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"Clinico-pathological and biological characterization of a cohort of 140 patients affected by relapsed/refractory Diffuse Large B Cell Lymphoma: looking for prognostic factors"

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1. ABSTRACT

Diffuse Large B Cell Lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma. Although most patients respond efficaciously to the first line therapy, about 30-40% are primary refractory or present relapse after an initial response, constituting a group with poor prognosis. Many efforts have been made to find prognostic factors, able to identify high-risk patients; however, the currently available scores are often inadequate to this purpose.

In this study we compared the clinical and biological features of a cohort of relapsed/refractory patients (R/R, n = 140), with those a cohort of patients not affected by relapse after at least 5 years of follow-up (controls, n = 45). We divided the R/R patients according to the time of relapse in three subgroups – *refractory* (characterized by persistence of disease or recurrence within 9 months from diagnosis, n = 72), *early relapsed* (with recurrence of disease within 10 and 24 months, n = 35) and *late relapsed* (with recurrence of disease beyond 24 months from diagnosis, n = 33). We also performed gene expression profiling (GEP) analysis on a subgroup of patients, aiming at recognizing differentially expressed genes; through this analysis, we identified the B1 subunit of NADH:Ubiquinone Oxidoreductase (*NDUFB1*) as a gene with enhanced expression in R/R patients. We further verified *NDUFB1* expression and protein levels in DLBCL cell lines and performed *ad hoc* immunohistochemistry on patients' samples.

Our results show that the R/R subgroups differentiate in terms of clinical and biological features, but also in terms of outcomes, with an inferior post-relapse overall survival (OS) for refractory patients. In the whole R/R cohort, we confirmed the prognostic value of the International Prognostic Index (IPI) and the Revised-IPI (R-IPI), even when calculated at relapse, and of well-known adverse factors (B-symptoms, advanced stage, lactate dehydrogenase (LDH) increase, bulky disease, extra-nodal involvement), but we also found a novel correlation between male sex and inferior progression-free survival (PFS) and OS, and between inflammatory indexes as neutrophils/lymphocytes (N/L) ratio, Systemic Immune-Inflammation Index (SII) and C-reactive protein value/albumin value (CAR) and outcome. As for immunohistochemistry data, high Ki67 values correlated with reduced OS, while NDUFB1 overexpression caused a PFS disadvantage. We detected a trend of more frequent altered expression of P53 in the R/R cohort, in which all patients with enhanced expression were refractory, while all cases with a "null" phenotype belonged to the late relapsed group. In the second line setting, 23% of patients underwent autologous stem cell transplant (ASCT); transplanted patients had a post-relapse OS significantly superior to patients who did not receive transplant.

Globally, we assessed the importance of the time of relapse for prognosis prediction, and identified some subgroup-specific features and variables impacting on outcome. These results, if validated in

larger cohorts could contribute to the formulation of new prognostic scores, improving risk-stratification in DLBCL.

1.1 RIASSUNTO

I linfomi diffusi a grandi cellule B (DLBCL) costituiscono il più comune tipo di Linfoma non Hodgkin; sebbene circa il 60-70% dei pazienti risponda alla terapia di prima linea con una remissione duratura, i restanti risultano refrattari o sviluppano recidiva dopo un'iniziale risposta alla terapia e sono caratterizzati da prognosi generalmente infausta. Nonostante gli sforzi volti alla ricerca di fattori prognostici in grado di identificare precocemente i pazienti ad alto rischio, gli *score* comunemente usati nella pratica clinica si rivelano spesso inadeguati a questo fine.
In questo studio abbiamo analizzato caratteristiche cliniche e biologiche (principalmente immunoistochimiche) di una coorte di pazienti recidivati o refrattari (R/R, n = 140) *versus* una coorte di pazienti risultati responsivi alla terapia di prima linea e non recidivati dopo un follow-up minimo di 5 anni (controlli, n = 45). Abbiamo diviso il gruppo dei R/R in base al tempo di ricaduta in 3 sottogruppi - i *refrattari* (caratterizzati da persistenza di malattia o da ricaduta entro 9 mesi dalla diagnosi, n = 72), i *ricaduti precoci* (affetti da ricaduta tra i 10 e 24 mesi, n = 35) e i *ricaduti tardivi* (con ripresa di malattia oltre i 24 mesi dalla diagnosi, n = 33). Abbiamo inoltre condotto delle analisi di *gene expression profiling* su un ristretto sottogruppo di pazienti con lo scopo di identificare, nel confronto tra R/R e controlli, geni con diversa espressione. Queste analisi ci hanno portato a selezionare la Subunità B1 della NADH:Ubichinone Ossidoreduttasi (*NDUFB1*), come gene maggiormente espresso nei R/R; l'espressione genica e proteica di NDUFB1 sono state successivamente verificate in linee cellulari di DLBCL e tramite studi di immunoistochimica sui campioni bioptici disponibili.
I nostri dati hanno evidenziato che i suddetti sottogruppi differiscono non solo in termini di caratteristiche cliniche e biologiche, ma anche per quanto concerne la sopravvivenza dalla ricaduta, risultando questa significativamente ridotta nei refrattari. Considerando globalmente la coorte dei R/R, abbiamo confermato il valore prognostico di IPI e R-IPI (anche calcolati alla ricaduta) e di noti fattori sfavorevoli (presenza di sintomi B, stadi avanzati, aumento della lattato-deidrogenasi (LAD), malattia *bulky* e con interessamento extra-nodale); tuttavia abbiamo anche osservato una correlazione tra il sesso maschile e inferiore progressione libera da malattia (PFS) e sopravvivenza (OS), come pure tra gli indici infiammatori – il rapporto neutrofili/linfociti (N/L), l'indice immuno-infiammatorio sistemico (SII) e il rapporto proteina C-reattiva/albumina (CAR) e gli indicatori di *outcome*. Considerando le analisi di immunoistochimica, abbiamo trovato correlazione tra i valori di Ki67 e OS e tra l'espressione di NDUFB1 e la PFS. Abbiamo rilevato tendenzialmente una più frequente alterata espressione di P53 nel gruppo dei R/R, con un'aggregazione dei casi con aumentata espressione nel sottogruppo dei refrattari e dei casi con espressione soppressa del

sottogruppo dei ricaduti tardivi. Nell'ambito della terapia di seconda linea, il 23% dei pazienti è stato sottoposto ad autotrapianto di cellule staminali; tali pazienti hanno dimostrato tassi di OS successivi alla ricaduta superiori rispetto ai pazienti non trapiantati.
Complessivamente, abbiamo documentato che il tempo di ricaduta influenza la prognosi; abbiamo inoltre identificato variabili caratterizzanti i diversi sottogruppi analizzati e con impatto sull' *outcome*. Questi dati, se validati in altre coorti, potrebbero contribuire alla formulazione di nuovi *score* prognostici, migliorando la stratificazione del rischio nei pazienti affetti da DLBCL.

2. BACKGROUND

2.1 DLBCL - The edge of a new era?

Diffuse Large B Cell Lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma, representing about 30% of all the diagnoses (1). It is a heterogenous entity, whose characterization is based on histo-morphological, immunohistochemical, molecular and clinical features.
In the last years many attempts have been made to categorize DLBCL into distinct subgroups: in 2022 the World Health Organization Classification was updated (2), followed by the release of the International Consensus Classification (ICC) (3), which constitutes another proposal for the classification of hematologic malignancies. Other attempts have been made to classify DLBCL on the basis of genetic profile (4,5).
In addition, the therapeutical scenario of DLBCL is substantially changing and expanding, with chimeric antigen receptor (CAR) T-cell therapy offered from the second line and new agents available in monotherapy (*e.g.* bispecific antibodies) or in combination (*e.g.* Polatuzumab Vedotin, Tafasitamab).

2.1.1 New classifications, old pathologies

A comparison between **4th WHO classification** (WHO-HAEM4), **5th WHO classification** (WHO-HAEM5) and **ICC 2022** is reported in Table 1. Both WHO-HAEM5 and ICC 2022 presents several changes, in relation to WHO-HAEM4. ICC still considers provisional entities, while these are no longer present in WHO-HAEM5; both classifications have confirmed as "defined entities" some provisional proposals included in WHO-HAEM4. Considering the simultaneous publication of two classifications, the indication for the pathologist is to report the diagnosis according to both WHO-HAEM5 and ICC 2022.
As for DLBCL and large cell B lymphoma (LCBL), we can point out the following differences:

- The entity "Transformation of indolent B-cell lymphomas" is only recognized by WHO-HAEM5, and comprises the cases of emergence of aggressive lymphoma with a previous or synchronous diagnosis of a clonally-related indolent lymphoma.
- For DLBCL, not otherwise specified (NOS), both the classification recommend the specification of the cell of origin (COO) *germinal center B-cell* (GCB) type, *activated B-cell* (ABC) type and *unclassified*; since in routine practice the performance of gene expression

profiling is still uncommon, the definition according to immunohistochemical algorithm is accepted.

- The provisional entity “Burkitt-like lymphoma with11q aberrations” was retained in ICC 2022, while it was redefined “High grade B-cell lymphoma with 11q aberrations” in WHO-HAEM5 in consideration of the intermediate/blastoid morphology.
- The provisional entity “Large B-cell lymphoma with *IRF4* rearrangement” has been upgraded to defined entity in both the classifications; in spite of morphology, it is generally a localized disease, with favorable outcome.
- The “Primary diffuse large B cell lymphoma of central nervous system (CNS)” was maintained as a distinct entity (comprising also the “Primary large B-cell lymphoma of vitreoretinal”) by ICC 2022, while these 2 entities, together with the new entity “Primary diffuse large B-cell lymphoma of testis” were comprised under the umbrella “Primary large B-cell lymphoma of immune-privileged sites” by WHO-HAEM5, in consideration of their localization in immune sanctuaries and their common genetic background (mutations of *MYD88* and *CD79b*). ICC 2022 also recognizes the new entity “Primary diffuse large B-cell lymphoma of testis”.
- The new entity “Fluid overload-associated large B-cell lymphoma”, defined by WHO-HAEM5, corresponds to the provisional one “HHV8 and EBV-negative primary effusion-based lymphoma” defined by ICC; they are characterized by exclusive involvement of body cavities and are generally favored by conditions leading to fluid overload and subsequent chronic serosal inflammation.
- The provisional entity “EBV-positive mucocutaneous ulcer” has been upgraded by both ICC and WHO-HAEM5, although the latter includes this entity in the category “Lymphoid proliferations and lymphoma associated with immune deficiency and dysregulation”, which comprises also “EBV-positive polymorphic B-cell lymphoproliferative disorder” (still considered provisional by ICC). EBV-positive mucocutaneous ulcer typically affects elderly and immunocompromised patients and is characterized by favorable outcome. All these entities differ from EBV-positive DLBCL, diagnosed with > 80% EBV+ cells.
- The “Fibrin-associated LBCL”, still considered a provisional subtype of DLBCL- associated with chronic inflammation, was upgraded by WHO-HAEM5 to a definite entity.
- The High-grade B-cell lymphoma (HGBCL), NOS is an entity recognized by all the classifications, characterized by blastoid cytology, absence of double-hit cytogenetics (although a consistent number of cases carry single-hit *MYC* rearrangement) and aggressive clinical behavior. Instead, the entity “HGBCL with *MYC* and *BCL2* and/or *BCL6*

rearrangements (double-hit or triple-hit)", identified by WHO-HAEM4, has undergone some changes: in WHO-HAEM5, lymphomas with *MYC* and *BCL2* rearrangements are split into both DLBCL and HGBCL with *MYC* and *BCL2* rearrangements, while ICC does not make this distinction. DLBCL and HGBCL with *MYC* and *BCL2* rearrangements show a blastoid/intermediate morphology and may derive from follicular lymphoma, whose mutational signature – comprising mutations of *CREBBP*, *BCL2*, *KMT2D*, *MYC*, *EZH2* and *FOXO1* - can be maintained. These patients can harbor also *MYC* hotspot mutations, which preventing MYC degradation may contribute to their aggressive phenotype. HGBCL with *MYC* and *BCL6* rearrangements is considered by WHO-HAEM5 a genetic subtype of DLBCL and HBCL, NOS, in reason of a less remarkable difference in biological and clinical features; nevertheless, ICC still considers it as a provisional entity (3).

Although not yet impacting the clinical practice, we cannot neglect the importance of two recent whole exome sequencing studies, which proposed the categorization of DLBCL into genetic subtypes. ***Schmitz et al*** combined whole exome and transcriptome sequencing, deep targeted amplicon sequencing and DNA copy number analysis, identifying 4 subgroups: MCD (characterized by concomitant mutations of *MYD88* (L265P) and *CD79*), N1 (carrying *NOTCH1* mutations), BN2 (defined by *NOTCH2* mutations and *BCL6* fusions), and EZB (with *EZH2* mutations and *BCL2* translocations). The first two subgroups are characterized by worse outcome, while BN2 and EZB show better response to chemo-immunotherapy (5). Simultaneously, ***Chapuy et al*** recognized 5 subgroups: Cluster 1 (corresponding to low risk ABC-DLBCL of extrafollicular/marginal zone origin, with *BCL6* and *NOTCH2* mutations, and characterized by immune escape mechanisms), whose counterpart is Cluster 5 (high risk ABC-DLBCL, with frequent extra-nodal involvement, *MYD88*, *CD79b* and *BCL2* mutation, and 18 gain); Cluster 3 (constituted by GCB-DLBCL with unfavorable outcome, characterized by chromatin modifiers and *BCL2* mutations, and *PTEN* inactivation), counterbalanced by Cluster 4 (GCB-DLBCL showing better outcome, with mutations of genes belonging to NF-kB or BCR/PI3K pathways and of genes involved in immune escape). Finally, Cluster 2, not related to COO, characterized by biallelic *P53* inactivation, loss of *CDKN2A* and genetic instability (4). These studies represent a breakthrough in the comprehension of the DLBCL pathogenesis and at same time suggest possible drug-targetable pathways, which could eventually lead to personalized therapy (6); a comparison between the two classifications is reported in Table 2.

2.1.2 New therapeutical perspectives

For over 20 years the standard-of-care for DLBCL in the first line setting has been the association between the anti-CD20 monoclonal antibody Rituximab and the combination of Cyclophosphamide, Vincristine, Doxorubicin and Prednisone (R-CHOP) (7). Dose of therapy, number of cycles and the addiction of radiotherapy vary according to age and comorbidities of patients, stage and bulky disease (8). Many attempts have been made to improve this backbone; unfortunately, combinations with Ibrutinib (PHOENIX) (9), Bortezomib (PYRAMID) (10) and Lenalidomide (ROBUST) (11) failed to reach better outcome when compared with R-CHOP plus placebo. On the contrary, the recent POLARIX trial succeeded in demonstrating an improvement in progression-free survival (PFS) in patients with intermediate or high International Prognostic Index (IPI) treated with Polatuzumab Vedotin (a CD79b-directed antibody-drug conjugate)-R-CHP *vs* standard R-CHOP, with subsequent approval of the combination therapy (12). An increase in overall survival (OS) has not been yet detected, but a longer follow-up is needed.
Many trials are exploring the combination of R-CHOP with other agents; hypothetically, in the future we could have more R-CHOP-X schemes, to select according to patients' risk and lymphoma biology.

As regards second and further lines, several new therapies have recently been approved.

- **CAR-T** have reshaped the therapeutical scenario of DLBCL. Three products (Axi-Cel, Tisa-Cel and Liso-Cel) are approved for the third and subsequent lines, having demonstrated overall response rates (ORR) between 54% and 84%, with CR rates of 40-58% (13). Despite the initial concerns about CAR's related toxicities – *i.e.* cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) - the extensive use of Tocilizumab and steroids have made these therapies safe and manageable. Recently, Axi-Cel and Liso-Cel have moved from the third to the second line, following randomized trial, which compared CAR-T products *vs* the standard of care treatment, namely high dose salvage chemotherapy plus autologous stem cell transplant (ASCT). Thus, ZUMA-7 trial (Axi-Cel) (14) and TRANSFORM trial (Liso-Cel) (15) reported an advantage in PFS for patients who underwent CAR-T therapy; besides, few months ago, the updated follow-up of ZUMA-7 showed also a benefit in terms of OS, paving the way for a substantial paradigm shift in the treatment of these lymphomas (16). However, we have to remember that, differently from the aforementioned studies, the BELINDA trial failed to demonstrate the

superiority of the CAR-T product (Tisa-Cel) in comparison with ASCT (17); these contrasting results are probably related to different study design and patients' characteristics.

- Another promising therapeutical approach derives from the introduction of **bispecific antibodies**; these molecules recognize two different antigens (usually CD20 and CD3) and enable the engagement of effector cells (specifically T-cells), to kill malignant B-cells. Four conjugates have undergone phase II studies for R/R DLBCL (Glofitamab, Epcoritamab, Mosunetuzumab and Odronextamab) and both Glofitamab and Epcoritamab have been recently approved by the Food and Drug Administration (FDA) for the use in the third and further lines of treatment. These latter drugs have demonstrated similar efficacy (ORR 52% and 63%, CR 40% and 39%, respectively), although with different administration schemes (18)(19). Mosunetuzumab, which was the first antibody entering a clinical trial, proved to be more effective in follicular lymphoma (20), while the development of the phase II protocol for Odronextamab was delayed, due to high CRS rates, which required a remodulation of the step-up dosing.
- **Polatuzumab-Vedotin** was initially approved in the R/R setting, in combination with **Rituximab and Bendamustine**, after the publication of the phase II GO29365 trial, which demonstrated significantly improved response rates in the comparison with R-Bendamustine (ORR 45% *vs* 17.5%, CR 40% *vs*17.5%, respectively) (21); these data were substantially confirmed by real-life studies.
- **Tafasitamab** is an Fc-modified anti-CD19 antibody, which enhances antibody-dependent cellular cytotoxicity (ADCC); as single-agent it did not show pronounced efficacy, but a synergistical anti-tumoral activity was observed in combination with the immunomodulating drug Lenalidomide (ORR = 47%, CR = 32% in phase II trial L-MIND) (22). However, real-world studies from the US reported lower PFS (2.8 *vs* 11.6 months) and OS rates, compared to the L-MIND trial, probably due to patients' more unfavorable features and higher risk disease (23).
- **Loncastuximab-Tesirine**, a CD19-directed antibody-drug conjugate, demonstrated in the LOTIS trial single-agent antiblastic activity, achieving durable responses (ORR = 48%, CR = 23%), with an acceptable safety profile (24).
- **Selinexor** is an oral inhibitor of nuclear export (through Exportin 1 blockage), that determines an increase in nuclear tumor suppressor, leading to apoptosis; in the phase II study SADAL, moderate anti-lymphoma activity was reported (ORR = 28%, CR = 12%) (25)

However, even if the therapeutical options are increasing in number and efficacy, relapse still constitutes a critical and often fatal event for patients affected by DLBCL, as we will discuss in the following paragraph.

2.2 Relapse, still an unmet clinical need

Despite the therapeutical progresses of the last years, relapse still constitutes an unmet clinical need. CAR-T in second line have improved PFS and, as recently demonstrated, OS (16), but are not worldwide available in this setting. Moreover, bispecific antibodies have shown promising results in heavily pretreated patients in third or further lines of treatment (18–20), but they still lack EMA approval and can be administered just in the contest of clinical trials or compassionate use.

In the last three decades, the standard of care for young and fit patients in second line mandated consolidation with auto-transplantation. This approach was actually established in the pre-Rituximab era (26) and, even if later studies pointed out that especially refractory and early relapsed patients obtain limited benefit from transplant (27)(28), it was considered the therapy of choice until nowadays and it is part of the most recent therapeutical algorithm for patients relapsing after 12 months from the termination of first line treatment (Figure 1)(29).

The study SCHOLAR-1 convincingly demonstrated the poor outcome of refractory DLBCL. Collecting data from 636 patients, it is the largest study in this setting and it documented that refractory patients who did not undergo transplant had a median OS of only 5 months, while transplanted patients reached 14.4 months of median OS, with a median OS in the whole population of 6.3 months (30).

It should be considered that about 30-40% of DLBCL patients will experience relapse; of these, half will be eligible to transplant, while the remaining 50% will be excluded, due to age or comorbidity. Again, just half of the transplant-eligible patients will obtain an adequate response to salvage treatment and will subsequently undergo transplant; finally, just half of the transplanted patients will show a durable response and could be considered “cured” (at the end they would be approximately 5% of patients) (29).
Clearly, these data are unsatisfactory and disclose the urgent need for new therapeutic approaches: CAR-T are thought to enhance the proportion of “cured patients” to about 20% (29), but these

predictions have to be confirmed in real-life, where they will face with feasibility and sustainability concerns; the way to efficiently solve the "relapse problem" is still long to go.

2.3 Time to relapse, what do we know?

The role of the time to relapse in determining the prognosis of patients is well-established in follicular lymphoma and mantle cell lymphoma and has become a matter of interest also in DLBCL. Similarly to the aforementioned pathologies, in DLBCL the event-free survival at 24 months (EFS24) turned out to be a crucial timepoint, since patients achieving this goal did not seem to have an impairment in OS, if compared to the general population (31).

Various and heterogenous studies have evaluated the clinical characteristics and outcomes of patients with early versus late relapse, often considering different cut-offs:

- ***Vose et al*** examined 162 patients treated in the pre-Rituximab era: 32 relapsed after more than 5 years of remission, and 130 had earlier relapses; although the former had better characteristics at diagnosis (limited stage, normal LDH), no difference in terms of post-relapse OS was detected (32).
- ***Modvig et al*** analyzed the Danish Lymphoma Group Registry and identified 818 relapsed patients, 78 of which could be defined as late relapse, occurring more than 5 years after CR; they appeared to have better IPI, performance status and OS, in relation to early relapsed patients. Radiotherapy displayed a protective role towards early but not late relapse, thus suggesting that early relapses could arise because of the persistence of minimal residual disease at the end of treatment (which radiotherapy could eradicate), while late relapses could be considered as *de novo* diseases derived from clonal instability (which radiotherapy could increase) (33).
- ***Vannata et al*** considered as late relapses those taking place after 5 years from initial diagnosis: in a cohort of 196 relapsed patients, late relapses (36 patients) were characterized by normal LDH levels, limited stage disease and low IPI when compared with early relapses (160 patients); late relapsing patients showed better 5-year OS (47% vs 25%). As previously demonstrated by others (34) and differently to what stated by *Maurer et al* (31), lymphoma patients (either relapsing at any time, or maintaining CR) had inferior OS rates in comparison with the general population, with the exception of patients younger than 50 years with persistent CR after more than 2 years (35).

- ***Wang et al*** focused on 78 patients relapsing 24 months after the initial diagnosis (after reaching EFS24): they showed that advanced stage and higher IPI at diagnosis were associated with increased risk of recurrence in this population. Patients with concurrent indolent lymphoma had a higher cumulative incidence of late relapse, although they were characterized by a better post-relapsed survival (not reached) when compared to patients with DLBCL alone (median post-relapse OS of 29.9 months) (36).
- ***Kang et al*** reported in a large study on 846 patients, that late relapses, defined as those occurring after 24 months from the obtainment of CR, were associated with lower IPI, LDH and beta-2-microglobulin level, lower incidence of B-symptoms, predominance of limited stages (I–II) and of GCB subtype at diagnosis, compared to early relapses; post-relapse OS was significantly longer, and the ORR after salvage chemotherapy was improved (37).

A first insight into the differences in genomic profiles between early and late relapses was provided by ***Broseus et al***, who demonstrated that patients relapsing within 12 months from the completion of first line therapy presented copy number variations (CNVs) in genes involved in transcriptional regulation, cell cycle and apoptosis, while, in patients relapsing after this cut-off, CNVs affected genes related to immune response, cell proliferation and transcriptional regulation. A limit of this study was the heterogeneous lymphoma sampling, in part at diagnosis and in part at relapse (38).

Interestingly, a recent study by ***Hilton et al*** aimed at correlating the time to relapse with genetic divergence, based on whole genome/exome sequencing. Three categories were defined:

- refractory disease: comprises primary refractory patients and those relapsing within 9 months from diagnosis (39);
- early relapsing disease: relapse occurring between 9-24 months from diagnosis;
- late relapsing disease: relapse after 24 months (40).

This analysis revealed that the degree of mutational divergence increased with time to relapse, and supported the hypothesis of a branching evolution from a common ancestor; thus, late relapses could be considered as *de novo* diseases, potentially retaining chemosensitivity, which is in line with the significantly superior post-ASCT PFS and OS shown by late relapsed patients in comparison to the other two groups. On the other hand, primary refractory patients seem to harbor an innate chemoresistance, since treatment does not determine substantial mutational changes (41). Considering the solid biological background of this work, we decided to adopt their classification for our study.

2.4 How to predict prognosis

As already described in the previous paragraphs, relapse is associated with adverse outcome. Thus, the identification of unfavorable biological features and the development of prognostic scores, able to promptly recognize high-risk patients, is of primary interest.

- The determination of the **COO**, as previously described, is nowadays a fundamental step in the formulation of a diagnosis of DLBCL. The classification into *germinal center B-cell* (GCB) subtype, *activated B-cell* (ABC) subtype and *unclassified* is based on GEP analysis on fresh frozen or formalin-fixed paraffin-embedded samples (using Lymph2Cx platform). Anyway, in the clinical practice it is usually determined using IHC algorithms (such as Hans' algorithm), which are less precise (giving a dichotomous categorization into *GCB* and *non-GCB*), but find faster and cheaper application (42). *ABC subtype* was reported to be characterized by inferior outcome (43).
- **Molecular features** associated with dismal outcome are MYC rearrangements (alone or combined with BCL2 rearrangements) (44)(45) and the double-hit signature (DHIT-sig); the latter is defined by gene expression profiling of a panel of 104 genes and patients DHIT-sig+ often harbor cryptic MYC and BCL2 rearrangements (46,47).
- We have already discussed the new **genetic classifications** proposed by Schmitz and Chapuy; the proposed categories are characterized by different outcome, in particular: BN2, EZB (Schmitz), C1 and C3 (Chapuy) seem to have a good prognosis, while MCD, N1 (Schmitz), C5 and C4 (Chapuy) have an adverse outcome (5)(4).
- **The International Prognostic Index (IPI)** was the first clinical scoring system, established in the pre-Rituximab era, but still widely used today. It defined 4 categories of patients (low, intermediate-low, high-intermediate and high), based on the assignment of 1 point to each of the following factors: age > 60 years, LDH increase, advanced Ann Arbor stage, Eastern Cooperative Oncology Group performance status > 2, ≥ 2 sites of extra-nodal involvement. For each category 2-year and 5-year PFS and OS were predicted (48). The **Revised-IPI (R-IPI)** was subsequently developed to estimate the outcome of individuals receiving Rituximab in association with chemotherapy; this latter score was based on the aforementioned factors but differentiated patients into three groups (very good, good, poor) (49). It was reported to better differentiate long term patients' outcomes, but its superior performance was questioned (50). To better identify patients with adverse outcome, the **National Comprehensive Cancer Network IPI (NCCN-IPI)** was

proposed: the same factors of IPI and R-IPI were employed, but better specified and differently weighted. This score outperformed IPI and R-IPI, particularly in the discrimination of OS, but it failed to recognize of a very poor risk subgroup characterized by 5-year OS < 50% (51).

- About 70% of patients affected by DLBCL are older than 65 years and their outcome remains poor because of comorbities, which impair access to some treatment options, and increased therapy toxicity; for this reason, the *Fondazione Italiana Linfomi* elaborated a new **Elderly Prognostic Score (EPI)**. The score is based on a simplified geriatric assessment - that classifies patients as fit, unfit or frail according to age, activities of daily living (ADL), instrumental activities of daily living (IADL) and the Cumulative Illness Rating Scale for Geriatrics (CIRS-G) - hemoglobin levels and IPI. It leads to the identification of 3 risk groups: low (with estimated 3-year OS of 87%), intermediate (with estimated 3-year OS of 69%) and high (with estimated 3-year OS of 42%) (52).
- The role of predictor of some **metabolic parameters** has also been explored; for example, since DLBCL have been recognized to be addicted to lipids metabolism for proliferation (53), the effect of serum lipids on prognosis was examined by *Yu et al*. They proposed a new score, the IPI-A, which conjugated the pre-treatment serum value of apolipoprotein-A-I with IPI and resulted in a significantly improved risk prediction in comparison to IPI (54).
- The **nutritional state** is known to impact on the prognosis of various diseases, including lymphoproliferative disorders; the Controlling Nutritional Status (CONUT) score is one of the indexes considered for prognosis prediction in DLBCL. It is based on serum albumin, total cholesterol and lymphocytes values, reflecting protein and caloric reserve and immune status, respectively; high CONUT score was reported to be related with inferior OS and PFS (55).
- **PET-CT** is an important tool to determine stage and therapy response, which is generally evaluated according to the Deauville score (DS). Anyway, the predictive value of the DS, although reported by some authors (56), was not definitely confirmed and other measurements were then proposed. The total metabolic tumor volume (derived by the sum of all metabolic volumes – both nodal and extra-nodal) seemed adequate in predicting EFS and OS (57), and the delta SUVmax (SUVmax at interim scan – SUVmax at baseline) was also demonstrated to predict 2-year PFS and 2-year OS (58); unfortunately, escalating treatment on the basis of interim PET did not improved outcomes.
- In recent years, several prognostic **immune risk scores** have been proposed, taking into consideration the interplay between neoplasia, inflammation and micro-environment; we

will discuss them in the paragraph "*The role of inflammation*". Interestingly, *He et al* elaborated a prognostic model based both on metabolism-associated genes and on the immune signature, which proved to have a better prognostic value than the IPI score (59).

- **Circulating tumor DNA** (ctDNA) is constituted by DNA fragments derived from tumor cells and released in the bloodstream. It can be detected through NGS techniques able to identify clonal tumor heavy chain sequences or tumor specific mutations derived from disease-dedicated gene panels (60). High levels of ctDNA are reported to be associated with poor PFS, EFS and OS, as assessed by many studies (61) and *Herrera et al* documented a positive predictive value of 88.2% and a negative predictive value of 97.8% in anticipating relapse (62).

Given the urgency of reaching a better risk stratification in DLBCL, it is not surprising that many new risk scores are crowding the literature; anyway, despite the frequent validation of these scores in external cohorts, none of the new proposals has firmly entered into the clinical practice until now. Obviously, the identification of high-risk patients should be associated to risk-stratified therapeutical algorithms, which would provide differentiated strategies according to the patients' risk profile.

2.5 Back to the molecular level

We have already discussed the importance of the new genetic classifications. Here we report the principal genes and pathways involved in DLBCL lymphomagenesis.

- **B cell development and differentiation**: **BCL-6** is the master regulator of germinal center reaction, regulating cell cycle, DNA damage response, cell death, plasma cell differentiation and cell migration. *BCL-6* could be involved in translocation leading to overexpression, or harbor mutations, which impair its negative autoregulation; enhanced BCL-6 activity can also depend on the dysfunction of its inhibitors (EP300, CREBBP and FBXO11) or on increased activity of MEF2B, which stimulates *BCL-6* transcription (63).
  PRDM1 function is to abrogate BCR signaling and promote plasma cell differentiation; its inactivating mutations determine differentiation block and NF-kB activation (64).
- **BCR and TLR signaling**: the B cell receptor (BCR) is essential for regulating non-malignant B cell survival and differentiation; it is implied in lymphomagenesis in two different ways: in ABC-DLBCL we generally find a chronic active BCR, which resembles antigen-dependent activation; while GCB-DLBCL show a tonic signaling (antigen-

independent) (65). Mutations can involve subunits of the BCR signaling complex as *CD79A* and *CD79B* (increasing BCR expression and impairing the negative feedback), but also downstream enzymes, as *CARD11*, *BCL-10* and *MALT1*, with the final consequence of promoting the activation of the NF-kB pathway. The same result can be obtained through an hyperactivation of the toll-like receptor signaling, often due to *MYD88* mutations (especially L265P) (6).

- **NF-kB** pathway regulates growth and survival of B-cells; its increased activation in DLBCL is often related to enhanced expression of the proto-oncogene REL or to inactivation of the ubiquitin-modifying protein TNFAIP3, which normally downregulates NF-kB pathway (66).
- **PI3K-AKT-mTOR** pathway has a crucial role in growth and survival of B cell malignancies. Activating mutations of PI3K subunits, *AKT* of *mTOR*, but also loss of function of the inhibitor *PTEN* can lead to constitutive activation of the pathway (4,5).
- **P53**: the role of P53 in DLBCL pathogenesis will be further discussed.
- **Cell apoptosis**: the implications of the BCL-2 family members in DLBCL pathogenesis will be further discussed.
- **NOTCH pathway**: NOTCH signaling regulates proliferation, differentiation and cell death; mutations determining increased stability of NOTCH1 and NOTCH2, but also inactivating mutation of the inhibitor SPEN are described in DLBCL (5).
- **Cell migration**: regularly, germinal center B cells are not able to survive outside the germinal center (GC) niche and their confinement into the GC is ensured by the Gα13 signaling; in DLBCL this pathway is perturbed in about 30% of cases and these alterations often co-occur with *BCL2* translocations (67).
- **Epigenetic regulators** are frequently involved in lymphomagenesis: activating mutations in *EZH2* lead to decreased expression of genes involved in cell cycle regulation and plasma cell differentiation (68); on the other hand, inactivating mutations of *KMT2D* results in the downregulation of several tumor suppressor genes (69). Finally, CREBBP and EP300 inactivation causes increased levels of BCL-6 and decreased p53 activity, but also reduction in the expression of genes involved in plasma cell differentiation and in immune response (70).
- **Immune escape** is sustained by deletions or inactivating mutations of HLA genes, *beta2-microglobulin* and *CIITA* resulting in loss of MHC class I and II expression; besides, mutations or deletions of *CD58*, causing impairment of NK cells adhesion (71), and gains or amplifications of *PDL1* and *PDL2*, determining T-cell exhaustion (72), are implied in immune evasion.

- **MYC**: the dysregulation of the transcription factor MYC is a hallmark of cancer and it influences cell growth and proliferation, as well as metabolism; *MYC* can undergo translocations, but also gains and amplifications, which determines overexpression (73).

2.6 Back to the molecular level - focus on P53

P53 is a master tumor suppressor gene, which plays a role in many cell functions (DNA repair, regulation of cell cycle and of the pro-apoptotic pathway, cellular senescence). Through its central DNA-binding domain it modulates the transcriptional activation of target genes. Normally, MDM2 (murine double minute 2 homolog) binds P53 and promotes its degradation in the cytosol, but cellular stresses determine the loss of the binding of MDM2 to P53 and thus lead to P53 activation (74). Once activated, P53 mediates the transcription of pro-apoptotic BCL2 proteins, of FAS, FasL and TRAIL-R2 (extrinsic apoptotic pathway proteins), and of effector caspases 9 and 6 (Figure 2) (75).

Inactivation of P53 is a hallmark of cancer and it is common also in DLBCL. *TP53* mutations are reported in more than 20% of DLBCL and in 90% of cases represent loss-of-function mutations (76,77); they mostly cluster within exon 5-8 (78) and their impact on survival in DLBCL is still controversial (78)(79). Less frequently, mutated P53 gains oncogenic properties (80). Finally, chromosome 17p13.1 results deleted in 10% of DLBCL, apparently without impact on survival (77).
Other mechanisms leading to P53 disfunction are:

- MDM2 overexpression (40% of DLBCL) (81);
- amplification of *MDM4* and *RFWD2* (two other P53 inhibitors) through gains of chromosome 1q23.3 (15% of DLBCL) (82);
- amplification of *BCL2L12*, a P53 and caspases 3/7 inhibitor (10% of DLBCL) (82);
- *CDKN2A* deletions, with subsequent decreased expression of ARF (p14), a MDM2 inhibitor (83) (19–35% of DLBCL) (84);
- loss of TP53 positive modifiers KDM6B and RPL26 (they could also be targeted by 17p13.1 copy loss) (85)(86);
- single nucleotide variants at the *TP53* 3' UTR (miRNA binding site): miR-34s, miR-125b, miR-504, miR-25 and miR-30d normally repress *TP53* and their impaired binding can lead to a better outcome if *TP53* is wild type (WT); by contrast, if *TP53* is mutated, its enhanced expression may confer inferior outcome (87);

- abnormal expression of P53 isoforms (Marcel 2011);
- deletions of P53 target genes *PERP* (caspase 8 activator, 27% of DLBCL) and *SCOTIN* (pro-caspase 3/7 activator, 8% of DLBCL) (82).

P53 plays also a role in the promotion of proinflammatory genes (88) and in the regulation of the flux of glucose thorough the glycolytic pathway; in fact, P53 loss of function is implied in the switch from oxidative phosphorylation to glycolysis (89), which is generally fundamental in cancer growth (90).

R/R DLBCL are reported to harbor *TP53* mutations observed in about 50% of cases; these mutations appear to be present in DLBCL subclones at diagnosis, which are then selected during treatment (91,92).

Recently, *Liu et al* proposed the incorporation of p53 mutational status and of two PET-based parameters - total metabolic tumor volume and the largest distance between two lesions - into a nomogram, which resulted to have higher prognostic power for 1-year PFS than IPI, aa-IPI and NCCN-IPI (93).

2.7 Back to the molecular level - Focus on the BCL-2 family

Escaping apoptosis is a hallmark of lymphoid malignancies. Here we will focus on the intrinsic (mitochondrial) apoptotic pathway, which is generally triggered by cellular stress and is crucial in the development of cancer. In fact, oxidative stress, hypoxia or DNA damage can lead to alteration of the mitochondrial outer membrane permeability, which determines the transition of pro-apoptotic molecules from mitochondria to cytosol and in the end causes caspases activation. The mitochondrial outer membrane permeability is regulated by a group of protein, collectively defined as “the BCL-2 family”, which comprises:

- anti-apoptotic (multi-domain) proteins: B-cell leukemia/lymphoma-2 (BCL-2), myeloid cell leukemia-1 (MCL-1), B-cell lymphoma-extra-large (BCL-XL), B-cell lymphoma-W (BCL-W) and others;
- pro-apoptotic multi-domain effector proteins: BCL-2–associated X protein (BAX), BCL-2 antagonist/killer 1 (BAK1) and BCL-2 homologous antagonist killer (BOK);
- pro-apoptotic BCL-2 homology (BH)3 domain-only proteins: BCL-2-interacting mediator of cell death (BIM), BCL-2 antagonist of cell death (BAD), p53 upregulated modulator of apoptosis (PUMA) and others (94).

The interplay between pro- and anti-apoptotic proteins determines cell fate: according to the direct activation model, anti-apoptotic proteins bind and inhibit the pro-apoptotic effectors, whose function would be the creation of pore-like structures in the outer mitochondrial membrane. The BH3 domain-only proteins can act both as direct activators – directly interacting with the effectors – or as sensitizers – through an interaction with the anti-apoptotic molecules, which leads to the release of the BH3 domain-only activators (Figure 3) (95).

As in the majority of B-NHL, also in DLBCL the proteins belonging to the BCL-2 family are frequently involved in the pathogenesis of the disease and BCL-2, in particular, has been extensively studied in this setting. Indeed, translocations t(14;18)(q32;q21) can be detected in approximately 30% of GCB DLBCL, while about 20% of ABC DLBCL carries amplifications of 18q21 locus, with subsequent BCL-2 overexpression (96)(97); globally, BCL-2 expression is found in 49%-67% of DLBCL (95). As for MCL-1, it is reported to be highly expressed in 84% of DLBCL (98), more often ABC-type (99); similarly, BCL-W is frequently overexpressed in DLBCL, with no difference between ABC and GCB-type (100). Finally, BCL-XL appears to be highly expressed in approximately 95% of DLBCL (101).

Recently, *Roh et al* published a BCL-2 signature score derived from unsupervised hierarchical cluster analysis and based on the expression of BOK, BCL2L15 and BCL2; in the study cohort of 157 patients, the score showed significative association with EFS (102).

Besides, *De Jong et al* demonstrated that, although DLBCL cells frequently express several anti-apoptotic proteins at the same time, they are usually functionally dependent on BCL-2 or MCL-1; thorough a dynamic BH3 profiling, they verified that CHOP therapy can alter this dependency towards BCL-XL in at least 3 DLBCL cell lines (103).

Obviously, given the important role of the BCL-2 family members in lymphomagenesis, many efforts have been made to generate target drugs; after some unsatisfactory attempts (Oblimersen sodium, Obatoclax, Navitoclax), Venetoclax, a highly selective BCL-2 inhibitor entered the clinical practice for the treatment of chronic lymphocytic leukemia (CLL) (104). Venetoclax monotherapy in DLBCL resulted disappointing (ORR 18% and CR 12%) (105) and was thus tested in combined regimens with R-CHOP or dose-adjusted etoposide, prednisolone, vincristine, doxorubicin, cyclophosphamide, rituximab (DA-EPOCH-R), showing improved CR rates in BCL-2 positive DLBCL and in double-hit lymphomas (106); a phase 2/3 trial testing these combinations in double-

hit and double-expressor lymphomas was started in 2019 but in currently suspended (NCT03984448).

Several phase 1 trials with MCL-1 inhibitors are now ongoing (95), while BCL-XL inhibitors did not were excluded from clinical use because of severe thrombocytopenia induced by BCL-XL inhibition in platelets; to mitigate this relevant side effect the proteolysis-targeting chimera (PROTAC) technology has been applied to develop a BCL-XL PROTAC, which would avoid BCL-XL degradation in platelets (107).

2.8 What about metabolism?

Metabolic reprogramming is a crucial step in tumor growth and proliferation; initially, great attention was paid to the glycolytic pathway (following the hypothesis of the Warburg effect), but later the involvement of oxidative phosphorylation, the utilization of different metabolites as energy sources and the interplay with the micro-environment gained more and more relevance (108).
The Consensus Cluster Classification (CCC) in 2005 identified, on the basis of transcriptional profiles, three clusters: the B-cell receptor/proliferation cluster (BCR-DLBCL, characterized by increased expression of the components of the BCR pathway), the oxidative phosphorylation cluster (OxPhos-DLBCL, with an enhanced expression of genes involved in the mitochondrial electron transport chain (ETC), particularly subunits of complexes I and V), and the host response cluster (HR-DLBCL, marked by a T-cell-rich inflammatory immune cell infiltrate) (109). BCR-DLBCLs appeared to rely principally on glycolysis, while OxPhos-DLBCLs showed a prevalent mitochondrial energy production, reflecting the exploitation of different survival strategies (108). Deeper analysis based on proteomics, metabolomics and mitochondrial respirometry, showed that OxPhos-DLBCLs are characterized by increased oxidative phosphorylation, elevated entry of glucose and fatty acid-derived carbons in the tricarboxylic acid cycle, marked fatty acid oxidation (which could represent a source of NADPH to generate glutathione), increased efficiency of ETC complexes and higher glutathione levels (with supposed greater detoxification capacity) (110).
Regardless of the aforementioned classification, the metabolic pathway which are generally involved in order to sustain malignant proliferation in DLBCL are the following (Figure 4) (111):

- **Aerobic glycolysis**: this is a major way for energy production and for the rapid generation of metabolic intermediates; it is sustained by MYC, which increases the glycolysis flux through overexpression of lactate dehydrogenase, monocarboxylate transporter 1 (MCT1), glucose transporter 1 (GLUT1) and pyruvate dehydrogenase kinase (112); the transcription

of these factors is also enhanced by the hypoxia-inducible factor 1 alpha (HIF-1alpha) (113). Besides, the inactivation od P53 promotes the expression of GLUT1, of the hexokinase 2 and of oncogenes as MYC itself (90)(114). NF-KB signaling stimulates the membrane localization of GLUT1 (115) and PI3K/AKT/mTOR pathway contributes to glycolysis as well (116).

- **Amino acid metabolism**: in this setting, glutaminolysis plays a central role to ensure energy supply, redox homeostasis and antioxidative protection (117); once more, MYC and P53 are implied in the promotion of this pathway.
- **Fatty acid metabolism**: lipid synthesis in crucial for proliferating cells, which need to rapidly generate new cell membranes and organelles; the overexpression of the fatty acid synthase (FASN) was reported to be an unfavorable prognostic factor in DLBCL (118) and its transcription is regulated by the PI3K/AKT and MAPK signaling (116).

Obviously, considering the importance of the energy supply pathways for the survival of malignant cells, targeted drugs are under development. Recently, the combined use of an inhibitor of monocarboxylate transporter 1 (MCT1) with a Complex I inhibitor showed promising results, with synergistic DLBCL cell death *in vitro* and anti-tumor effect in xenografts; this suggested that addressing simultaneously both the glycolytic and the mitochondrial pathway could prevent from the activation of compensatory mechanisms, resulting in greater efficacy (119).

2.9 Focus on NADH:Ubiquinone Oxidoreductase Subunit B 1 (NDUFB1), a Complex I subunit

As we will further explain, on the basis of gene expression analysis, we focused our attention on the NADH:Ubiquinone Oxidoreductase Subunit B 1 (NDUFB1). NDUFB1 is a 58 amino acids protein, which belongs to the mitochondrial membrane respiratory chain, and particularly to the NADH dehydrogenase (Complex I), located in the mitochondrial inner membrane. Complex I is responsible for the transport of 2 electrons from NADH to Ubiquinone (first step of the electron transport through the respiratory chain) (Figure 5) and it is also involved in the production of reactive oxygen species; apparently, NDUFB1 does not take part to the catalytic process and its function has not been fully elucidated. As for the majority of the accessory subunits, the knockout of NDUFB1 results in the loss of an assembled Complex I (120). The gene appears to be overexpressed in heart and in peripheral blood mononuclear cells (as for normal tissues), in various cancer cell lines (Figure 6) and tumor samples, including malignant lymphomas (Figure 7, data extrapolated from Genecards, Uniprot and Genevestigator datasets). Nevertheless, until now, the

role of NDUFB1 in cancer has not been extensively investigated; available data derive from a study on lung squamous cell carcinoma, where *NDUFB1* gene expression resulted to be a positive prognostic factor (121), and from Clear-Cell Renal-Cell Carcinoma, where the down-regulation of NDUFB1 and other Complex I subunits' RNA was hypothesized to contribute to the Warburg shift and finally to inferior outcomes (122). We still lack the necessary knowledge to conciliate these contrasting data, and further studies are needed to get a more defined overview of the interplay between metabolic pathways and cancer development.

2.10 The role of inflammation

Various types of lymphoma, and particularly DLBCL, have been reported to be associated with inflammatory conditions. Thus, autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, Sjogren's syndrome and chronic thyroiditis, especially if characterized by a severe development, lead to an increased risk of NHL. Two main mechanisms are supposed to be involved in lymphomagenesis: long-standing systemic inflammation probably contribute to the emergence of non-localized lymphoma (as DLBCL), while persistent stimulation of self-reactive B cells may lead to organ-specific lymphoproliferative disorders (as marginal zone lymphomas) which can eventually evolve into DLBCL (123). On the other hand, we have to consider that because of their immune nature, lymphomatous cells can have a wide range of interactions with the immune system, which can constitute a supportive micro-environment and sustain lymphoma growth or turn into a suppressive milieu, from which the tumor cells have to escape (Figure 8)(124).

In many solid and hematologic tumors, a correlation between circulating inflammatory parameters and adverse prognosis was observed, with subsequent elaboration of cumulative prognostic scores (125). As for DLBCL, the following prognosticators were identified:

- erythrocyte sedimentation rate (ESR): ESR was reported to be related to unfavorable characteristics (advanced stage, high IPI extra-nodal involvement, MYC expression) and to inferior PFS and OS; its periodic evaluation could even help in relapse prediction (126);
- the neutrophils to lymphocytes ratio (NLR): neutrophils can sustain tumor growth through the production of cytokines and growth factors and their increase can be favored by the granulocyte colony-stimulating factor, released by the tumor cells; on the contrary, lymphocytes (particularly T cells) should recognize and destroy tumor cells and their decrease corresponds to the failure of immune surveillance; thus, NLR reflects neoplasia and micro-environment interactions and the patients' immunologic response (127)(128);
- the Systemic Immune-Inflammation Index (SII), calculated as

(neutrophils' count x platelets' count) / lymphocytes' count: SII has been shown to correlate with tumoral activity and inflammatory conditions (several cut-offs have been proposed by various studies) (127)(129);

- the CAR Index, calculated as C-reactive protein (CRP) value / albumin value: elevated CRP appears to be linked with cancer-related production of inflammatory cytokines, while the albumin value is a known marker of nutritional status and is related to chronic inflammation, thus CAR mirrors both inflammatory and nutritional conditions (127);
- the lymphocytes to monocytes ratio (LMR) and the LMR/LDH ratio: it has been reported that LMR can adequately reflect the patient immune status and both high LMR and high LMR/LDH are associated with shorter PFS (130);
- the immune risk score, based on a complex formula, which takes into account memory B cells, follicular T helper cells, T gamma-delta cells, NK cells, eosinophils and macrophages (subdivided into M0-M1-M2 classes); this equation was derived using the CIBERSORT algorithm and the 8 cell populations were inferred from gene expression analysis (131).

Some of these factors have been combined with other clinical features into nomograms, aimed at ameliorating prognosis prediction, with encouraging results (127)(129)(131), which need to be validated in further studies.

2.11 Focus on CD5+ DLBCL

As previously reported, DLBCL is a heterogenous disease and comprises many different entities. CD5+ DLBCL represents about 5-22% of all DLBCL cases and are reported to have a more aggressive behavior and a worse outcome, compared to the CD5- counterpart (132). Generally, CD5+ diseases affect elderly patients and are characterized by high LDH levels, development of B-symptoms and extra-nodal involvement (133), with frequent CNS and bone marrow localizations (134). As for morphology, differently from classical DLBCL, four variants are identified: common (76%), polymorphic (12%), giant-cell (11%, associated with worst prognosis), and immunoblastic (1%) (135). The co-expression BCL-2 and MYC is detected in 27.6% of CD5+ patients vs 3% in CD5- (136) and age, IPI and MYC expression are independent outcome predictors (137).

Recent studies investigated the biology and the molecular subtyping of this subgroup of lymphomas; in particular, *Ma et al* revealed a higher incidence of ABC subtype (considering COO determination with Lymph2Cx) and of MCD subtype (determined by next-generation sequencing and classified according to *Schmitz et al*); in addition to *MYD88* and *KMT2D* mutations, also *PIM1* and *CDKN2A* are frequently mutated in CD5+ DLBCL. Increased mRNA expression of MME and

SERPINA9 correlates with improved outcome, while increased expression of CYB5R2 is associated with inferior outcome (138). Besides, these lymphomas are characterized by the overexpression of integrin beta1 and the downregulation of extracellular matrix genes, which probably contribute to the aggressive nature of the disease (139). The biological mechanisms which underlay the aggressiveness of CD5+ has been investigated, although not fully elucidated. One pathway consists on the inhibition of BCR signaling; in fact, CD5 acts, through the recruitment of SHP-1, as an inhibitor of BCR, thus eluding programmed cell death and enhancing B-cell survival. In addition, the activation of ERK pathway and of the transcription factors STAT3 and NFAT2, determines the overexpression of interleukin (IL)-10 (the principal cytokine involved in B-cell proliferation), BCL-2, Cyclin D2, and CXCR4, which sustain survival and dissemination (132).

An attempt to ameliorate the outcomes of this disease came from the phase II PEARL5 study, which combined 8 cycles of DA-EPOCH-R with 2 cycles of HD-MTX, administered between the 4^{th} and 5^{th} DA-EPOCH-R (140). The 5-year follow-up of this study was published last year: 5-year PFS and OS resulted 72% and 79%, respectively, at a median follow-up of 6 years; 5-year CNS relapse rate was 9%, inferior in comparison to historical data (13%), establishing the role of DA-EPOCH-R/HD-MTX as an effective first line regimen for CD5+ DLBCL (141). Recently, a Japanese real-world analysis based on the comparison of 11 CD5+ patients treated with DA-EPOCH-R/HD-MTX vs 52 CD5- patients showed similar results in terms of ORR and CR (90% vs 82.9% and 90% vs 80.5%); analogously, there was no significative difference in 2-year PFS and OS (81.8% vs 78.8% and 81.8% vs 59.1%), confirming the efficacy of this treatment (142). To further improve the outcome of CD5+ DLBCL, the incorporation of drugs targeting BCL-2 and CXCR4 into combination regimens appears a promising approach, but clinical studies in this setting are not currently ongoing (132).

3. AIM OF THE STUDY

As discussed before, DLBCL are the most frequent type of NHL and relapses – occurring in about one third of cases – are characterized by a dismal outcome (30). Thus, the prompt recognition of patients at high risk of relapse – based on validated and reproducible prognostic scores - is of primary interest. Once identified, these high-risk patients could undergo intensified or target therapy, as an effort to revert their poor prognosis.

The principal purpose of this study was to obtain a global characterization of a large cohort of relapse/refractory DLBCL patients, collecting both clinical and biological features, as follows:

- clinical characteristics: age, sex, presence of B-symptoms, LDH increase, extra-nodal, bone marrow and CNS localization, ECOG performance status, stage, bulky disease, prevalence of HBV, HCV and HIV infections, dosage of VES, Immunoglobulin G, calculation of primary and secondary IPI and R-IPI, R/R IPI, SII and CAR, SUV max at diagnosis and relapse, kind of first line therapy and subsequent response, incidence of infection during first line treatment;
- biological characteristics: cell-of-origin determination, Ki67 value, expression of CD5, P53, NDUFB1, MCL1, BCL-XL, BCL-W.

On the basis of recent evidences (41), we further categorized the R/R patients into three different subgroups, according to their different time of relapse, with the objective of highlighting features specific of each group.
Besides, we aimed at comparing the whole R/R cases with a group of controls – defined as patients not experiencing relapse after at least five years from diagnosis and first line treatment - in order to identify differentially distributed variables and their impact on patients' outcome.
Both the subgroups analysis and the match with controls should pave the way for the identification of new prognostic markers in DLBCL.

Finally, as far as the biological analysis are concerned, they could also reveal proteins and pathways implied in disease persistence and recurrence, thus providing possible new pharmacological targets.

4. MATERIALS AND METHODS

4.1 Patients and evaluation of clinical features

We retrospectively analyzed the medical charts of patients referred to the Hematology Unit of Padua University Hospital – Department of Medicine (DIMED) - between October 1999 and March 2023; we selected 140 R/R DLBCL cases and 45 patients diagnosed with DLBCL between January 2011 and January 2018, not affected by relapse until August 2023 (controls' cohort). The R/R patients were divided into three different subgroups, according to their different time of relapse, thus identifying the *refractory* (characterized by persistence of disease or recurrence within 9 months from diagnosis), the *early relapsed* (with recurrence of disease within 10 and 24 months) and the *late relapsed* (with recurrence of disease beyond 24 months). All patients signed at diagnosis an informed consent for the use of their clinical and biological data to research purposes, in accordance with the Declaration of Helsinki. Clinical data concerning diagnosis, treatment and follow-up were available for most patients, while the histological formalin-fixed paraffin-embedded (FFPE) samples of diagnosis and relapse were not always available, or adequate for further analysis. For the following continuous variables, we set thresholds – derived from literature – to discriminate between increased and normal values: SUV at diagnosis and relapse was considered elevated when ≥ 15 (143), VES when > 37.5mm/h (126), N/L ratio if ≥ 3.5 (128)(144), SII when > 1684.09(127), CAR if > 0,21 (127).

4.2 Histological evaluation and preparation of tissue micro arrays (TMA)

The histological analysis, performed by the Unit of General Pathology and Cytopathology of Padua University Hospital – Department of Medicine (DIMED), was carried out on slices of formalin-fixed paraffin-embedded (FFPE).
Tissue microarrays were prepared for cases with sufficient biological material. The original slides were reviewed by two pathologists (M.P. and F.S.) to confirm the diagnosis of DLBCL. Representative tumor areas were selected and two tissue cores (diameter: 2 mm) were obtained for each case. Appropriate positive and negative controls from lymphoid and non-lymphoid tissues were also included. TMA blocks were prepared using the Galileo TMA CK3500 arrayer (Integrated System Engineering, Milan, Italy).

4.3 Immunohistochemical analysis

Immunohistochemical analysis was run on 3–4-μm-thick tissue sections, using the following primary antibodies in the BondMAX automated immunostainer (Leica Biosystems): CD10 (clone DAK-CD10; Dako), BCL6 (clone LN22; Leica Biosystems), BCL2 (clone 123; Dako), c-MYC (clone EP121; Epitomics), MUM1 (clone MUM1p; Dako); Ki67 (clone SP6; Cell Marque), CD5 (clone 4C7; Leica Biosystems); P53 (clone DO-7; Leica Biosystems), NDUFB1 (Polyclonal; Proteintech); MCL1 (clone D5V5L; Cell Signaling), BCL-XL, (clone 54H6; Cell Signaling), BCL-W (clone 31H4; Cell Signaling). The positivity for BCL6, CD10, BCL2, MUM1 and Myc was assessed using cut-offs reported in the literature (≥30% of positive neoplastic cells for BCL6, CD10 and MUM1; ≥40% of positive neoplastic cells for MYC; ≥50% of positive neoplastic cells for BCL2). As for P53, expression was assumed enhanced over 70% of positive neoplastic cells.

4.4 Gene expression analysis in selected patients' samples

Gene expression analysis on FFPE tissue sections was performed in collaboration with the Pediatric Hematology Unit, Oncology and Stem Cell Transplant Division, University Hospital of Padua, using the one-Cycle cDNA Synthesis Kit, the IVT Labeling Kit and the Human Clariom S arrays (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's indications. Bioinformatic analysis was conducted using R software and designated libraries (R Development Core Team).

4.5 Analysis of NDUFB1-gene expression in cell lines

To verify the expression of the gene coding for NDUFB1 in cell lines (provided by Leibniz Institute DSMZ) we employed the reverse transcription kit Reverse Transcription System (Promega), kit Luna (New England BioLabs) as reagents for Real-Time PCR and primers by Sigma- Merck; the amplification was performed by QuantStudio5 thermocycler (ThermoFisher).

4.6 Western blotting analysis

Protein extraction: whole cell extracts were obtained by lysis with 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA supplemented with 0,5% Triton X-100 (Merck), protease inhibitor cocktail (Merck), phosphatase inhibitor cocktail (Thermo Scientific), 1 μM okadaic acid (Merck), 1mM DTT (Merck).
In all Western Blot (WB) 20μg of lysate was subjected to SDS-PAGE and transferred to PVDF membrane. Densitometric analysis was conducted using Quantity One Software (Biorad). As

secondary antibodies HRP conjugated, we used Anti-Rabbit IgG (Cell signalling, 7074), anti-mouse IgG (Jackson Immuno Research). WB membranes were probed with the following primary antibodies: anti-β-ACTIN (Sigma; cat. A2228; dilution 1:3000); anti-GAPDH (Millipore; cat. MAB374; dilution: 1:3000); anti-NDUFB1 (Proteintech; cat. 16902-1-AP; dilution 1:500).

4.7 Statistical analysis

The comparison of clinical and immunohistochemical variables, between the R/R cohort vs controls and between the subgroups of patients stratified by the time of relapse, were performed with Kruskall-Wallis, Fisher's exact or Chi-square test, when appropriate. Survival curves were elaborated using the Kaplan-Meier method; median time from the Kaplan-Meier curve was provided along with the corresponding 95% confidence interval (CI) estimated using the Brookmeyer-Crowley method. Overall survival (OS) was considered as time from diagnosis to death and progression free survival (PFS) as time from diagnosis to recurrence of disease or death; last follow-up date was considered for patients not presenting events. Hazard ratio (HR) and confidence interval (CI) at 95% were obtained for each group with univariate Cox proportional hazards models. All tests were bilateral and data were retained statistically significant for p values <0.05. Statistical analysis was conducted using RStudio (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA).

5. RESULTS

5.1 Our starting point

In 2020 we completed the first collection of R/R DLBCL patients (about 100 cases) treated at our Institution and tried to divide them - according to the time to relapse – in four categories: *primary refractory* (not responsive to first line therapy), *early relapsed* (experiencing relapse within 12 months from the end of first line therapy), *intermediate relapsed* (experiencing relapse between 12 and 60 months from first line therapy) and *late relapsed* (experiencing relapse after 60 months from first line therapy). As discussed before, the theme of the "time to relapse", widely explored in other lymphoproliferative disease, was progressively getting more attention also in the contest of DLBCL; we then decided to go further with this research project, setting new subgroups on the basis of more recent evidences (41).
Besides, we had also the opportunity of having a more complete immunohistochemical characterization of our original cohort and of performing GEP on a restricted group of patients. Finally, we were able to enlarge the study cohort (reaching a total of 140 R/R cases) and to collect and analyze more clinical features; all these data will be presented in the following sections.

5.2 Data from the Lab

5.2.1 Gene expression analysis

We had the possibility of performing gene expression analysis on 19 DLBCL samples collected at diagnosis (13 R/R and 6 controls) (Figure 9); for 13 R/R patients we had also the tissue from relapse available. However, results were partly conditioned by RNA degradation. Upon correction for sensitivity thresholds, among a group of genes differentially expressed between R/R patients and controls, we identified the NADH:Ubiquinone Oxidoreductase Subunit B 1 (NDUFB1) as the most significative (log2FC 0.33) and worthy of further studies on cell lines.

5.2.2 Analysis of NDUFB1-gene expression in cell lines

Real time PCR was performed on healthy B lymphocytes (from buffycoat) and on DLBCL, MCL and plasmoblastic lymphoma cell lines. As shown in Figure 10, *NDUFB1* gene expression,

appeared enhanced in most lymphoma cell lines, particularly in plasmoblastic and some DLBCL coltures, namely OCI-Ly1, OCI-Ly18, Pfeiffer, OCI-Ly7, OCI-Ly10, U2932.

5.2.3 Western blotting analysis

We tested the anti-NDUFB1 antibody (Proteintech) obtaining the expected band at a 6 kDa weight. As reported in Figure 11, we found a concordant increased expression trend between mRNA and protein in the cell lines OCI-Ly1, OCI-Ly18 and OCI-Ly10, while in Pfeiffer, OCI-Ly7 and U2932 the protein level was not enhanced. On the contrary, the protein level was high in RI-VA, without a corresponding increase in mRNA expression; cell lines characterized by high levels of NDUFB1 protein had also elevated replication rate.

5.3 Patients' data

As described previously, we collected 140 R/R cases and 45 controls; the R/R patients were further divided into *refractory* (characterized by persistence of disease or recurrence within 9 months from diagnosis, 72 patients), *early relapsed* (with recurrence of disease within 10 and 24 months, 35 patients) and *late relapsed* (with recurrence of disease beyond 24 months from diagnosis, 33 patients). We present here most relevant data about the distribution of clinical and biological features in the whole R/R group – compared with the controls' cohort - and in the various R/R subgroups; impact on PFS and OS will also be reported.
Variables will be grouped in 5 main paragraphs according to the category of interest; data are then collectively displayed in Tables 3-6 and Figures 12-14.

5.3.1 Baseline data

Median age was 68 years in the whole R/R group (67 in the refractory, 68 in the early relapsed and 71 in the late relapsed) and 55 in the controls' cohort; males and females were well-balanced between cohorts, with the exception of a remarkable prevalence of males among the refractory patients (72%) (*p .005*). B-symptoms were more frequent in the R/R patients (41% *vs* 22%, *p .02*), particularly in the refractory (53%) (*p .01*); lactic dehydrogenase (LDH) increase was more frequent in the R/R (59% *vs* 51%, *p 0.36*, not significative), even if in the late relapsed cohort, it was less common (30%) (*p .002*). Extra-nodal, bone marrow and CNS localization were more often recorded in the R/R group (75% *vs* 56% (*p .009*), 36% *vs* 15.4% (*p .016*), 6% *vs* 0% (*p 0.1*, not significative),

respectively), but, once more, late relapsed were characterized by reduced bone marrow infiltration (16%, *p 0.12*, not significative) and CNS was never involved (*p 0.2*, not significative). Advanced stages were more frequent in the R/R (75% *vs* 62%, *p 0.13*, not significative), and among them, in the refractory (86%) (*p .0001*); SUV max at diagnosis ≥ 15 distributed evenly between groups. Finally, bulky disease was equally present in R/R and controls, but it was significantly reduced among late relapse (*p .003*) and the prevalence of HBV, HCV and HIV infections were similar between cohorts.

As for prognostic scores, we calculated IPI and R-IPI: low and intermediate-low IPI and very good and good R-IPI were prevalent in the controls' group (38% and 47% *vs* 19% and 30% (*p .0001*), 16% and 69% *vs* 7% and 41% (*p .0001*), respectively), while intermediate-high and high IPI and poor R-IPI were reported for most R/R patients (34%, 17% and 52%, respectively, *p .0001*); unfavorable IPI and R-IPI characterized in particular refractory patients (*p .008* and *p .017* - not significative, respectively). CNS-IPI was high in 31 R/R patients and in 3 controls.

As for the R/R cohort, we analyzed the impact on median PFS (mPFS) and median OS (mOS): males were characterized by inferior mPFS (7 vs 19 months, *p .0063*, 2-year PFS 16.9% *vs* 33.3%) and mOS (22 vs 74 months, *p .0085*). The presence of B-symptoms affected both mPFS (7 *vs* 13.5 months, *p .0009*) and mOS (17 *vs* 50 months, *p .0288*); similarly, LDH increase determined worse mPFS (7 *vs* 15 months, *p .0000*) and mOS (24 *vs* 75 months, *p .0393*) and advanced stage as well (with mPFS of 7 *vs* 18.5 months, *p .0000* and mOS 27 *vs* 83 months, *p .0079*). As expected, both IPI and R-IPI influenced mPFS and mOS with statistical significance (*p .0003* and *.0013* for mPFS and *p .0269* and *.0072* for mOS, respectively). Comparing IPI *vs* R-IPI in a multivariate model we found that IPI was the best PFS predictor and R-IPI the best OS predictor in our study.
Other variables with impact on mPFS were bone marrow involvement (7 *vs* 9 months, *p .0202*), CNS localization at diagnosis (5 *vs* 9 months, *p .0011*), bulky disease (6 *vs* 11.5 months, *p .0005*), and positive HBV-serology (7 *vs* 9 months, *p .0088*). Finally, extra-nodal involvement conditioned mOS (25 *vs* 76 months, *p .0395*).
Mortality rate was higher in R/R *vs* controls (71.3% vs 6.7%, *p .0001*) and it reached 85.5% in the refractory subgroup (*p .0001*); 58.76% of deaths among R/R were due to lymphoma progression.

Karyotype data were available for 50 patients (36%) among the R/R and 16 controls (36%): in the R/R group it resulted normal in 37 cases, while in 13 (26%) it was altered (8 could be defined as

complex karyotype); among the controls, karyo was normal in 14 patients and altered in 2 (13%), without cases of complex karyotype (*p 0.262*).
Considering all the 15 abnormal cases together, we observed that 8/15 (53%) were associated with other hematologic or non-hematologic malignancies, which resulted statistically significant when compared with patients carrying a normal karyo (*p .024553*).
Ki67 was available in 9/15 altered cases; we found that in 7/9 cases (77%) it was ≥ 70% (*p 0.317*); the determination of the cell-of-origin according to the Hans' algorithm was possible in 8/15 cases, and in 7/8 it resulted non-GCB type (significative, *p .015*).
When considering the R/R patients with altered karyo, 8/13 resulted primary refractory, 4/13 early relapsed and 1/13 late relapsed *(p 0.808*); all data are reported in detail in Table 7.

5.3.2 Inflammatory indexes

As previously reported, a ESR > 37.5mm/h was considered increased: it resulted beyond this threshold in 47% of R/R and in 40% of controls, without a significative difference.
When evaluating the N/L ratio, we assessed it as abnormal if ≥ 3.5: this occurred in 48% of R/R and in 38% of controls (no statistically significative); considering the R/R subgroups, the percentage fell to 26% among the late relapsed (*p .024*).
SII was retained altered when > 1684.09: this was the case in 20% of both R/R patients and controls.
Finally, CAR was considered elevated if > 0.21: again, there was no significative difference between R/R and controls (it was recorded in 64% and 54% of cases, respectively).
As for the R/R subgroups, both SII and CAR were more frequent altered in the refractory and rarely in the late relapsed group (*p .024* and *p .002*, respectively).

Considering the R/R cohort, N/L ratio ≥ 3.5 was associated with reduced mPFS (6 vs 12 months, *p .0019*); analogously, SII > 1684.09 conferred an inferior mPFS (5 *vs* 10 months, *p .0002*). CAR > 0.21 impacted both on mPFS (7 *vs* 15 months, *p .0004*) and on mOS (20 *vs* 87 months, *p .0003*).

5.3.3 Immunohistochemistry

Cell-of-origin could be determined, using Hans' algorithm, in half of the patient (95); no difference was found between R/R and controls or among the R/R subgroups, confirming the results of our

original analysis (2020). In that study we analyzed also the expression of BCL2 and MYC, finding no difference in the distribution between R/R and controls or among the R/R subgroups.
We then wondered if the proliferation index Ki67 could reflect the aggressiveness of the underlying disease and set two different cut off, at 70 and 80%; no differences emerged between R/R and controls or among the R/R subgroups, but in the R/R cohort, Ki67 showed an impact on mOS for both the considered thresholds with mOS of 18 *vs* 32 months (*p .0142*) and of 18 *vs* 24 months (*p .0328*), for patients with Ki67 < or ≥ 70% and < or ≥ 80%, respectively.

We extended our expression analysis to CD5, P53, NDUFB1, MCL1, BCL-XL, BCL-W (Figure 15); unfortunately, not all the patients' samples could be analyzed and these data are available for about half of them. In the comparison between R/R and controls, the most relevant output of this analysis was a more frequent (although not significative) altered expression of P53 in the R/R cohort (21% *vs* 5%, *p 0.1*); on the contrary, we did not find an increased NDUFB1 expression in R/R patients, as it had been suggested by GEP and also the other proteins tested did not reveal a differential expression.
When focusing on the R/R subgroups, CD5 and NDUFB1 appeared overexpressed just in refractory patients (but always below the level of statistical significance); as regards CD5+ patients, 3/5 were elderly (>75 years), 4/5 had increased LDH and 5/5 had extra-nodal involvement.
On the basis of the data from the analysis in cell lines, we looked for a correlation between NDUFB1 expression and Ki67 value: in 4/9 overexpressed cases it was not available, in 1 it was about 60% and about 70% in the remaining 4 patients. Noteworthy, NDUFB1 enhanced expression in the R/R cohort was associated with a mPFS disadvantage (4 *vs* 9 months, *p .0005*).
As far as P53 is concerned, all patient with enhanced expression were refractory, while all the cases negative for P53 belonged to the late relapsed group. Karyotype was available just for 3/10 patients with altered P53 expression and in 2/3 it was altered (one hyperdiploid clone and one complex karyotype).

For 15 patients we had immunohistochemical data of both diagnosis and relapse: in 7 cases there was no relevant differences as regards the expression of the above-mentioned proteins. In 5 cases, samples at relapse showed an increase expression of BCL-XL (3/5 were late relapse), one case revealed an enhanced MCL1 positivity and another BCL-W expression; finally, one case gained expression of p53, NDUFB1 and MCL1.

5.3.4 First line therapy - kind of regimen, response and toxicity

Considering all the 185 patients analyzed, 77% received as first line treatment CHOP-like regimens (91% of controls and 74% of R/R, respectively, *p .0490*); the combination of Etoposide, Cyclophosphamide, Mitoxantrone and Prednisone (VEMP) was another scheme which was quite often administered, especially in aged or unfit patients; given the older median age of the R/R, in this group the regimen was indeed more frequently used (16% *vs* 7%, *p 0.049*), without significative differences among R/R subgroups. Rituximab was associated to chemotherapy in all but 10 R/R patients.
Radiotherapy (RT) was administered to 40/185 patients (22%); there was no correlation with the presence of bulky disease, while we found a significative association with limited stage (50% of patients with limited stage disease received RT versus 10% of patients with advanced stage, $p <$ *.00001*). Due to the higher frequency of limited stage disease among the controls, we registered a more common (although not significative) employment of RT in this group (31% *vs* 19%).

All controls obtained a complete response (CR) at the end of first line treatment, while in the R/R group 35% of patients showed stable or progressive disease (PD), 18% reached a partial response (PR) and 47% a CR. As expected, we found a significative difference in terms of chemotherapy response among the R/R subgroups, with a higher rate of CR in early and late relapsed and, on the contrary, a prevalence of SD and PD in the refractory (*p .0001*).
We found that patients treated with R-VEMP had inferior responses, if compared with R-CHOP-like regimens and other therapies (*p .0001*). We have to considered that patients treated with the former regimen are usually more compromised; anyway, the kind of therapy did not impact on PFS and OS in the R/R cohort.
We also looked for possible relationship between pre-CT SUV ≥15 and response to first line therapy, but no association was found.

Collectively, 58 patients (35%) had their treatment complicated by infections, without significative difference between R/R and controls or among the R/R subgroups.
In 26 patients, pre-treatment immunoglobulins G (IgG) were below normal range (6g/L) and 60% of the 25 evaluable cases developed infections during first line therapy *vs* 31% of patients with normal IgG (*p .005*). Post-treatment IgGs were reduced in 60 patients (32%); no significative differences between R/R and controls, and no association with infection during treatment was found.

Lastly, the kind of first line regimen showed no relation with the development of infections and infective complications did not condition PFS and OS.

5.3.5 Data at relapse

Considering data at relapse, SUV max ≥ 15 showed the same distribution in all three subgroups of R/R, while LDH was more often elevated in refractory patients.
We calculated secondary IPI and R-IPI, which kept showing a prevalence of the adverse scores in the refractory patients (*p .022* and *p .007*, respectively), as seen for "primary" IPI and R-IPI. In terms of post-relapse OS, secondary IPI retained its prognostic power, with high and intermediate-high scores associated with poor survival (4 and 6 months, respectively, *vs* 14 and 59 months for intermediate-low and low risk, *p .0000*).
We also looked for validation of the R/R IPI (Figure 16)(145), which resulted evaluable in 87 patients; as reported in Table 8, we calculated the score according to the author's indication and matched the observed 2-year post-relapse OS with the predicted 2-year post-relapse OS, finding just partial concordance; overall, our survival data appeared to be more favorable than the predicted ones.

Ten patients (7%) had a CNS localization of disease at relapse (in two of them CNS was actually involved also at diagnosis). CNS-IPI was high just in 3/10 cases, patients were mostly refractory to first line (6/10) and progressed even after second line therapy (8/10); data are displayed in detail in Table 9.

Second line therapy was highly heterogeneous, as 40 (29%) patients received R-DHAOx/DHAP regimens, 17 (12%) Lenalidomide (+/- Rituximab), 13 (9%) Bendamustine (+ anti-CD20 antibody), 8 (6%) R-CHOP-like regimens, 6 (4%) R-VEMP and 4 (3%) R-IVAC; other kind of treatment was administered in 40 (29%) cases, while 9 (6%) received best supportive care. Infections occurred in 36 patients (28%).
Response to second line therapy was significantly inferior in refractory patients (CR rate of 13.8%), when compared to early and late relapsed (CR rate of 51.7% and 45.4%, respectively, *p .006*).
Considering the time to relapse, the refractory patients showed a post-relapse OS significantly reduced, if compared to early and late relapsed (8 months, *vs* 38 and 30 months, *p .00009*).

Consolidation with ASCT was performed in 32 R/R patients (23%), mainly early relapsed (difference not statistically relevant among the R/R subgroups); 12 patients (38%) received it after the completion of second line therapy and obtained a durable CR (maintained until last follow-up); other 4 patients underwent ASCT after further lines of treatment but still reached stable CR. The remaining 16 patients relapsed after ASCT: 7 received it after the second line of treatment and 9 after further lines. Despite the incidence of relapse post-ASCT, transplanted patients had a median post-relapse OS significantly superior to patients who did not undergo transplant (67 *vs* 8 months, *p* *.0000*).

6. DISCUSSION

6.1 Gene expression profiling and analysis on cell lines

As previously stated, an important limitation to our GEP analysis was RNA degradation, which surely affected our results. It is well known that FFPE tissue in not an optimal source of nucleic acids, particularly of RNA; therefore, it would be desirable, when planning a GEP analysis, send fresh frozen tissue to the lab, or perform freeze-drying, which is less detrimental for RNA. Nowadays, often due to the retrospective design of many studies, we are constrained to use samples from our archives (mostly FFPE), but in a long-range planning, the employment of freeze-drying could be helpful for future studies. Besides, the kind of array used could be more sensitive to degradation in comparison to other technologies.

NDUFB1 gene expression, appeared enhanced in OCI-Ly1, OCI-Ly18, Pfeiffer, OCI-Ly7, OCI-Ly10, U2932 DLBCL cell lines; of those, just Pfeiffer is reported do belong to the OxPhos-DLBCLs (119). This means that the increased gene expression of *NDUFB1* is not exclusive of OxPhos-DLBCLs, which are known to depend upon mitochondrial energy production, and it constitutes also an input to analyze the other OxPhos-DLBCL cell lines - WSUDLCL2, K422 and Toledo - which were not tested (119)(146,147). We have also to consider that we found just partial correspondence between gene expression analysis and Western blot and, unexpectedly, Pfeiffer cell line did not show an enhancement of the protein.
Finally, cell lines characterized by high levels of NDUFB1 protein had also elevated replication rate; this association was also found in the samples from our patients, since in 4/5 NDUFB1 positive cases tested also for Ki67, this proliferation marker resulted positive on average in around 70% of neoplastic cells.

6.2 Impact of clinical variables on outcome

It was not surprising that variables as B-symptoms, elevated LDH, advanced stage, extra-nodal (and bone marrow) involvement, bulky disease, IPI and R-IPI correlated with patients' outcome.
Data also confirmed, as we previously verified in 2020, that **male sex** predominates among the refractory patients and correlates with inferior PFS and OS; this is in accordance with many recent evidences, which demonstrate that women may benefit more from R-CHOP (148–150) but also from CAR-T treatment (151), as if they were more sensitive to immunotherapy.

Besides, it was noteworthy that **positive HBV-serology** was associated with reduced PFS; this result is in line with previous works reported that the positivity for HBsAg is an independent prognosticator for OS (129). Moreover, the correlation between hepatitis B virus (HBV) and B-cell lymphoma, including DLBCL, is well-established. HBV-associated DLBCL present generally advanced clinical stage, poor response to therapy and adverse prognosis (152); these lymphomas are also characterized by peculiar mutation targets and by the involvement of specific tumorigenic pathways (153).

6.3 Karyotype

As reported, karyotype data were available for 35.6% of patients. Among these, we observed that 8/15 (53%) of cases with altered karyotype presented other hematologic or non-hematologic malignancies (*p .024553*); to our knowledge, these data have not been already reported in the literature. Thus, altered karyotype could represent an indication for closer follow-up and monitoring also for the development of solid cancers.
Besides, although the determination of the COO was possible just in few cases, in the 87.5% (7/8) of patients it resulted non-GCB type (*p .015*). This observation could be partially supported by the evidence that ABC-DLBCL are generally affected by dysregulation of both canonical and noncanonical NF-κB pathway and the latter is responsible for genomic instability (154); anyway, other studies did not detect such difference (155). Even if the role of altered (and complex) karyotype in DLBCL is not well established as in other lymphoproliferative disorders, a recent study showed correlation with chromosomal abnormalities and outcome (156), encouraging further analysis in this setting.

6.4 Inflammatory indexes

As previously described, the validation of new prognostic scores is a hot topic in DLBCL and the proposals encompass clinical and biological factors. The inflammatory indexes, which reflect the immune status of the patient and the inflammatory milieu where the neoplasia develops, are lately gaining more interest in DLBCL.
We evaluated some of them, namely ESR, N/L, SII and CAR. As for ESR we did not obtain significant results in terms of distribution between subgroups and impact on PFS and OS, differently from what has been described by others (126).

The **N/L ratio**, despite a similar increase in R/R and controls, appeared less frequently altered in the late relapsed cases, among the R/R subgroups (*p .024*); besides, it was also associated with reduced PFS, in agreement with other reports (127)(128).

The **SII** takes into account not only neutrophils and lymphocytes, but also platelets, which are generally reported to enhance tumor cell adhesion and proliferation; thus, this score should give a more precise estimation of tumor activity and inflammatory status if compared to N/L (129). We set as a cut-off value 1684.09, which was reported by previous studies to be related with advanced stage, abnormal LDH and IPI (127); in our R/R cohort the SII resulted significantly increased in the refractory group (*p .024*) and it associated with inferior PFS, as described in literature (127).

Similarly to the SII, also **CAR** score was significantly increased in the refractory subgroup (*p .002*) and impacted both on PFS and OS, in line with data from the literature (127); in comparison to the aforementioned indexes, the CAR score adds other pieces of information, as an indicator of both underlying inflammation and nutritional status of the patients. Thus, it constitutes a *trait d'union* between the inflammatory indexes and the nutritional scores.

6.5 Data from Immunohistochemistry

Our data showed that in the R/R cohort, Ki67 correlated with reduced OS, considering both the proposed thresholds (≥ 70%, ≥ 80%), with slightly increased significance for the 70% limit (*p .0142 vs p .0328*). In literature, supporting data are reported for both cut-offs (157,158), and even an intermediate value (75%) was proposed with encouraging results (159). Nevertheless, other studies questioned the impact of Ki67 on outcome, but lower thresholds were considered (160,161).

Collectively, we detected 6 **CD5+** cases (1 control and 5 R/R – all refractory, data available for 69/185 patients). With the exception of the not relapsed patient, all others were characterized by extra-nodal involvement, as reported by the literature (133) and 4/5 of the refractory had an OS < 12 months. All patients were treated with CHOP-like regimen, instead of DA-EPOCH + HD-MTX (140), which nowadays would be our first choice.

As for **NDUFB1**, differently from what suggested by GEP analysis, we did not find an enhanced protein expression in the R/R group; on the contrary, its expression was increased in 5/20 controls and 4/47 R/R – all refractory (25% and 8.5%, respectively, considering the available cases).

Nevertheless, increased NDUFB1 expression in the R/R cohort had a negative impact on mPFS (*p .0005*). Of note, all the patients with expression of NDUFB1 and evaluable for the expression of members of the BCL-2 family (4 refractory and 1 control) showed concomitant expression of MCL1; 3/4 refractory revealed in addition increased expression of BCL-XL and the case lacking BCL-XL showed increased expression of P53. Three out of four of the refractory patients had an OS < 12 months. Thus, our data suggest that the combined overexpression of these proteins – involving a dysregulation of the apoptotic pathway and presumably also of the oxidative phosphorylation – may contribute to an aggressive phenotype. Anyway, these considerations derived from the analysis of few cases and should be confirmed in larger cohorts.
Besides, as previously reported, the role of NDUFB1 in tumorigenesis is still unclear: in fact, although it is overexpressed in various cancers, in a study on lung squamous cell carcinoma it was reported to be a positive prognostic factor (121) and analogously in Clear-Cell Renal-Cell Carcinoma, its down-regulation was associated with inferior outcomes (122). These apparently contrasting results probably reflect the complexity of the interaction between metabolic pathways and cancer development and need further analysis to be fully clarified.

P53 inactivation is a hallmark of cancer; we detected its altered expression in 11/67 of the available samples (16.4%), which, considering the cut-off we set for over-expression (70%), could be compared with other published data (162); anyway, matches with other studies is not easy, since many used lower thresholds (10-30-50%) (162–164). In detail, P53 showed null- or over-expression 1 control and 10 R/R and, although the difference does not reach the significance level (*p 0.1*), we can at least recognize a trend. Interestingly, the patterns of altered expression seemed to "compartmentalize" in R/R subgroups: all patient with enhanced expression resulted refractory (7/10, *i.e.* 25% of the tested refractory patients show P53 overexpression), while all the cases with "null" expression were part of the late relapsed (3/10, *i.e.* 37.5% of the tested late relapsed patients show P53 "null" expression); as far as we know, a similar clustering of abnormalities of P53 expression has not been previously described in DLBCL. Roughly translating genetics into immunohistochemical data, we could assume that P53 overexpression corresponds to mutations, which stabilize an altered protein, able to acquire proto-oncogenic activity (80), that contribute to the refractory phenotype. On the other hand, we argue that P53 "null" expression would be associated with mutations, which compromise P53 synthesis with loss of its gate-keeper functions and subsequent accumulation of mutations that lead to late relapse. In order to verify these speculations, it would be worthy enlarging the sample size – as the percentages of mutated cases are not negligible – and associating genetic analysis to immunohistochemistry.

Aware of the correlation between P53 dysregulation and chromosomal instability, we evaluated the karyotype of the patients with P53 null- or over-expression: unfortunately, it was available just for 3/11 patients, and in 2/3 it was altered (one hyperdiploid clone and one complex karyotype).

The **BCL-2 family members** regulate the intrinsic apoptotic pathway and their involvement in lymphomagenesis is common, as described in the dedicated paragraph. Here we did not detect association of the expression of BCL2 family members with PFS and OS, but the comparison between samples at diagnosis and relapse (unfortunately possible just for 15 patients) led to intriguing results: in fact, in 5 cases, samples at relapse showed an increase expression of BCL-XL, a finding that is in line with what demonstrated by *De Jong et al*, namely that CHOP therapy can change the dependency of DLBCL cell lines from BCL-2 or MCL-1 towards BCL-XL (103). In accordance with what reported by the same author, we also confirmed that one third of patients presenting overexpression of a BCL-2 family member expressed more anti-apoptotic proteins at the same time. BCL-XL was not the only protein with acquired overexpression at relapse; in fact, also MCL1 and BCL-W were found increased (in 2 cases MCL and in 1 case BCL-W), highlighting the important pro-survival stimulus which is mediated by these proteins and its role in supporting relapse since a BCL-2 family member is involved in more than 50% of relapsed samples.

6.6 Data from the first line setting

As reported, the **R-VEMP regimen** was employed in 25 (13.5%) of our patient, principally R/R. This treatment was reported to be safe and significantly active in DLBCL, also relapsed after a first line anthracycline-containing regimen (165). We used it especially in elderly (median age 77.4 years) or younger patients with comorbidities, which discouraged the administration of anthracyclines. This is probably the reason why it found more frequent application among the R/R (68 *vs* 55 years) and it resulted to be less effective than R-CHOP-like regimens; it is necessary to collect a larger cohort of patients treated with R-VEMP and to compare it with an age-adjusted group treated with reduced-dose R-CHOP to have a clearer overview of the efficacy of this scheme of therapy.

Infectious complications as chemo-immunotherapy related toxicities and corresponding prophylaxis have not been widely explored in the setting of DLBCL. The American Society of Clinical Oncology end the Infectious Disease Society of America (ASCO/IDSA) Guidelines do not give definite indications for prophylaxis in lymphoma, with exception of patient who undergo

ASCT (166). On the other hand, according to NCCN Guideline, lymphoma patients are classified at intermediate risk of developing infections overall and thus should receive prophylaxis with antibiotics such as fluoroquinolones, antifungal and antiviral agents when neutropenic, and continuous anti Pneumocystis jiroveci pneumonia prophylaxis; anyway, clear recommendations still lack (167). As for factors associated with increased risk of infections, three categories have been identified (168):

- patient-related: elderly (> 65 years), comorbidities, compromised performance status;
- disease-related: bone marrow involvement, advanced stage disease;
- treatment-related: dose-dense or high-dose treatments (for example grade 3-5 infective events were reported to be significantly higher with DA-EPOCH than with CHOP (169)).

In our study, we found a significant correlation between reduced pre-treatment IgG levels and infections, identifying a population at increased risk of infective complications, which possibly could benefit from intensified use of granulocyte colony stimulating factor (G-CSF) and antibiotic prophylaxis with fluroquinolones; as far as we know, this association was not previously described. On the contrary, we detected no impact of treatment (but none of our patients received DAEPOCH in first line) and of bone marrow involvement (*p 0.07*).
Finally, we did not observe an impairment of survival due to infections, differently from what reported by others (170).

6.7 Prognostic scores at relapse

The role of **secondary IPI** and **R-IPI** is not widely recognized and also their definition is still ambiguous. *Lee et al* considered as secondary IPI, the IPI score calculated at the end of the first line therapy for patients obtaining a PR, and used it together with the Deauville score of the end-of-treatment PET to predict post-PR OS and PFS (171). On the other hand, *Gisselbrecht et al* reported that the secondary IPI calculated at relapse was the main prognostic factor for the response to the second line treatment (172). In our R/R cohort we confirmed the value of the secondary IPI was a reliable predictor for post-relapse OS.

The **R/R IPI** was the first score conceived for the relapse setting; it combines age and time to relapse, two easily accessible variables, to predict the 2-year OS. A reliable life expectancy prediction for R/R patients could be helpful in second line therapy selection and in the evaluation of the efficacy of new agents (145). We obviously calculated the score retrospectively, to verify if the predicted 2-year OS corresponded to our real-life experience: defining three risk categories, we

compared the 2-year OS recorded *vs* expected and realized that R/R IPI had the tendency to underestimate survival, which was actually reported also in one of the validation cohorts. The evaluable patients in our cohort were just 87 (62%), because of age cut-offs (40-80 years) to the application of the score, which represents a limit for a universal use of the R/R IPI. Another limiting factor was the anthracycline-based first line therapy, which further reduced our assessable patients; anyway, we included 12 patients treated with VEMP regimen (thus, with the anthracycline derivative Mitoxantrone), considering the treatment comparable to a CHOP-like regimen. Lastly, we have to remind that this model was based on historical data and it will certainly underestimate survival of patients treated after the approval of new effective therapy, both in the first and in the second line setting.

6.8 Time to relapse

Determining the impact of the time to relapse on prognosis was one of the main objectives of our study. At variance with what we evidenced in our previous cohort in 2020, with the increase of the sample size and the redefinition of the subgroups *refractory*, *early relapsed* and *late relapsed*, we demonstrated that patients belonging to the first group were characterized by a significantly reduced post-relapse OS. This could reflect a different chemosensitivity of the redefined subgroups, as proposed by *Hilton et al* (41); anyway, it is remarkable that early and late relapsed showed a similar survival, in contrast with the studies which set PFS24 as unique threshold to distinguish between early and late relapses (36,40). This could depend by the fact that few late relapsed patients underwent transplant in comparison with early relapsed (12.5% *vs* 34.3%), but the absence of difference according to the time to relapse was also demonstrated by other authors (32). Surely, the three identified subgroups show clear distinctions, with a predominance of adverse factors in the refractory, foreshadowing impaired outcome.

6.9 The role of ASCT

Our data showed that 23% of the R/R patients underwent transplant, and of these just half obtained a durable response, in line with reports from the literature (29). Besides, 8/16 patients in stable remission after ASCT were refractory to first line therapy, assessing a certain degree of chemosensitivity in a proportion of these patients; anyway, it corresponds to just 11% of the entire refractory population. This, together with the disappointing data of response to second line therapy reported in this subgroup (13.8% of CR), confirms the need of different therapeutical approaches

for these patients. As previously described, to address this issue, in the US and in many European countries, two CAR-T products (Axi-Cel and Liso-Cel) have been approved as second line treatment in primary refractory or in patients relapsing within 12 months from the completion of first line therapy, thus comprising a population comparable to our refractory and partially to our early relapsed subgroups. The pivotal trial ZUMA-7 (14) and TRANSFORM (15,17) showed a relevant improvement in PFS for patients treated with CAR-T in comparison to SOC; on the contrary, the BELINDA trial (17), probably enrolling more compromised patients, failed to prove the superiority of the CAR-T approach. The lastly published update of ZUMA-7 demonstrated a significant advantage in terms of OS for patients receiving Axi-cel in second line *versus* salvage therapy and ASCT (16); anyway, real world data have still to come.

Simulating the application of the algorithm proposed by *Westin and Sehn* (reported in Figure 1) to our cohort, it would result that 41 patients relapsed after 12 months from the conclusion of first line therapy (29% comparable to the estimated 25% of the R/R), 8/41 patients were transplanted (just 20% rather than the 50% hypothesized) and 5/41 were supposed to be cured (12%, namely the 3.5% of the whole cohort of R/R *versus* the expected 5%). Thus, data highlight that also in this subset of patients, although theoretically more chemo-sensitive, transplantation is determinant just in a small percentage; remembering that collectively, patients not subjected to transplant had a significantly reduced OS compared to the transplanted, the inevitable conclusion is that new treatments are urgently required.

7. CONCLUSIONS

In this study we evaluated 140 R/R patients, divided according to the time of relapse in three subgroups – refractory, early- and late relapsed; we compared them to 45 patients, not-relapsed after 5-year follow-up, with the aim of obtaining a detailed clinical and immunohistochemical characterization of the cohorts, and of identifying potentially prognostic factors.
Pursuing this objective, we also employed GEP analysis in a subgroup of patients; although limited by RNA degradation and by the small number of tested patients, this analysis brought us to the identification of a differentially expressed gene, namely a subunit of NADH:Ubiquinone Oxidoreductase (NDUFB1).
Our results show that the aforementioned groups differentiate in terms of clinical and biological features, but also in terms of outcomes, with an inferior post-relapse OS for refractory patients. In the whole R/R cohort, we confirmed the prognostic value of IPI and R-IPI (even when calculated at relapse) and, in addition to the impact on prognosis of well-known adverse factors (*i.e.* advanced stage and LDH increase), we found that males were characterized by inferior PFS and OS, and that inflammatory indexes as N/L ratio, SII and CAR resulted associated with outcome. As for immunohistochemistry data, high Ki67 values correlated with reduced OS, while NDUFB1 overexpression caused a PFS disadvantage. We detected a trend of more frequent alteration in the expression of P53 in the R/R cohort, where all patients with enhanced expression were refractory, while all the cases with a "null" phenotype belonged to the late relapsed group.
Considering first line therapy regimens, patients treated with R-VEMP had inferior responses, if compared with R-CHOP-like approaches, but this did not translate into an impact on OS or PFS.
In the second line setting, just 23% of patients underwent ASCT; transplanted patients had a median post-relapse OS significantly superior to patients who did not receive transplant.
Finally, we observed a correlation between altered karyotype and non-GCB type COO and with the occurrence of other hematological and non-hematological malignancies and a higher rate of infections during first line treatment among patients with pre-treatment IgG below normal range.
Thus, collectively, we recognized some subgroup-specific features and variables impacting on outcome. Our study, especially with regards to immunohistochemistry analysis, was limited by the small sample size and by the lack of confirmatory molecular analysis; our aim is to overcome these limitations by expanding our cohort and performing sequencing or expression studies, contributing to the global effort at reaching a better risk stratification in DLBCL.

8. TABLES AND FIGURES

Table 1. Comparison between 4th WHO classification, 5th WHO classification and ICC 2022 (3).

4th WHO classification	5th WHO classification	ICC 2022
Transformations of indolent B-cell lymphomas		
Not included as entity	Transformations of indolent B-cell lymphomas	Not included as entity
Large B-cell lymphomas		
Diffuse large B-cell lymphoma, NOS	Diffuse large B-cell lymphoma, NOS	Diffuse large B-cell lymphoma, NOS
Germinal Center B cell subtype	Recommended	Germinal Center B cell subtype
Activated B cell subtype	Recommended	Activated B cell subtype
Burkitt-like lymphoma with 11q aberration (provisional)	High grade B-cell lymphoma with 11q aberration	Large B-cell lymphoma with 11q aberration (provisional)
Large B-cell lymphoma with IRF4 rearrangement (provisional entity)	Large B-cell lymphoma with IRF4 rearrangement (upgraded to distinct entity)	Large B-cell lymphoma with IRF4 rearrangement (upgraded to distinct entity)
Nodular lymphocyte predominant B-cell lymphoma (not included in this category)	Nodular lymphocyte predominant B-cell lymphoma (not included in this category)	Nodular lymphocyte predominant B-cell lymphoma
T-cell/histocyte-rich large B-cell lymphoma	T-cell/histocyte-rich large B-cell lymphoma	T-cell/histocyte-rich large B-cell lymphoma
Primary large B-cell lymphoma of immune-privileged sites		
Primary large B-cell lymphoma of CNS	Primary large B-cell lymphoma of CNS	Primary large B-cell lymphoma of CNS
Not included as entity	Primary large B-cell lymphoma of testis (new entity)	Primary large B-cell lymphoma of testis (new entity)
Included in primary large B-cell lymphoma of CNS	Primary large B-cell lymphoma of vitreoretina	Included in primary large B-cell lymphoma of CNS
Primary cutaneous diffuse large B-cell lymphoma, leg-type	Primary cutaneous diffuse large B-cell lymphoma, leg-type	Primary cutaneous diffuse large B-cell lymphoma, leg-type
Intravascular large B-cell lymphoma	Intravascular large B-cell lymphoma	Intravascular large B-cell lymphoma
Not included as entity	Fluid overload-associated large B-cell lymphoma (new entity)	HHV8 and EBV-negative primary effusion-based lymphoma (provisional)
EBV-positive mucocutaneous ulcer (provisional)	EBV-positive mucocutaneous ulcer (not included in this category, but in lymphoid proliferations and lymphomas associated with immune deficiency and dysregulation)	EBV-positive mucocutaneous ulcer (upgraded to distinct entity)
EBV-positive DLBCL, NOS	EBV-positive DLBCL, NOS	EBV-positive DLBCL, NOS
DLBCL associated with chronic inflammation	DLBCL associated with chronic inflammation	DLBCL associated with chronic inflammation
Fibrin-associated large B-cell lymphoma (subtype of DLBCL associated with chronic inflammation)	Fibrin-associated large B-cell lymphoma (new entity)	Fibrin-associated large B-cell lymphoma (subtype of DLBCL associated with chronic inflammation)
Lymphomatoid granulomatosis	Lymphomatoid granulomatosis	Lymphomatoid granulomatosis
Not included as entity	Described in lymphoid proliferations and lymphomas associated with immune deficiency and dysregulation (not considered as entity)	EBV-positive polymorphic B-cell lymphoproliferative disorder, NOS (provisional)
ALK-positive large B-cell lymphoma	ALK-positive large B-cell lymphoma	ALK-positive large B-cell lymphoma
Plasmablastic lymphoma	Plasmablastic lymphoma	Plasmablastic lymphoma
High grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements	DLBCL/High grade B-cell lymphoma with MYC and BCL2 rearrangements	High grade B-cell lymphoma with MYC and BCL2 rearrangements
Not included as entity	Not included as entity	High grade B-cell lymphoma with MYC and BCL6 rearrangements (provisional)
High grade B-cell lymphoma, NOS	High grade B-cell lymphoma, NOS	High grade B-cell lymphoma, NOS

Table 2. Comparison between genomic classifications proposed by *Chapuy et al* (4) and *Schmitz et al* (5)(173).

Chapuy et al	*Schmitz et al*	Predominant COO subtype
C0 (4%) No defining genetic driver		
C1 (19%) BCL-6 rearrangements, MYD88 mut (not L265P), FAS, NOTCH2, NF-kB pathway mutations	**BN2 (15%)** BCL6 fusions and NOTCH2 mutations	GCB- and ABC-DLBCL, DLBCL NOS
C2 (21%) TP53 mutations, 17p/TP53, 9p21.3/CDKN2A/13q14.2/RB1 deletions		GCB- and ABC-DLBCL,
C3 (18%) BCL-2 mutations and translocation, PTEN inactivation, mutations in chromatin modifiers, alteration in BCR and PI3K signaling	**EZB (22%)** EZH2 mutations and BCL2 translocations	GCB-DLBCL
C4 (17%) Mutations in NF-kB modifiers, immune evasion molecules, core histone genes and RAS/JAK/STAT pathway		DLBCL NOS
C5 (21%) 18q gains, CD79B and MYD88(L265P) mutations	**MCD (8%)** MYD88(L265P) and CD79B mutations	ABC-DLBCL
	N1 (2%) NOTCH1 mutations	ABC-DLBCL
	Other (54%)	

Figure 1. Therapeutical algorithm for R/R DLBCL proposed by Westin and Sehn (29).

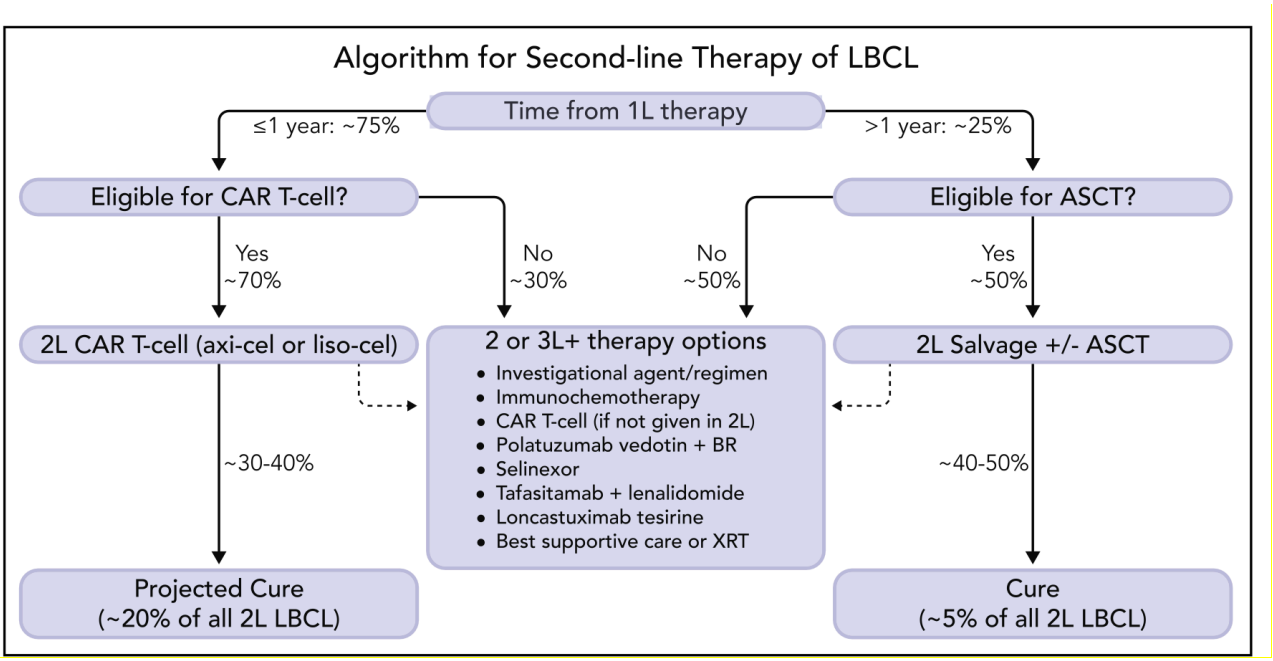

Figure 2. Overview of the apoptotic pathways in DLBCL (173).

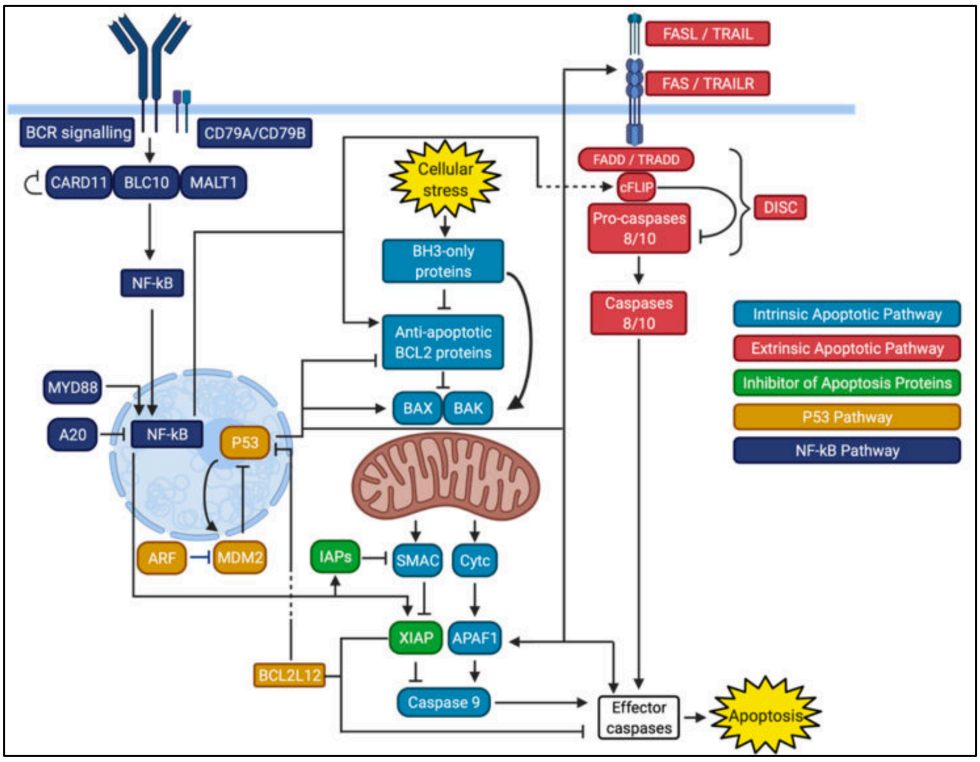

Figure 3. Interplay between pro- and anti-apoptotic proteins belonging to the BCL2-family. BH3 domain-only proteins which act as direct activator are marked in orange, while the sensitizers are marked in pink (95).

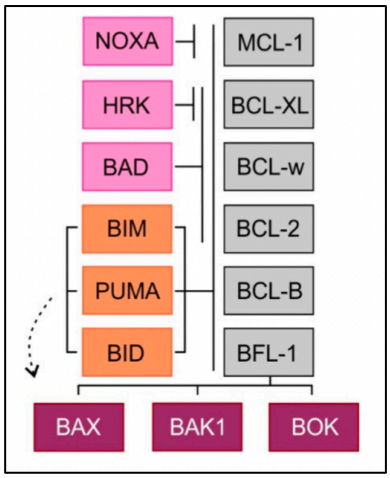

Figure 4. Metabolic pathway promoting malignant cells survival (111).

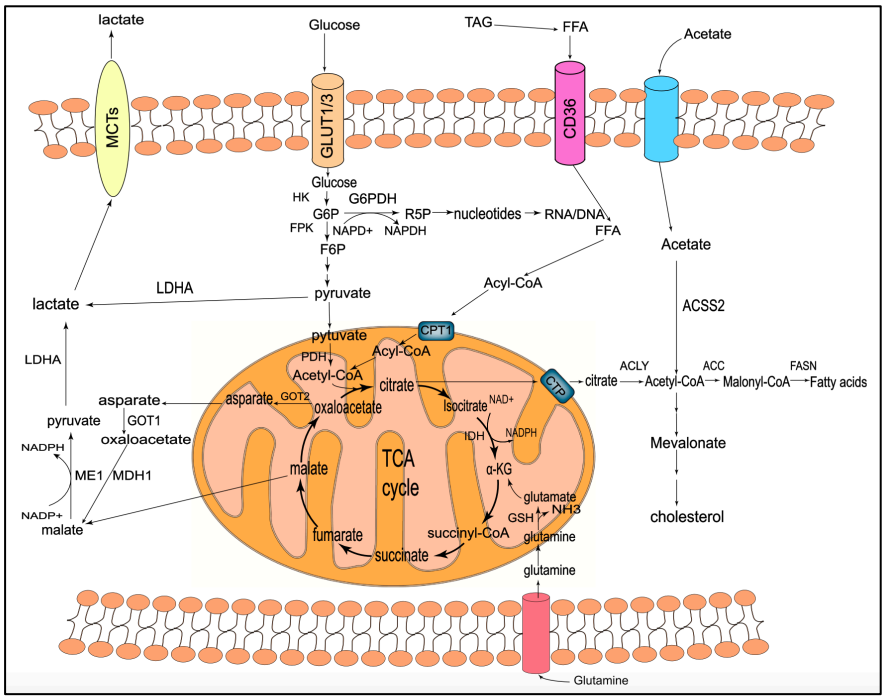

Figure 5. The electron respiratory chain (Reactome).

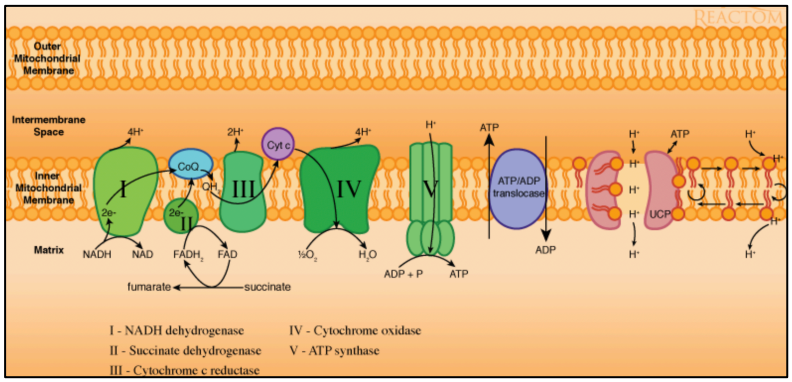

Figure 6. Expression of NDUFB1 in cancer cell lines (Genecards).

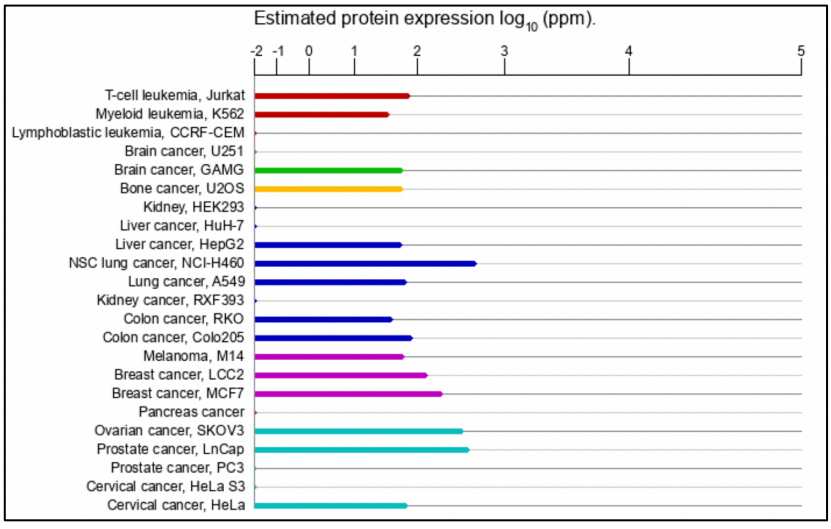

Figure 7. Expression of NDUFB1 in cancers (Genevestigator).

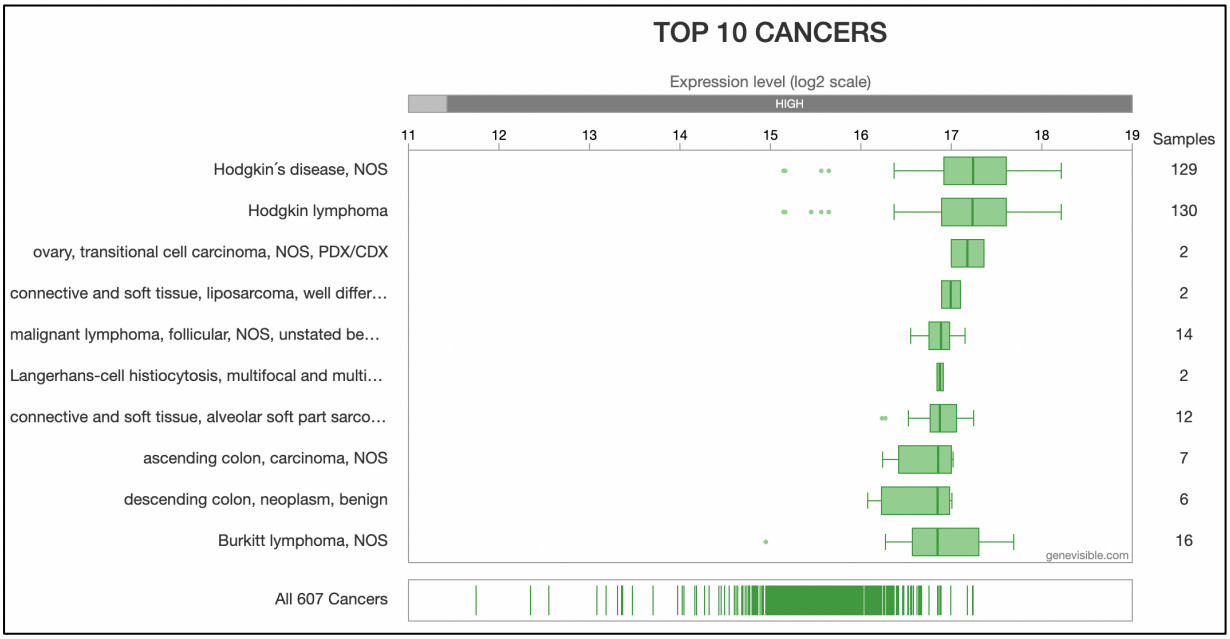

Figure 8. Representation of the interplay between lymphoma cells and the immune system (124).

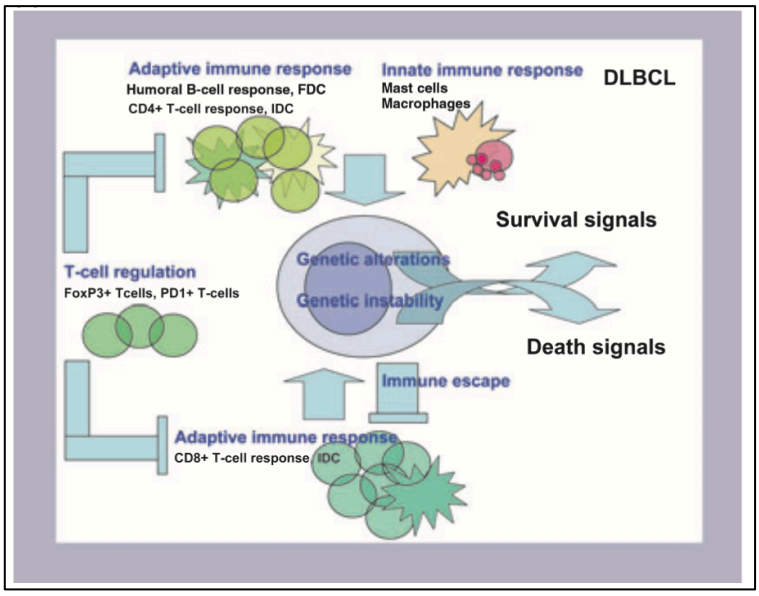

Figure 9. Heat-map showing the comparison between gene expression profile of controls and R/R patients.

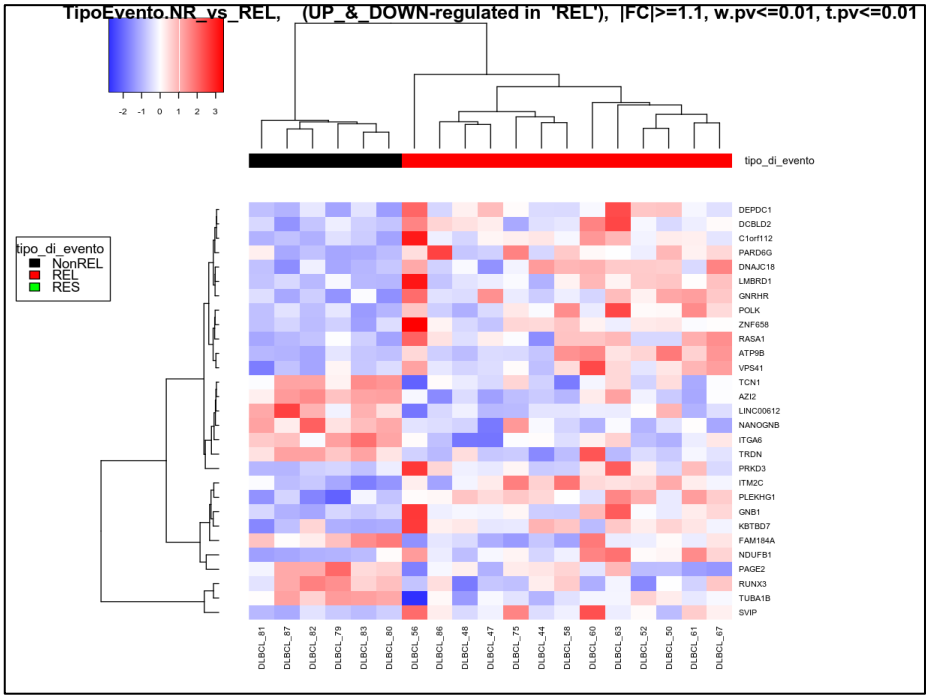

Figure 10. NDUFB1-gene expression in cell lines.

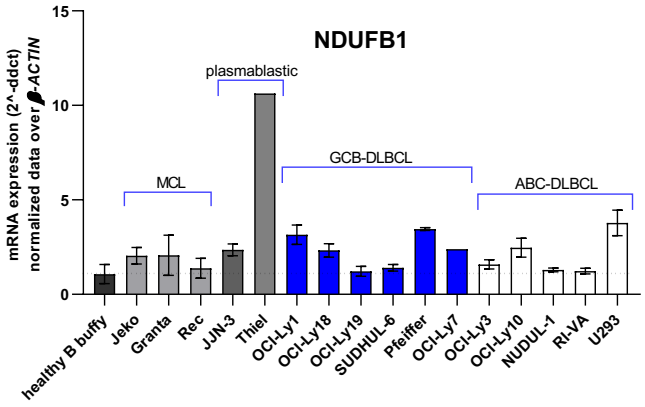

Figure 11. Western blotting analysis for NDUFB1 in cell lines.

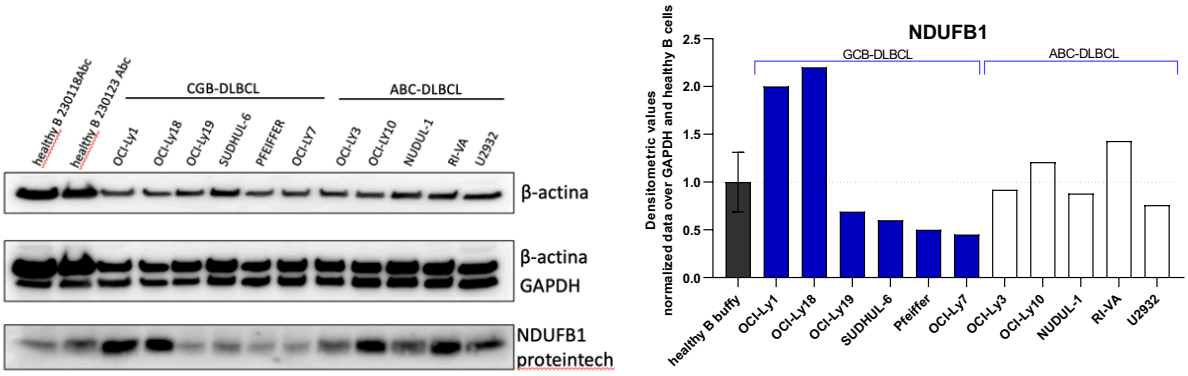

Table 3. Patients' characteristics - comparison between controls and R/R.

Variables		Controls (N=45)	R/R (N=140)	Total (N=185)	*p value*
Age	Median (Q1, Q3)	55.0 (45.0, 64.0)	68.0 (53.8, 76.2)	65.0 (50.0, 75.0)	
Sex	F	21 (46.7%)	57 (40.7%)	78 (42.2%)	*0.4820*
	M	24 (53.3%)	83 (59.3%)	107 (57.8%)	
Outcome	Missing	0	4	4	***0.0001***
	Alive	42 (93.3%)	39 (28.7%)	81 (44.8%)	
	Dead	3 (6.7%)	97 (71.3%)	100 (55.2%)	
Other hemopathies	No	39 (86.7%)	114 (81.4%)	153 (82.7%)	*0.4190*
	Yes	6 (13.3%)	26 (18.6%)	32 (17.3%)	
Other malignancies	No	36 (80.0%)	114 (81.4%)	150 (81.1%)	*0.8310*
	Yes	9 (20.0%)	26 (18.6%)	35 (18.9%)	
B-Symptoms	Missing	0	7	7	***0.0210***
	No	35 (77.8%)	78 (58.6%)	113 (63.5%)	
	Yes	10 (22.2%)	55 (41.4%)	65 (36.5%)	
LDH increase	Missing	0	11	11	*0.3630*
	No	22 (48.9%)	53 (41.1%)	75 (43.1%)	
	Yes	23 (51.1%)	76 (58.9%)	99 (56.9%)	
Extra-nodal involvement	Missing	0	3	3	***0.0090***
	No	20 (44.4%)	33 (24.1%)	53 (29.1%)	
	Yes	25 (55.6%)	104 (75.9%)	129 (70.9%)	
BM involvement	Missing	6	32	38	***0.0160***
	No	33 (84.6%)	69 (63.9%)	102 (69.4%)	
	Yes	6 (15.4%)	39 (36.1%)	45 (30.6%)	
CNS involvement (diagnosis)	Missing	0	1	1	*0.1000*
	No	45 (100.0%)	131 (94.2%)	176 (95.7%)	
	Yes	0 (0.0%)	8 (5.8%)	8 (4.3%)	
Stage	Missing	0	2	2	*0.1330*
	I-II	17 (37.8%)	36 (26.1%)	53 (29.0%)	
	III-IV	28 (62.2%)	102 (73.9%)	130 (71.0%)	
Bulky disease	Missing	1	6	7	*0.3290*
	No	26 (59.1%)	90 (67.2%)	116 (65.2%)	
	Yes	18 (40.9%)	44 (32.8%)	62 (34.8%)	
HBV serology	Missing	3	9	12	*0.5740*
	Negative	33 (78.6%)	108 (82.4%)	141 (81.5%)	
	Positive	9 (21.4%)	23 (17.6%)	32 (18.5%)	
HCV serology	Missing	3	9	12	*0.6260*
	Negative	40 (95.2%)	122 (93.1%)	162 (93.6%)	
	Positive	2 (4.8%)	9 (6.9%)	11 (6.4%)	
HIV serology	Missing	9	40	49	*0.2930*
	Negative	36 (100.0%)	97 (97.0%)	133 (97.8%)	
	Positive	0 (0.0%)	3 (3.0%)	3 (2.2%)	
VES > 37.5mm/h	Missing	10	59	69	*0.4920*
	No	21 (60.0%)	43 (53.1%)	64 (55.2%)	
	Yes	14 (40.0%)	38 (46.9%)	52 (44.8%)	
N/L > 3.5	Missing	0	18	18	*0.2230*
	No	28 (62.2%)	63 (51.6%)	91 (54.5%)	
	Yes	17 (37.8%)	59 (48.4%)	76 (45.5%)	
COO	Missing	13	77	90	*0.1700*
	Non-GCB	20 (62.5%)	30 (47.6%)	50 (52.6%)	
	GCB	12 (37.5%)	33 (52.4%)	45 (47.4%)	
Ki67 ≥ 70%	Missing	17	64	81	*0.7050*
	No	11 (39.3%)	33 (43.4%)	44 (42.3%)	
	Yes	17 (60.7%)	43 (56.6%)	60 (57.7%)	
Ki67 ≥ 80%	Missing	17	64	81	*0.8670*
	No	20 (71.4%)	53 (69.7%)	73 (70.2%)	
	Yes	8 (28.6%)	23 (30.3%)	31 (29.8%)	
Infective toxicity	Missing	0	22	22	*0.4670*
	No	27 (60.0%)	78 (66.1%)	105 (64.4%)	
	Yes	18 (40.0%)	40 (33.9%)	58 (35.6%)	
SUV ≥ 15	Missing	13	56	69	*0.8960*
	No	8 (25.0%)	22 (26.2%)	30 (25.9%)	
	Yes	24 (75.0%)	62 (73.8%)	86 (74.1%)	
IPI	Missing	0	12	12	***0.0001***
	Low	17 (37.8%)	24 (18.8%)	41 (23.7%)	
	Low-int	21 (46.7%)	38 (29.7%)	59 (34.1%)	
	High-int	4 (8.9%)	44 (34.4%)	48 (27.7%)	

	High	3 (6.7%)	22 (17.2%)	25 (14.5%)	
R-IPI	Missing	0	12	12	***0.0001***
	Very good	7 (15.6%)	9 (7.0%)	16 (9.2%)	
	Good	31 (68.9%)	53 (41.4%)	84 (48.6%)	
	Poor	7 (15.6%)	66 (51.6%)	73 (42.2%)	
Kind of CT	Missing	0	2	2	***0.0490***
	R-VEMP	3 (6.7%)	22 (15.9%)	25 (13.7%)	
	R-CHOP/COMP	41 (91.1%)	102 (73.9%)	143 (78.1%)	
	Other regimens	1 (2.2%)	14 (10.1%)	15 (8.2%)	
Reduced IgG	Missing	4	24	28	*0.6990*
	No	35 (85.4%)	96 (82.8%)	131 (83.4%)	
	Yes	6 (14.6%)	20 (17.2%)	26 (16.6%)	
Reduced post-CT IgG	Missing	2	36	38	*0.0930*
	No	30 (69.8%)	57 (54.8%)	87 (59.2%)	
	Yes	13 (30.2%)	47 (45.2%)	60 (40.8%)	
CD5	Missing	23	93	116	*0.4030*
	Negative	21 (95.5%)	42 (89.4%)	63 (91.3%)	
	Positive	1 (4.5%)	5 (10.6%)	6 (8.7%)	
P53	Missing	25	93	118	*0.1000*
	Negative	1 (5.0%)	10 (21.3%)	11 (16.4%)	
	Positive	19 (95.0%)	37 (78.7%)	56 (83.6%)	
NDUFB1	Missing	25	93	118	*0.0700*
	Negative	15 (75.0%)	43 (91.5%)	58 (86.6%)	
	Positive	5 (25.0%)	4 (8.5%)	9 (13.4%)	
MCL1	Missing	30	96	126	*0.4150*
	Negative	10 (66.7%)	34 (77.3%)	44 (74.6%)	
	Positive	5 (33.3%)	10 (22.7%)	15 (25.4%)	
BCL-XL	Missing	32	96	128	*0.5510*
	Negative	10 (76.9%)	37 (84.1%)	47 (82.5%)	
	Positive	3 (23.1%)	7 (15.9%)	10 (17.5%)	
BCL-W	Missing	31	96	127	*0.9670*
	Negative	13 (92.9%)	41 (93.2%)	54 (93.1%)	
	Positive	1 (7.1%)	3 (6.8%)	4 (6.9%)	
SII > 1684.09	Missing	0	19	19	*0.9810*
	No	36 (80.0%)	97 (80.2%)	133 (80.1%)	
	Yes	9 (20.0%)	24 (19.8%)	33 (19.9%)	
CAR > 0.21	Missing	4	39	43	*0.2360*
	No	19 (46.3%)	36 (35.6%)	55 (38.7%)	
	Yes	22 (53.7%)	65 (64.4%)	87 (61.3%)	

Table 4. Patients' characteristics - focus on R/R: comparison between R/R subgroups.

Variables		**0 (N=72)**	**1 (N=35)**	**2 (N=33)**	**Total (N=140)**	***p value***
Age	Median (Q1, Q3)	67.0 (52.8, 77.0)	68.0 (56.5, 70.0)	71.0 (50.0, 77.0)	68.0 (53.8, 76.2)	*0.8670*
Sex	F	20 (27.8%)	18 (51.4%)	19 (57.6%)	57 (40.7%)	***0.0050***
	M	52 (72.2%)	17 (48.6%)	14 (42.4%)	83 (59.3%)	
Outcome	Missing	3	0	1	4	***0.0001***
	Alive	10 (14.5%)	14 (40.0%)	15 (46.9%)	39 (28.7%)	
	Dead	59 (85.5%)	21 (60.0%)	17 (53.1%)	97 (71.3%)	
CT response	CR	6 (8.3%)	28 (80.0%)	32 (97.0%)	66 (47.1%)	***0.0001***
	PR	20 (27.8%)	4 (11.4%)	1 (3.0%)	25 (17.9%)	
	SD	8 (11.1%)	3 (8.6%)	0 (0.0%)	11 (7.9%)	
	PD	38 (52.8%)	0 (0.0%)	0 (0.0%)	38 (27.1%)	
B-Symptoms	Missing	0	0	7	7	***0.0120***
	No	34 (47.2%)	24 (68.6%)	20 (76.9%)	78 (58.6%)	
	Yes	38 (52.8%)	11 (31.4%)	6 (23.1%)	55 (41.4%)	
LDH increase	Missing	3	2	6	11	***0.0020***
	No	22 (31.9%)	12 (36.4%)	19 (70.4%)	53 (41.1%)	
	Yes	47 (68.1%)	21 (63.6%)	8 (29.6%)	76 (58.9%)	
Extra-nodal involvement	Missing	0	0	3	3	*0.1710*
	No	13 (18.1%)	12 (34.3%)	8 (26.7%)	33 (24.1%)	
	Yes	59 (81.9%)	23 (65.7%)	22 (73.3%)	104 (75.9%)	
BM involvement	Missing	11	7	14	32	*0.1250*
	No	36 (59.0%)	17 (60.7%)	16 (84.2%)	69 (63.9%)	
	Yes	25 (41.0%)	11 (39.3%)	3 (15.8%)	39 (36.1%)	
CNS involvement (diagnosis)	Missing	0	0	1	1	*0.2420*

	No	66 (91.7%)	33 (94.3%)	32 (100.0%)	131 (94.2%)	
	Yes	6 (8.3%)	2 (5.7%)	0 (0.0%)	8 (5.8%)	
Stage	Missing	0	0	2	2	***0.0001***
	I-II	10 (13.9%)	11 (31.4%)	15 (48.4%)	36 (26.1%)	
	III-IV	62 (86.1%)	24 (68.6%)	16 (51.6%)	102 (73.9%)	
Bulky disease	Missing	1	1	4	6	***0.0030***
	No	41 (57.7%)	22 (64.7%)	27 (93.1%)	90 (67.2%)	
	Yes	30 (42.3%)	12 (35.3%)	2 (6.9%)	44 (32.8%)	
HBV serology	Missing	3	3	3	9	*0.0560*
	Negative	55 (79.7%)	24 (75.0%)	29 (96.7%)	108 (82.4%)	
	Positive	14 (20.3%)	8 (25.0%)	1 (3.3%)	23 (17.6%)	
HCV serology	Missing	4	2	3	9	*0.7420*
	Negative	64 (94.1%)	31 (93.9%)	27 (90.0%)	122 (93.1%)	
	Positive	4 (5.9%)	2 (6.1%)	3 (10.0%)	9 (6.9%)	
HIV serology	Missing	15	13	12	40	*0.6200*
	Negative	55 (96.5%)	22 (100.0%)	20 (95.2%)	97 (97.0%)	
	Positive	2 (3.5%)	0 (0.0%)	1 (4.8%)	3 (3.0%)	
VES > 37.5mm/h	Missing	25	19	15	59	*0.3460*
	No	26 (55.3%)	6 (37.5%)	11 (61.1%)	43 (53.1%)	
	Yes	21 (44.7%)	10 (62.5%)	7 (38.9%)	38 (46.9%)	
N/L > 3.5	Missing	5	3	10	18	***0.0240***
	No	28 (41.8%)	18 (56.2%)	17 (73.9%)	63 (51.6%)	
	Yes	39 (58.2%)	14 (43.8%)	6 (26.1%)	59 (48.4%)	
COO	Missing	35	19	23	77	*0.6760*
	GCB	16 (43.2%)	9 (56.2%)	5 (50.0%)	30 (47.6%)	
	Non-GCB	21 (56.8%)	7 (43.8%)	5 (50.0%)	33 (52.4%)	
Ki67 ≥ 70%	Missing	24	14	26	64	*0.3850*
	No	18 (37.5%)	11 (52.4%)	4 (57.1%)	33 (43.4%)	
	Yes	30 (62.5%)	10 (47.6%)	3 (42.9%)	43 (56.6%)	
Ki67 ≥ 80%	Missing	24	14	26	64	*0.3940*
	No	31 (64.6%)	17 (81.0%)	5 (71.4%)	53 (69.7%)	
	Yes	17 (35.4%)	4 (19.0%)	2 (28.6%)	23 (30.3%)	
Infective toxicities	Missing	5	6	11	22	*0.7920*
	No	46 (68.7%)	18 (62.1%)	14 (63.6%)	78 (66.1%)	
	Yes	21 (31.3%)	11 (37.9%)	8 (36.4%)	40 (33.9%)	
SUV ≥ 15	Missing	22	12	22	56	*0.7480*
	No	13 (26.0%)	7 (30.4%)	2 (18.2%)	22 (26.2%)	
	Yes	37 (74.0%)	16 (69.6%)	9 (81.8%)	62 (73.8%)	
IPI	Missing	2	2	8	12	***0.0080***
	Low	7 (10.0%)	6 (18.2%)	11 (44.0%)	24 (18.8%)	
	Low-int	21 (30.0%)	12 (36.4%)	5 (20.0%)	38 (29.7%)	
	High-int	28 (40.0%)	8 (24.2%)	8 (32.0%)	44 (34.4%)	
	High	14 (20.0%)	7 (21.2%)	1 (4.0%)	22 (17.2%)	
R-IPI	Missing	2	2	8	12	***0.0170***
	Very good	1 (1.4%)	3 (9.1%)	5 (20.0%)	9 (7.0%)	
	Good	27 (38.6%)	15 (45.5%)	11 (44.0%)	53 (41.4%)	
	Poor	42 (60.0%)	15 (45.5%)	9 (36.0%)	66 (51.6%)	
Kind of CT	Missing	0	0	2	2	*0.2080*
	R-VEMP	15 (20.8%)	2 (5.7%)	5 (16.1%)	22 (15.9%)	
	R-CHOP/COMP	52 (72.2%)	27 (77.1%)	23 (74.2%)	102 (73.9%)	
	Other regimens	5 (6.9%)	6 (17.1%)	3 (9.7%)	14 (10.1%)	
Reduced IgG	Missing	9	4	11	24	*0.1970*
	No	51 (81.0%)	24 (77.4%)	21 (95.5%)	96 (82.8%)	
	Yes	12 (19.0%)	7 (22.6%)	1 (4.5%)	20 (17.2%)	
Reduced post-CT IgG	Missing	17	9	10	36	*0.4030*
	No	27 (49.1%)	15 (57.7%)	15 (65.2%)	57 (54.8%)	
	Yes	28 (50.9%)	11 (42.3%)	8 (34.8%)	47 (45.2%)	
CD5 expression	Missing	44	24	25	93	*0.1500*
	Negative	23 (82.1%)	11 (100.0%)	8 (100.0%)	42 (89.4%)	
	Positive	5 (17.9%)	0 (0.0%)	0 (0.0%)	5 (10.6%)	
P53 expression	Missing	44	24	25	93	*0.1070*
	Negative	7 (25.0%)	0 (0.0%)	3 (37.5%)	10 (21.3%)	
	Positive	21 (75.0%)	11 (100.0%)	5 (62.5%)	37 (78.7%)	
NDUFB1 expression	Missing	44	24	25	93	*0.2270*
	Negative	24 (85.7%)	11 (100.0%)	8 (100.0%)	43 (91.5%)	
	Positive	4 (14.3%)	0 (0.0%)	0 (0.0%)	4 (8.5%)	
MCL1 expression	Missing	45	26	25	96	*0.3870*
	Negative	19 (70.4%)	8 (88.9%)	7 (87.5%)	34 (77.3%)	

	Positive	8 (29.6%)	1 (11.1%)	1 (12.5%)	10 (22.7%)	
BCL-XL expression	Missing	45	26	25	96	*0.2760*
	Negative	21 (77.8%)	9 (100.0%)	7 (87.5%)	37 (84.1%)	
	Positive	6 (22.2%)	0 (0.0%)	1 (12.5%)	7 (15.9%)	
BCL-W expression	Missing	45	26	25	96	*0.6500*
	Negative	25 (92.6%)	8 (88.9%)	8 (100.0%)	41 (93.2%)	
	Positive	2 (7.4%)	1 (11.1%)	0 (0.0%)	3 (6.8%)	
SII > 1684.09	Missing	5	4	10	19	***0.0240***
	No	48 (71.6%)	27 (87.1%)	22 (95.7%)	97 (80.2%)	
	Yes	19 (28.4%)	4 (12.9%)	1 (4.3%)	24 (19.8%)	
CAR > 0.21	Missing	12	11	16	39	***0.0020***
	No	15 (25.0%)	9 (37.5%)	12 (70.6%)	36 (35.6%)	
	Yes	45 (75.0%)	15 (62.5%)	5 (29.4%)	65 (64.4%)	
Response to 2nd line therapy	Missing	7	6	11	24	***0.0060***
	CR	9 (13.8%)	15 (51.7%)	10 (45.5%)	34 (29.3%)	
	PR	6 (9.2%)	2 (6.9%)	2 (9.1%)	10 (8.6%)	
	SD	4 (6.2%)	2 (6.9%)	1 (4.5%)	7 (6.0%)	
	PD	46 (70.8%)	10 (34.5%)	9 (40.9%)	65 (56.0%)	
Secondary IPI	Missing	2	3	4	9	***0.0220***
	Low	11 (15.7%)	12 (37.5%)	9 (31.0%)	32 (24.4%)	
	Low-int	18 (25.7%)	6 (18.8%)	13 (44.8%)	37 (28.2%)	
	High-int	26 (37.1%)	9 (28.1%)	3 (10.3%)	38 (29.0%)	
	High	15 (21.4%)	5 (15.6%)	4 (13.8%)	24 (18.3%)	
Secondary R-IPI	Missing	2	3	4	9	***0.0070***
	Very good	1 (1.4%)	4 (12.5%)	2 (6.9%)	7 (5.3%)	
	Good	28 (40.0%)	14 (43.8%)	20 (69.0%)	62 (47.3%)	
	Poor	41 (58.6%)	14 (43.8%)	7 (24.1%)	62 (47.3%)	
SUV ≥ 15 (relapse)	Missing	32	11	15	58	*0.9830*
	No	16 (40.0%)	10 (41.7%)	7 (38.9%)	33 (40.2%)	
	Yes	24 (60.0%)	14 (58.3%)	11 (61.1%)	49 (59.8%)	
ASCT	Missing	0	0	1	1	*0.1040*
	No	56 (77.8%)	23 (65.7%)	28 (87.5%)	107 (77.0%)	
	Yes	16 (22.2%)	12 (34.3%)	4 (12.5%)	32 (23.0%)	

Table 5. Patients' characteristics – focus on R/R: impact on overall survival.

Variables		**n**	**events**	**median OS**	**0.95LCL**	**0.95UCL**	***logrank***
Sex	F	56	37	**74**	38	99	***0.0085***
	M	80	60	**22**	17	38	
Other hemat. malignancies	No	110	78	**38**	22	65	*0.5306*
	Yes	26	19	**42**	10	89	
Other malignancies	No	110	78	**42**	25	72	*0.3721*
	Yes	26	19	**22**	10	64	
B-Symptoms	No	78	52	**50**	25	76	***0.0288***
	Yes	52	40	**17**	11	38	
LDH increase	No	51	37	**75**	36	89	***0.0393***
	Yes	75	54	**24**	17	38	
Extra-nodal involvement	No	32	20	**76**	38	147	***0.0395***
	Yes	101	75	**25**	19	42	
BM involvement	No	66	44	**53**	21	83	*0.0520*
	Yes	39	29	**27**	10	50	
CNS involvement (diagn.)	No	128	91	**38**	24	61	*0.6090*
	Yes	7	5	**24**	8	NA	
Stage	I-II	35	23	**83**	45	147	***0.0079***
	III-IV	99	72	**27**	19	38	
Bulky disease	No	88	61	**45**	26	75	*0.2092*
	Yes	43	31	**22**	16	61	
HBV serolgy	Negative	106	75	**42**	24	72	*0.9503*
	Positive	22	14	**38**	11	NA	
HCV serology	Negative	119	83	**38**	24	64	*0.7217*
	Positive	9	6	**99**	4	NA	
HIV serology	Negative	94	64	**32**	19	50	*0.5918*

	Positive	3	2	**38**	20	NA	
VES > 37.5mm/h	No	41	31	**38**	19	65	*0.6143*
	Yes	38	30	**22**	12	38	
N/L > 3.5	No	61	45	**45**	21	83	*0.3729*
	Yes	58	42	**26**	17	38	
COO	GCB	29	15	**49**	21	NA	*0.0551*
	Non-GCB	32	25	**19**	11	37	
Ki67 ≥ 70%	No	33	19	**32**	20	NA	***0.0142***
	Yes	40	35	**18**	11	24	
Ki67 ≥ 80%	No	51	33	**24**	17	49	***0.0328***
	Yes	22	21	**18**	11	27	
Infective toxicity	No	75	53	**32**	19	53	*0.6172*
	Yes	40	29	**46**	19	75	
SUV ≥ 15	No	22	13	**50**	11	NA	*0.4349*
	Yes	60	45	**25**	19	53	
IPI	Low	23	16	**89**	45	147	***0.0269***
	Low-int	37	24	**36**	20	75	
	High-int	43	32	**25**	11	50	
	High	22	18	**20**	10	38	
R.IPI	Very good	8	4	**213**	17	NA	***0.0072***
	Good	52	36	**52**	21	83	
	Poor	65	50	**24**	12	38	
Kind of CT	R-VEMP	22	18	**19**	8	61	*0.3020*
	RCHOP/COMP	98	67	**38**	24	72	
	Other regimens	14	10	**38**	10	118	
Reduced IgG	No	94	70	**37**	21	64	*0.8156*
	Yes	19	12	**32**	11	89	
Reduced post-CT IgG	No	56	39	**45**	22	76	*0.1526*
	Yes	46	35	**24**	13	65	
CD5 expression	Negative	41	35	**27**	17	50	*0.0518*
	Positive	5	5	**10**	4	NA	
P53 expression	Negative	10	9	**17**	4	37	*0.1596*
	Positive	36	31	**26.5**	17	72	
NDUFB1 expression	Negative	42	37	**26.5**	17	42	*0.8321*
	Positive	4	3	**9**	8	NA	
MCL1 expression	Negative	33	29	**27**	15	50	*0.5221*
	Positive	10	9	**18**	4	37	
BCL-XL expression	Negative	36	33	**23**	12	38	*0.2725*
	Positive	7	5	**37**	8	NA	
BCL-W expression	Negative	40	35	**21.5**	12	37	*0.7645*
	Positive	3	3	**42**	38	NA	
SII > 1684.09	No	95	70	**38**	24	64	*0.3044*
	Yes	23	16	**22**	11	72	
CAR > 0.21	No	33	18	**87**	35	NA	***0.0003***
	Yes	65	55	**20**	15	27	

Table 6. Patients' characteristics – focus on R/R: impact on progression-free survival.

Variables		**n**	**events**	**median PFS**	**0.95LCL**	**0.95UCL**	***logrank***	**2y PFS**	**Low.95% CI**	**Up.95% CI**
Sex	F	57	57	**19**	10	23	***0.0063***	**33.3%**	21.6%	45.5%
	M	83	83	**7**	6	9		**16.9%**	9.8%	25.6%
Other hemat. malignancies	No	114	114	**10.5**	8	15	*0.5035*	**23.7%**	16.4%	31.8%
	Yes	26	26	**5.5**	3	15		**23.1%**	9.4%	40.3%
Other malignancies	No	114	114	**9**	7	14	*0.6949*	**22.8%**	15.6%	30.9%
	Yes	26	26	**10.5**	6	16		**26.9%**	11.9%	44.5%
B-Symptoms	No	78	78	**13.5**	8	16	***0.0009***	**25.6%**	16.6%	35.7%
	Yes	55	55	**7**	5	9		**10.9%**	4.4%	20.7%
LDH.increase	No	53	53	**15**	8	24	***0.0000***	**35.9%**	23.3%	48.6%
	Yes	76	76	**7**	5	9		**10.5%**	4.9%	18.6%

Extra-nodal involvement	No	33	33	**15**	8	20	*0.1971*	**24.2%**	11.4%	39.6%
	Yes	104	104	**8**	6	11		**21.2%**	13.9%	29.4%
BM involvement	No	69	69	**9**	7	16	***0.0202***	**23.2%**	14.1%	33.6%
	Yes	39	29	**7**	5	10		**7.7%**	2.0%	18.7%
CNS involvement (diagn.)	No	131	131	**9**	7	15	***0.0011***			
	Yes	8	8	**5**	1	10				
Stage	I-II	36	36	**18.5**	14	34	***0.0000***	**41.7%**	25.6%	57.0%
	III-IV	102	102	**7**	6	9		**15.7%**	9.4%	23.4%
Bulky disease	No	90	90	**11.5**	8	16	***0.0005***	**30.0%**	20.9%	39.6%
	Yes	44	44	**6**	5	9		**4.5%**	0.8%	13.6%
HBV serology	Negative	108	108	**9**	7	16	***0.0088***	**26.9%**	18.9%	35.4%
	Positive	23	23	**7**	5	11		**4.3%**	0.3%	18.2%
HCV serology	Negative	122	122	**9**	7	13	*0.4301*	**22.1%**	15.3%	29.8%
	Positive	9	9	**18**	3	41		**33.3%**	7.8%	62.3%
HIV serology	Negative	97	97	**8**	6	11	*0.7762*	**20.6%**	13.2%	29.1%
	Positive	3	3	**7**	2	NA		**33.3%**	0.9%	77.4%
VES > 37.5mm/h	No	43	43	**6**	5	14	*0.9392*	**25.6%**	13.8%	39.1%
	Yes	38	38	**8**	7	14		**18.4%**	8.1%	32.0%
N/L > 3.5	No	63	63	**12**	8	16	***0.0019***	**27.0%**	16.8%	38.3%
	Yes	59	59	**6**	5	8		**10.2%**	4.1%	19.4%
COO	GCB	30	30	**9**	6	16	*0.2776*	**16.7%**	6.1%	31.8%
	Non-GCB	33	33	**7**	6	10		**15.2%**	5.5%	29.2%
Ki67 ≥ 70%	No	33	33	**9**	5	15	*0.2074*	**12.1%**	3.8%	25.5%
	Yes	43	43	**6**	5	8		**7.0%**	1.8%	17.1%
Ki67 ≥ 80%	No	53	53	**7**	6	11	*0.3856*	**9.4%**	3.5%	19.1%
	Yes	23	23	**6**	3	8		**8.7%**	1.5%	24.2%
Infective toxicity	No	78	78	**8**	6	11	*0.9739*	**18.0%**	10.4%	27.2%
	Yes	40	40	**9**	6	14		**20.0%**	9.4%	33.5%
SUV ≥ 15	No	22	22	**6**	3	16	*0.6963*	**9.1%**	1.6%	25.1%
	Yes	62	62	**8**	7	12		**14.5%**	7.1%	24.4%
IPI	Low	24	24	**18**	8	37	***0.0003***	**45.8%**	25.6%	64.0%
	Low-int	38	38	**9**	6	16		**13.2%**	4.8%	25.8%
	High-int	44	44	**7**	5	11		**18.2%**	8.5%	30.7%
	High	22	22	**6.5**	4	12		**4.6%**	0.3%	18.9%
R-IPI	Very good	9	9	**32**	3	105	***0.0013***	**55.6%**	20.4%	80.5%
	Good	53	53	**9**	7	16		**20.8%**	11.1%	32.5%
	Poor	66	66	**7**	5	9		**13.6%**	6.7%	23.0%
Kind of CT	R-VEMP	22	22	**7**	5	11	*0.7112*	**22.7%**	8.3%	41.5%
	RCHOP/COMP	102	102	**9**	7	14		**22.6%**	15.0%	31.0%
	Other regimens	14	14	**15.5**	3	19		**21.4%**	5.2%	44.8%
Reduced IgG	No	96	96	**8.5**	7	14	*0.3300*	**21.9%**	14.2%	30.6%
	Yes	20	20	**8.5**	5	15		**5.0%**	0.3%	20.5%
Reduced post-CT IgG	No	57	57	**12**	8	17	*0.3112*	**26.3%**	15.8%	38.1%
	Yes	47	47	**7**	6	11		**17.0%**	8.0%	29.0%
CD5 expression	Negative	42	42	**9**	6	14	*0.0621*			
	Positive	5	5	**7**	1	NA				
P53 expression	Negative	10	10	**5.5**	1	30	*0.5982*	**30.0%**	7.1%	57.8%
	Positive	37	37	**9**	7	14		**13.5%**	4.9%	26.4%
NDUFB1 expression	Negative	43	43	**9**	7	14	***0.0005***			
	Positive	4	4	**4**	1	NA				
MCL1 expression	Negative	34	34	**9**	7	15	*0.2577*	**20.6%**	9.1%	35.3%
	Positive	10	10	**6**	1	8		**10.0%**	0.6%	35.8%
BCL-XL expression	Negative	37	37	**9**	6	15	*0.4475*	**18.9%**	8.3%	32.8%
	Positive	7	7	**6**	1	8		**14.3%**	0.7%	46.5%
BCL-W expression	Negative	41	41	**8**	6	14	*0.2853*			
	Positive	3	3	**7**	4	NA				
SII > 1684.09	No	97	97	**10**	7	15	***0.0002***	**22.7%**	15.0%	31.4%
	Yes	24	24	**5**	3	7		**4.2%**	0.3%	17.6%
CAR > 0.21	No	36	36	**15**	6	23	***0.0004***	**33.3%**	18.8%	48.6%
	Yes	65	65	**7**	6	8		**7.7%**	2.8%	15.8%

Figure 12. Kaplan-Meier curves, reporting variables with impact on OS: A) sex; B) CAR index; C) IPI score; D) R-IPI score; E) Ki67 ≥ 70%; F) Ki67 ≥ 80%.

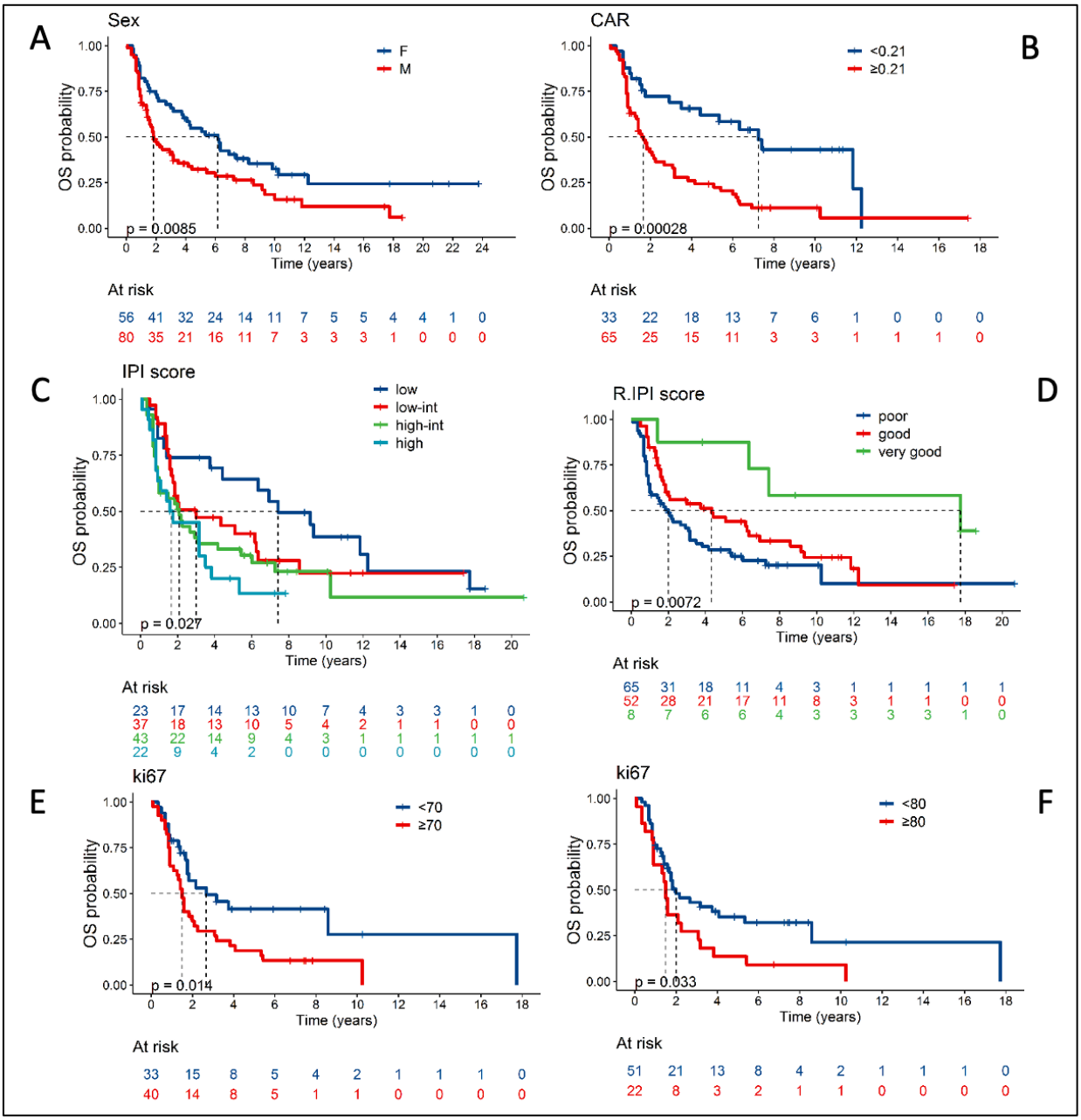

Figure 13. Kaplan-Meier curves, reporting variables with impact on PFS: A) sex; B) HBV serology; C) IPI score; D) R-IPI score; E) NDUFB1 overexpression; F) N/L ratio; G) SII; H) CAR index.

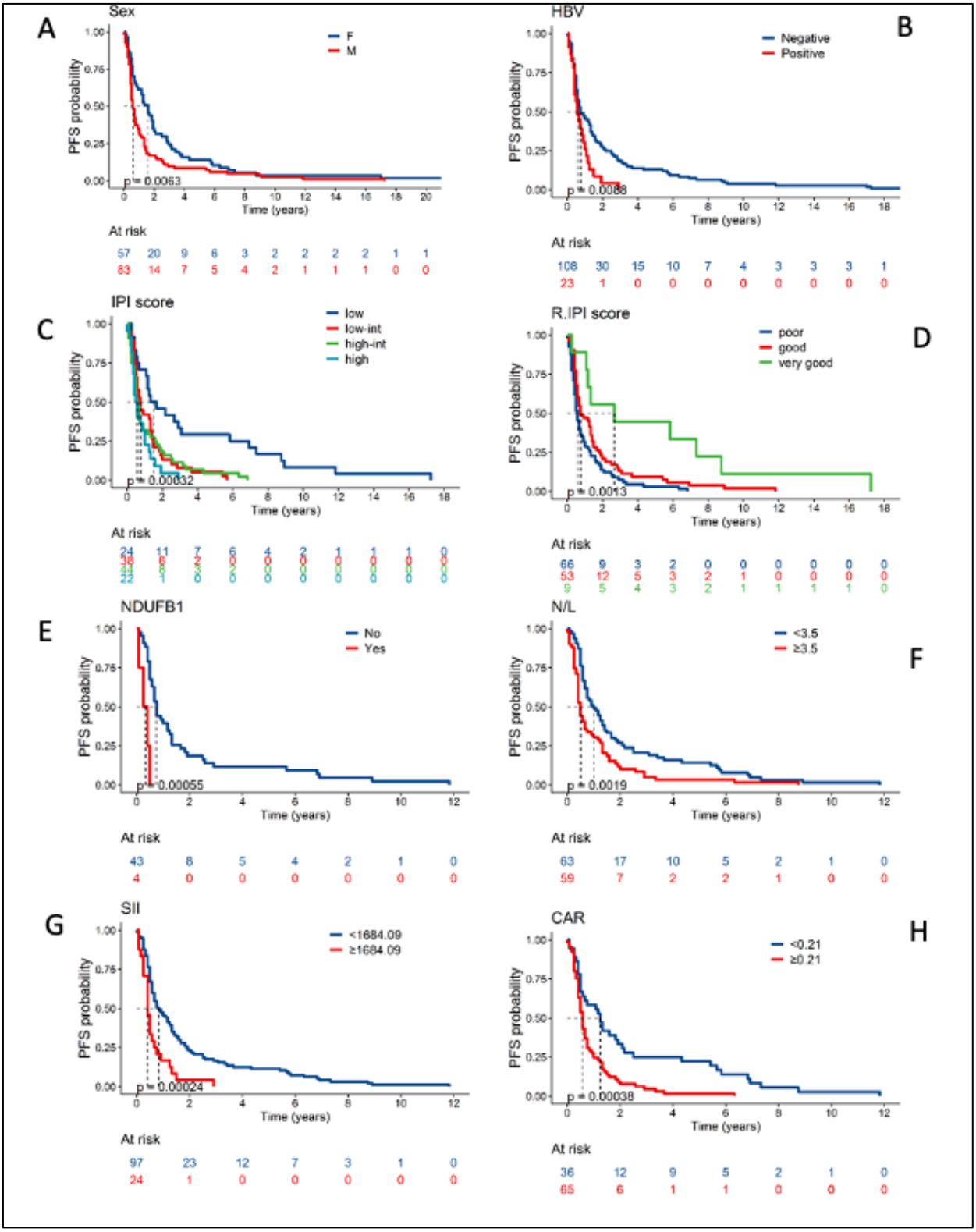

Figure 14. Kaplan-Meier curves, reporting variables with impact on post-relapse OS: A) R/R subgroups (refractory, early- and late relapsed); B) patients undergoing ASCT or not.

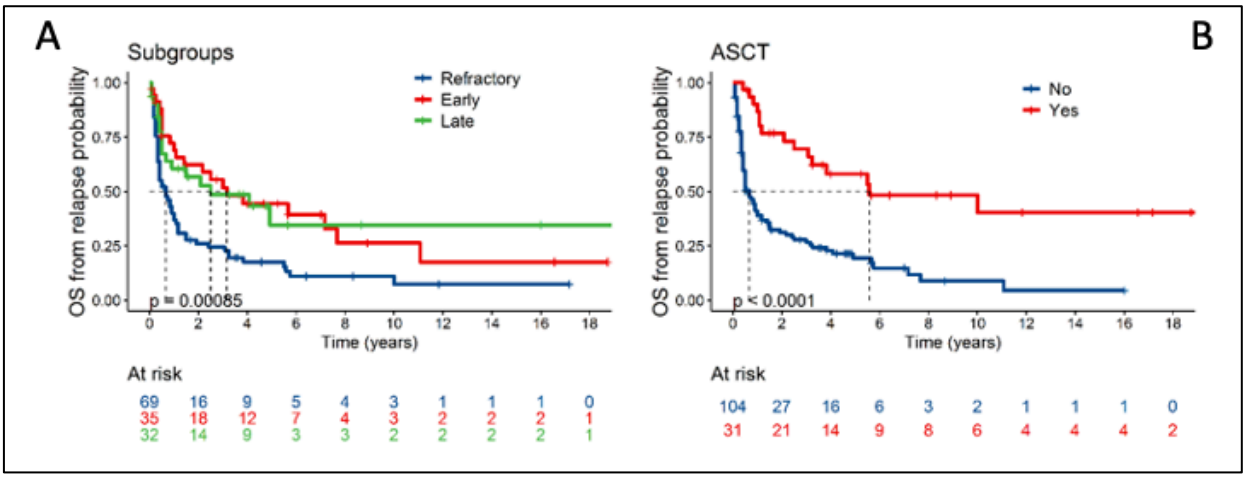

Table 7. Description of cases with altered karyotype.

Karyotype	R/R vs control, subgroup of R/R	GCB vs non-GCB	Ki67 (%)	Other malignancies
Tetraploid clone, with complex karyo	R/R, refractory	ND	ND	Monoclonal component, tongue carcinoma
45,X,-Y	R/R, refractory	ND	40	Colon adenocarcinoma
3 clones with complex karyo	R/R, refractory	Non-GCB	80	Other lymphoproliferative disease
Tetraploid clone	R/R, early relapsed	ND	30	Marginal zone lymphoma
Hyperdiploid clone, with complex karyo	R/R, refractory	Non-GCB	80	Cutaneous carcinoma
Complex karyo	R/R, refractory	ND	80	Myelodysplastic syndrome
Tetraploid clone, with complex karyo	R/R, late relapsed	Non-GCB	95	-
47, XY +add(3), add(22)	R/R, early relapsed	Non-GCB	70	-
Tetraploid clone, with complex karyo	R/R, early relapsed	Non-GCB	70	-
t(8;8)(p21;q23)	R/R, refractory	Non-GCB	70	-
Hyperdiploid clone	R/R, refractory	ND	ND	-
Triploid clone, with complex karyo	R/R, early relapsed	ND	ND	-
Hyperdiploid clone, with complex karyo	R/R, refractory	ND	ND	-
46, XY/ 45,X,-Y	Control	Non-GCB	ND	Prostatic cancer
47, XY + 11	Control	GCB	ND	Spinocellular carcinoma

Figure 15. Images from immunohistochemistry: A) staining for CD5 (null expression *vs* overexpression); B) staining for NDUFB1 (null expression *vs* overexpression); C) staining for P53 (null expression *vs* intermediate expression *vs* overexpression); D) staining for MCL1 (null expression *vs* overexpression); E) staining for BCL-XL (null expression *vs* overexpression); F) staining for BCL-W (null expression *vs* overexpression).

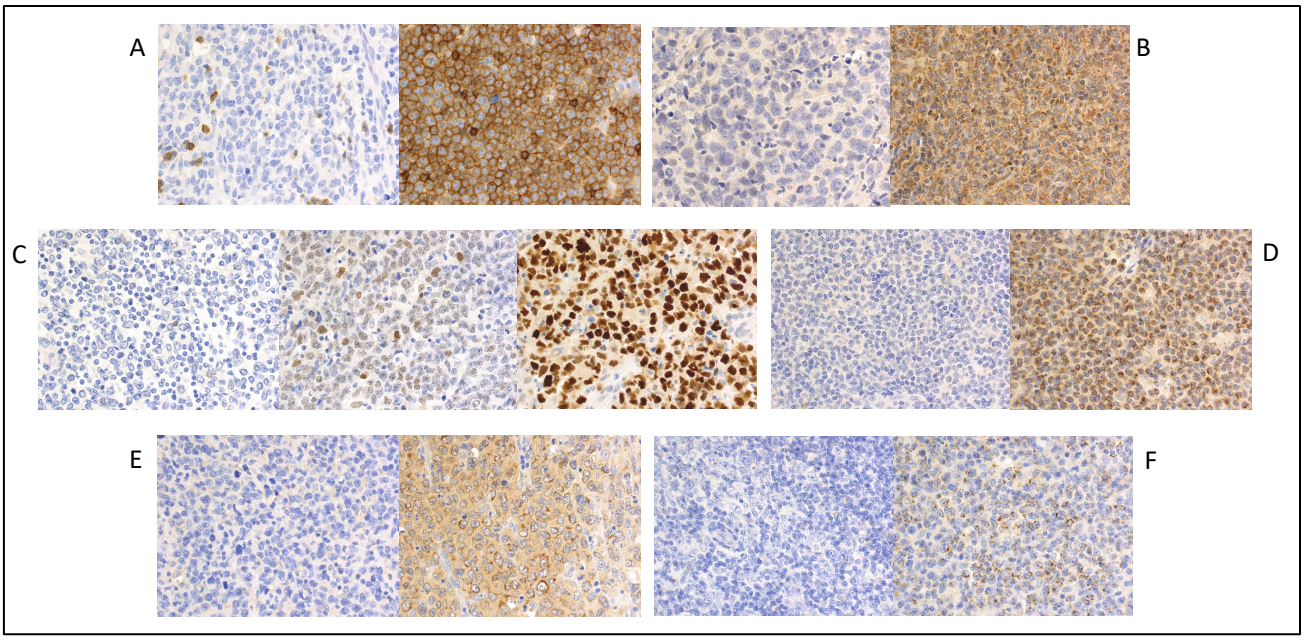

Figure 16. Nomogram for the R/R IPI model (145).

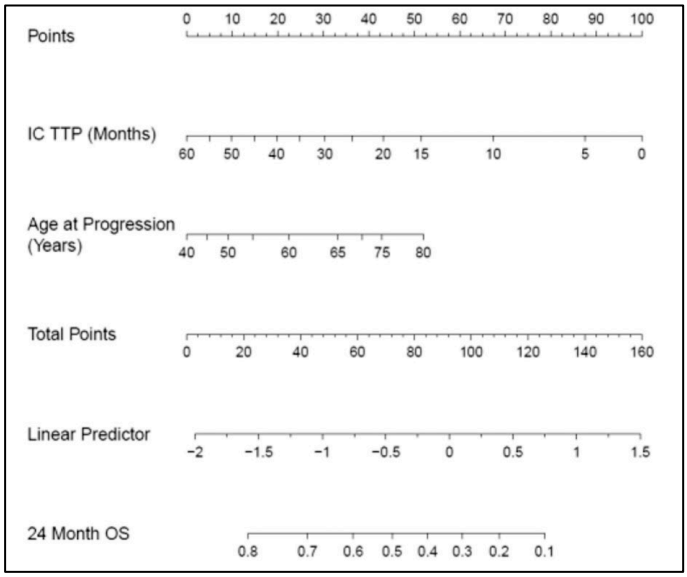

Table 8. Comparison between 2-year post-relapse OS observed in our cohort vs Expected 2-year post-relapse OS as per R/R IPI, stratified according to age at relapse and time to relapse according to *Maurer et al* (145).

Total points calculated as per (145)	**2-year post-relapse OS observed in our R/R cohort**	**Expected 2-year post-relapse OS as per** (145)
30-78	48.5%-88.3% (m 74.1%)	45%-75%
80-123	21.4%-50.4% (m 35.8%)	13%-40%
124-147	6.0%-37.7% (m 19.1%)	<10%

Table 9. Description of patient with CNS localization at relapse.

	CNS involved at diagnosis	CNS-IPI	CNS prophylaxis	Time of relapse	2nd line therapy	Response
1	yes	int	TIT	Refractory	MTX it, HD Cytarabine, ASCT	PD
2	no	high	TIT	Early relapsed	IVAC + TIT	PD
3	no	high	no	Refractory	TIT + MTX ev	PD
4	no	int	no	Refractory	Marietta	PD
5	no	low	no	Refractory	HD Cytarabine	PD
6	no	int	no	Late relapsed	MATRIX + ICE	PD
7	no	low	no	Refractory	ILL-SCNL1	PR
8	yes (liquor +)	high	TIT	Refractory	MTX, HD Cytarabine, TIT	PD
9	no	NA	no	Early relapsed	HD chemo + ASCT	CR
10	no	int	no	Refractory	RT	PD

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