follow-up periods. Although in an old study t(14;18) was associated to poor response to therapy and short survival (Yunis 1989), in the same period other authors observed no difference in prognosis between cases with and without the translocation (Levine 1988) and this observation was confirmed in subsequent years (Pezzella 1992, Maeshima 2013). Overall, it seems that there are no grounds for considering *BCL2* status to be a useful prognostic marker in clinical practice. However, as testing for t(14;18) is currently asked by hematologists to monitor response to therapy and detect recurrent disease, it becomes crucial to investigate *BCL2* rearrangement already at diagnosis, as the rationale behind this request fails in the presence of *BCL2*-negative FLs. Given its superior diagnostic sensitivity, FISH represents the front-line molecular test for t(14;18). PCR analysis is faster, less expensive to perform and has greater analytic sensitivity than FISH. However, this latter is not ordinarily required at the time of initial diagnosis, when lymphoma cells tend to be abundant, but becomes crucial in the setting of disease revaluation.

In summary, our results indicate that *BCL2* rearrangement in FL is not as frequent as previously reported, and its presence should not be taken for granted, with important consequences on both the diagnosis and follow up of the patients. It becomes evident that the genetic landscape of FL is more complex than previously thought and that other alternative genetic abnormalities may trigger the pathogenesis of this lymphoma (Magnoli 2019). Studies on larger and well characterized series of *BCL2*-negative FLs are required to identify their pathogenesis and molecular background in order to clarify their biological significance and to properly manage patients.

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In conclusion, what we learned from our studies is that even within an individual clinico-pathological entity, there is considerable heterogeneity with respect to genetic alterations, expression of commonly assayed markers and, most important, clinical outcome. Even if classification schemes are essential frameshift for the patients' management, we are not dealing with monolithic entities at all,

but rather puzzle pieces which only assembled together allow us to properly understand diseases and treat our patients. The personalized approach acknowledges this complexity and gives us tools for the continuous improvement of patients' care.

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