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ORIGINAL ARTICLE

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A new digital droplet PCR method for looking at epigenetics in diffuse large B-cell lymphomas: The role of *BMI1*, *EZH2*, and *USP22* genes

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Abstract

Introduction: Epigenetics has been shown to be relevant in oncology: *BMI1* overexpression has been reported in leukemias, *EZH2* mutations have been found in follicular lymphoma, and *USP22* seems to stabilize BMI1 protein. In this study, we measured the expression of *BMI1*, *EZH2*, and *USP22* in lymph nodes from 56 diffuse large B-cell lymphoma (DLBCL) patients.

Methods: A new multiplex digital droplet PCR (ddPCR) has been set up to measure the expression of 4 genes (*BMI1*, *EZH2*, *USP22*, and *GAPDH*) in the same reaction on RNA extracted from paraffin-embedded tissues.

Alessio Lusci Gemignani and Robel Papotti are co-first authors.

Results: The specificity of ddPCR was confirmed by a 100% alignment on the BLAST platform and its repeatability demonstrated by duplicates. A strict correlation between expression of *BMI1* and *EZH2* and *BMI1* and *USP22* has been found, and high expression of these genes was correlated with extra-nodal lymphomas. Progression-free survival (PFS) and overall survival (OS) were conditioned by IPI, bone marrow infiltration, and the complete response achievement. High levels of *BMI1* and *USP22* did not condition the response to therapy, but impaired the PFS, especially for patients defined at "high risk" based on the cell of origin (no germinal center [GCB]), high BCL2 expression, and IPI 3-5. In this subgroup, the probability of relapse/progression was twice higher than that of patients carrying low *BMI1* and *USP22* levels.

Conclusion: High expression of *BMI1* and of *USP22* might be a poor prognostic factor in DLBCL, and might represent the target for novel inhibitors.

KEYWORDS
BMI1, ddPCR, DLBCL, EZH2, USP22

1 | INTRODUCTION

Non-Hodgkin's lymphomas (NHLs) are the most common hematological malignancies worldwide, accounting for 3% of all cancers.[1] In this context, diffuse large B-cell lymphoma (DLBCL) is the most common aggressive form, representing approximately one third of all NHLs.[2] Although durable remissions can be achieved in more than half of cases, DLBCL still has some unmet clinical needs, with approximately 30% of patients not being cured by standard immunochemotherapies. Moreover, in relapsed/refractory patients, conventional treatments offer 26% of responses, with only 20% of patients still alive at 2 years.[3] The limits to adopt a treatment "fit for all" are, at least in part, related to the biological heterogeneity of disease, which can be recognized at the morphologic, genetic, and clinical level. For example, the forms with co-occurrence of *MYD88* and *CD79b* mutations seem to be associated with a different outcome in respect of those with *BCL6* fusions or with *NOTCH1*/*NOTCH2* mutations.[4] To this heterogeneity might also contribute the epigenetic tumor control, in particular the expression of genes belonging to the Polycomb repressive complexes (PRCs), like *EZH2* and *BMI1*. *BMI1* is essential for the assembly and catalytic activity of PRC1, while *EZH2* serves as the catalytic subunit of the PRC2. Together, these two complexes and relative genes are important for stem cell functionality and tissue homeostasis maintenance.[5] Moreover, Polycomb genes are important in the normal B-cell differentiation: *BMI1* is principally expressed in resting B lymphocytes, and in non-dividing centrocytes of the germinal center follicles. In murine models of B-lymphoproliferative diseases, *BMI1* has been reported to be an oncogene, because, by repressing p16Ink4a, p15Ink4b, Noxa and BIM, it prevents apoptosis and is sufficient to induce lymphoma.[6] *EZH2* is up regulated when B cells are activated and undergo rapid proliferation and is responsible for cells survive during the somatic hypermutation process. As occurs for *BMI1*, *EZH2* also represses p16Ink4, Cdkn1a/p21 and IRF4, all essential for B-cell development. Consequently, depletion of *EZH2* from lymphomas suppresses tumor proliferation.[7] When immature B cells enter the germinal center, *EZH2* levels increase, but decrease when B lymphocytes leave the germinal center and differentiate. An overexpression of *EZH2* results in an excessive silencing of its target genes, with block of differentiation, lymphocytes proliferation and survival.[7] Moreover, *IgH*::*BMI1* rearrangements, leading to the over-expression of *BMI1*, have been reported in chronic lymphocytic leukemia,[8] thus showing that in lymphoproliferative disorders, as well as in chronic myeloid leukemia,[9,10] *BMI1* over-expression is responsible for chemoresistance.[11] In addition, EZH2 protein is detected in Burkitt lymphoma, follicular lymphomas, and DLBCL, where its levels correlate with disease aggressiveness and represent a poor prognostic factor.[12] In about 20% of follicular lymphomas, *EZH2* mutations at tyrosine residues 641 and 677 correlate with a more aggressive disease phenotype,[13] and tazemetostat, an oral EZH2 inhibitor, offers more than 60% of responses in relapsed *EZH2*-mutated patients.[14]

The activities of PRC1 and PRC2 are strictly related, but it has been reported that the Polycomb genes might also interact with different oncogenes. Among them, *USP22*, a deubiquitinase that in cancer mediates the immune evasion—by stabilizing progressive disease (PD)-L1 and up-regulating *c-MYC* and *TP53*.[15,16] *USP22* has been reported to be involved in the immunoglobulin isotypic switch, and its repression results in the block of B-cell development and in an abnormal variable–diversity–joining rearrangement recombination.[17]

About the possible relationship among *BMI1*, *EZH2*, and *USP22*, it has been reported that *USP22* increases *BMI1*: in gastric cancer, *USP22* inactivation decreases the neoplasm growth, while high levels of *BMI1* accelerate the cell cycle via INK4a/ARF and AKT.[18] Moreover, a reciprocal interaction between *EZH2* and *USP22* has been described in the sepsis-induced myocardial dysfunction model: in this

case, *EZH2* represses *USP22* through the H3K27me3 modification.[19] In acute myeloid leukemia, the activation of *BAX* and the inhibition of *BCL2* lead to a reduced expression of *BMI1* and *EZH2*.[20] Finally, both *BMI1* and *EZH2* seem to be targets for the same miRNA, the miR200 that is a key regulator of the epithelial-mesenchymal transition; indeed, a reduced expression of these genes reduces the migration capacity of colon cancer cells.[21]

At the best of our knowledge, no data about the possible interactions among *USP22*, *BMI1* and *EZH2* and their prognostic impact on DLBCL patients' outcome have been reported in literature. Consequently, we decided to measure the expression of these 3 genes in lymph nodes from 56 DLBCL cases already characterized for the cell of origin (COO) in our pivotal study already published,[22] to investigate the eventual correlated expressions of *USP22*, *BMI1* and *EZH2* and to assess a possible prognostic value of these 3 genes in DLBCL.

To measure gene expression more accurately, we set up a novel digital droplet PCR assay (ddPCR), able to analyze simultaneously the 3 chosen genes (*BMI1*, *USP22*, *EZH2*) and one housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase or *GAPDH*), all amplified in the same reaction. Different experiments were conducted to optimize reaction parameters, including primers/probes concentrations, annealing temperature, and cycle numbers. The "multiplex ddPCR" is a new molecular technique that has been used in several contexts, including cancers[23] and infections,[24] with a clear advantage in terms of time and accuracy. In hematology, this technique has already been used for detecting *IDH2* mutations in acute myeloid leukemia[25] and *BCR::ABL1* expression in chronic myeloid leukemia.[26] Thus, also the application of this ddPCR is a novelty coming from the current study.

2 | MATERIALS AND METHODS

2.1 | Samples

The RNAs used in this study have already been extracted from 56 lymph nodes of patients affected by DLBCL at diagnosis by using the Maxwell® RSC simplyRNA Tissue kits with the Maxwell instrument (Promega, Italy), as already described.[22] In our pivotal study,[22] the lymph nodes were fixed in formalin and embedded in paraffin, and tissue sections were obtained to determine the COO using two different methods: the immunohistochemical approach (Hans's algorithm) and the Nanostring technique (gene expression profiling technique). Additionally, the expression levels of BCL-2, c-MYC, and BCL6 were measured by immunohistochemistry; FISH for c-MYC, BCL2 and BCL6 was performed in 12 cases.

2.2 | Reverse transcription

For reverse transcription the "Complete Whole Transcriptome Amplification Kit" from Sigma Aldrich (Italy) was used. This kit is designed for low-concentration samples or partially degraded samples.

2.3 | Multiplex ddPCR

To analyze in the same reaction the 4 genes chosen as target in our study, it was necessary to optimize different reaction parameters, including primers and probes concentrations, annealing and extension temperatures, and the number of amplification cycles. Indeed, in the multiplex PCR assays, targets are detected in the end-point reactions with probes conjugated with a single dye but employed at different final concentrations in the same droplet. The detection system was based on the TaqMan probes associated with 6-Carboxyfluorescein, single isomer and HEX fluorophores: the 4 targets were stained with the same fluorophore in pairs (*BMI1* and *EZH2* with FAM; *USP22* and *GAPDH* with *HEX*), and primers concentrations were used for optimizing the reaction conditions. The levels of all 3 genes were normalized by using *GAPDH*, and expressed as copies/uL. The conditions for ddPCR are reported in the Table S1.

In more detail, our novel method enables the measurement of three or more target genes per reaction, making possible to distinguish clusters in a 2-dimensions plot: (1) positive samples with *low* FAM signal = *y*; (2) positive samples with *high* FAM signal = 2*y*; (3) positive samples with *low* HEX signal = *x*; (4) positive samples with *high* HEX signal = 2*x* (see Figure S1A).

Initially, we studied the relative intensity of individual assays and then we analyzed the fluorescence intensity both individually and in duplex (FAM assay/HEX assay). Because the signal related to *EZH2* was higher than that of *BMI1*, we used higher doses of primers/probe for *EZH2* compared with *BMI1*, analogously for *USP22* in comparison with *GAPDH*. In each experiment we also analyzed NTC (No Template Control) and NRT (No Reverse Transcription control) to exclude RNA or DNA contamination, respectively. Additionally, a negative control has been included in each run to assess the presence of potential contaminations. We did not use a calibrator because ddPCR is an "end-point" reaction that does not require the use of a reference curve. For performing multiplex ddPCR, the Droplet Generator and the Droplet Reader instruments (BioRad, QX200) have been used. Analysis of results was conducted using the QuantaSoft Pro Software (BioRad, Italy). Each sample was analyzed in duplicate. At the end of the amplification, thanks to the "QuantaSoft Analysis Pro™" software, the concentrations (copies/µl) and relative expression of each gene of interest (geneX/GAPDH) were obtained. An example of final plot is depicted in the Figure S1.

2.4 | Patients

For this study we used RNA still available in our laboratory from the previous study where we analyzed the prognostic role of BCL2 protein levels in addition to the COO in DLBCL.[22] The inclusion criteria were diagnosis of DLBCL/not otherwise specified (NOS), no previous therapy, age >18 years, human immunodeficiency virus type 1 negativity, availability of clinical and laboratory data. For the present study, the only adopted inclusion criterium was the availability of RNA. The

TABLE 1 Clinical features and outcome of the enrolled DLBCL patients.

Parameter	Absolute number (percentage)
Age (median/range)	65 (57–76)
Sex	
F	26 (46%)
M	30 (54%)
Cell of origin (COO) nanostring	
GCB	23 (41%)
Non-GCB	24 (43%)
unclassifiable	9 (16%)
BCL-2 (immunohistochemistry)	
Positive	41 (73%)
Negative	15 (27%)
BCL-6 (immunohistochemistry)	
Positive	42 (75%)
Negative	14 (25%)
MUM1 (immunohistochemistry)	
Positive	36 (64%)
Negative	20 (36%)
c-MYC (immunohistochemistry)	
Positive	4 (7%)
Negative	52 (93%)
FISH for c-MYC, BCL2, BCL6	12 (21%)
Double hit	2 (4%)
Stage	
2	13 (23%)
3	15 (27%)
4	28 (50%)
IPI	
Low	4 (7%)
Low-intermediate	11 (20%)
High-intermediate	24 (43%)
High	17 (30%)
R-IPI	
Very good	2 (4%)
Good	29 (52%)
Poor	25 (44%)
Extra-nodal involvement	
No	26 (46%)
Yes	30 (54%)
Response	
CR	44 (79%)
PR	5 (9%)
SD/PD	7 (12%)
Relapse	
No	43 (77%)
Yes	13 (33%)
4year-OS	67%
4year-PFS	77%

Abbreviations: CR, complete response; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center; SD, stable disease; OS, overall survival; PFS, progression-free survival; PR, partial response.

clinical features and outcome of the 56 enrolled patients are presented in the Table 1.

In detail, we enrolled 26 female and 30 males, with median age of 65 (range, 57–76). Two patients presented with a "double hit" disease, and 50% of subjects were in stage 4. According to international prognostic index (IPI), 30% of patients were classified "at high risk," and the extra-nodal involvement was observed in 54% of them. According to COO, 41% of lymphomas were "germinal center" (GCB), 43% no-GCB, and the remaining 16% unclassifiable.

All patients except two received the R-CHOP treatment (rituximab with cyclophosphamide, daunorubicin, vincristine, and prednisone); the remaining two were treated according to the R-CVP scheme (rituximab with cyclophosphamide, vincristine, prednisone). At the end of treatment, 79% of patients achieved CR, but one third relapsed by 3 years.

Our pivotal study[22] was approved by Ethical Committee of Modena and Reggio Emilia in May 2016 with the reference number 44/16. In the informed consent, patients gave the RNA from samples of diagnosis for sustaining the research on DLBCL. All data were put in an electronic CRF conserved in the Pisa university cloud after data anonymization by each center.

2.5 | Statistical Analysis

Categorical data were described using absolute frequency and percentage, while continuous data were described by median and the interquartile range. To compare the gene expressions with categorical variables the Mann-Whitney *U* test method was applied. The analysis of the relationship among categorial variables was performed using the Chi-square test. For survival analysis, curves were constructed using the Kaplan–Meier method, and differences were calculated using the Log-Rank test. A significance level of $p < 0.05$ was set, and all analyses were performed using SPSS v.28 software (Bologna, Italy).

3 | RESULTS

3.1 | Multiplex ddPCR validation

The multiplex ddPCR set for the present study was further validated in a series of 43 samples from 13 patients affected by chronic myeloid leukemia at diagnosis; gene expression was measurable in all cases and once again *EZH2* and *BMI1* appeared strictly correlated. The

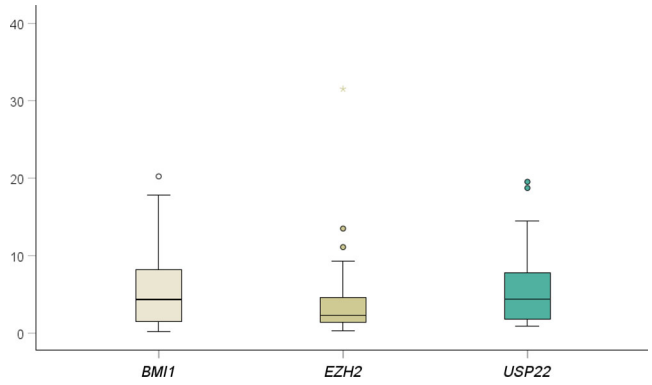

FIGURE 1 The Figure 1 represents *BMI1*, *EZH2*, and *USP22* expression values, including respective median and standard deviations. These values have been produced by digital droplet PCR.

specificity of the novel method was based on the use of already validated commercial single ddPCR assays (BioRad, Milan, Italy) that we combined in the multiplex ddPCR. We also aligned the sequence of each gene using the basic local alignment search tool (BLAST) platform finding a 100% alignment. Repeatability of our technique was demonstrated by duplicates analyzed. Nevertheless, it was not possible to perform a comparison with Real-Time PCR because the standards specific for our targets are not commercially available, and so quantitation by real-time PCR is not feasible.

3.2 | Measure of gene expression

BMI1 was the gene that presented the highest expression, followed by *EZH2* and *USP22* (*BMI1* median expression = 25.8 copies/uL; range, 14.3–32.3; *EZH2* median expression = 3.4 copies/uL; range, 2.5–6.0; *USP22* median expression = 3.9 copies/uL; range, 2.6–6.3) (see Figure 1).

When the Spearman's rank correlation has been applied, a significant correlation between *BMI1* and *EZH2* ($p < 0.001$) and between *BMI1* and *USP22* ($p = 0.008$) levels was found, whereas no significant correlation was observed between *EZH2* and *USP22* ($p = 0.406$). These relationships are represented in the Figure 2.

3.3 | Gene expression levels and patients' clinical features

First, patients were classified in two categories according to the score system that we already published[22]: it combines COO (one point for ABC/unclassified, zero points for GCB) with the IPI (one point for IPI 3–5, zero for IPI 1–2) and the BCL2 protein expression (one point for BCL2 ≥50%, zero for BCL2 <50%). According to this score, we identified 2 groups of patients: one at "low risk" (points 0–1)—22 patients—and one "at high risk" (points 2–3)—34 subjects. We tested if the expression of our target genes would correlate with these risk categories, but, notwithstanding a trend to a correlation between higher expression of *BMI1* and the "high risk", the statistical significance was not reached. Then, we analyzed the association among *BMI1*, *USP22*, and *EZH2* levels with BCL-2, c-MYC, and BCL6 positivity, but even in this case no significant correlations were observed. On the contrary, a significant correlation was observed between *BMI1* and *USP22* high expression (defined according to the median—under or above) and the extra-nodal lymphoma presentation: indeed, high *BMI1* levels were found in 73% of cases with extra-nodal lymphomas versus in 38% of patients with nodal presentation ($p = 0.03$). Analogously, high *USP22* levels were measured in 72% of patients with extra-nodal lymphomas and in 37% of nodal DLBCL ($p = 0.03$). Finally, no significant correlations were observed between the target genes expression and further clinical features, including IPI, R-IPI, presence of systemic symptoms, bone marrow involvement, disease stage, or lymphocytes count.

3.4 | Gene expression levels and outcome

Then, we investigated if *BMI1*, *EZH2* or *USP22* levels might condition the response to the treatment, including autologous transplantation, or the long-term outcome of our series. Concerning the response to R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) or R-CVP (rituximab, cyclophosphamide, vincristine, and prednisone), 80% of patients achieved a complete response (CR), 7% a partial response (PR), and 13% were stable or progressed, with a median observation time of 3 years. We did not find any correlation between *BMI1*, *USP22* or *EZH2* expression and the quality of response to chemo-immunotherapy. Because of relapse, 13 patients (23%) received autologous transplantation; this therapeutic option was not offered in different measure to patients with high or low *BMI1*, *EZH2* or *USP22* levels.

Concerning progression-free survival (PFS), the 4-year PFS of the whole population (51 evaluable cases) was 77% (95% CI, 64–92%) and it was conditioned by R-IPI (4-year PFS: 90% for patients at very good/good risk - $n = 32$; 95% CI, 75.7–103.1% versus 45% for those at poor risk - $n = 18$; 95% CI, 16.9%–43.12%; $p = 0.007$), bone marrow involvement (4-year PFS: 86% for patients without bone marrow infiltration - $n = 42$; 95% CI, 72.8%–99.2% versus 50% for those with bone marrow involvement - $n = 6$; 95% CI, 6.19%–34.5%; $p = 0.006$), and by the CR achievement (4-year PFS: 92% for patients in CR - $n = 40$; 95% CI, 90.8%–109.3% versus 22% for cases with PR or PD - $n = 11$; 95% CI, 0%–33.5%; $p < 0.001$).

Then, we divided our patients into two groups at "low" and "high" risk on the basis of our previous score (COO, BCL2, IPI); these two cohorts were subclassified again on the basis of BMI1, EZH2 and USP22 expression levels (high or low), thus creating 4 different groups.

Firstly, we observed that *EZH2* levels did not impact on PFS of the whole series, while high levels of *BMI1* or of *USP22* reduced 4 years-PFS up to 29% ($p = 0.05$). This difference was more evident in the subgroup of patients defined at "high risk" according to the score based on COO, BCL2, and IPI. Indeed, the 4-year PFS halved in these patients when they expressed also high levels of *BMI1* or of

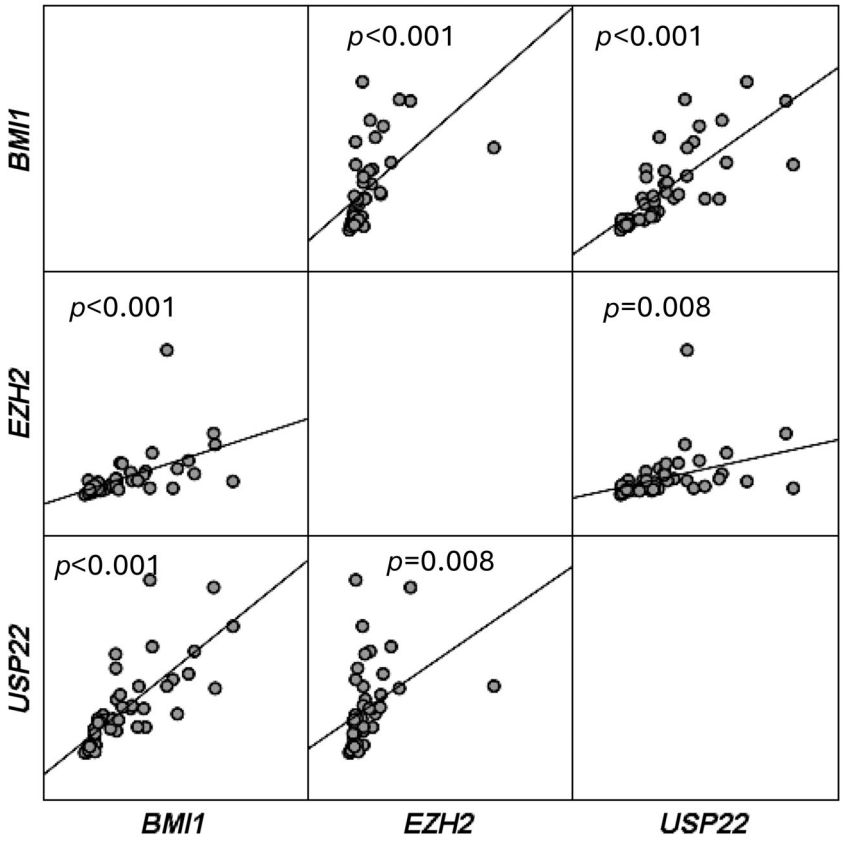

FIGURE 2 The Figure 2 shows the correlations among *BMI1*, *EZH2*, and *USP22* expression values. The expression of *BMI1* resulted significantly correlated with that of *EZH2* and *USP22*; on the contrary, no significant correlations between *EZH2* and *USP22* were observed.

USPP22 ($p = 0.016$, $p = 0.017$) (Figure 3A,B). Nevertheless, in multivariate analysis, the only parameter that retained statistical significance was the CR, whose absence increased of 13 times the probability of relapsing ($p < 0.01$).

About overall survival (OS), the 4-year OS of the whole series was 66% (CI 95%, 53.8–82.9), and it was significantly conditioned by the CR (4-year OS 95% for patients in CR—$n = 40$; 95% CI, 59.4–90.6% versus 15% for PR/PD—$n = 11$; 95% CI, 11.9%–55.2%; $p < 0.001$), the bone marrow involvement (4-year OS: 72% for patients without bone marrow infiltration - $n = 42$; 95% CI, 55.7–86.1% versus 50% for those with bone marrow involvement - $n = 6$; 95% CI, 12.4%–39.6%; $p = 0.02$).

High *BMI1* or *USP22* levels reduced of 16% the probability of survival, but this difference was not statistically significant ($p = 0.06$).

In multivariate analysis, the only parameter that retained its statistical significance was the CR whose absence increased of 6 times the probability of death ($p < 0.01$), while the low expression of *BMI1* or *EZH2* remained hierarchically the second most relevant factor (HR 0.5; $p = 0.11$).

4 | DISCUSSION

In the recent years, there has been a growing interest towards epigenetics that influences the expression of many genes as well as the molecular profile of many diseases. Studying alterations in the expression or mutations affecting epigenetic regulators could provide important therapeutic targets in different neoplasms and could contribute to further elucidate the pathogenic mechanisms or the resistance phenomenon.

The role of *BMI1*, *EZH2*, and *USP22* in lymphoproliferative disorders is still unclear and currently lacks a true clinical utility. Nevertheless, alterations in proteins coded by these genes have already been identified, and in the future they could represent new therapeutic targets. For instance, for *EZH2*-mutated cases tazemetostat has been successful, also when combined with further treatments such as R-CHOP[27] or venetoclax.[28] Also *BMI1* inhibitors have been developed and seem to be efficacious in acute myeloid leukemias,[29] multiple myeloma,[30] and mantle cell lymphoma.[31] About *USP22*, this gene has not still been studied in lymphoproliferative diseases; nevertheless, it represents a nodal point between immunity and epigenetics, as shown in other neoplasms. For example, in hepatocellular carcinoma *USP22* upregulates PD-L1 and induces the exhaustion of tumor infiltrating CD8+ T cells,[32] while in the colorectal carcinoma it targets BMI-1, c-MYC, CYCLIN D2, p16INK4a, and p14ARF, so exerting a crucial role in tumor proliferation.[33]

Based on these considerations, we investigated if epigenetics might have a role in the outcome of DLBCL patients by designing this study where 2 genes of the Polycomb family, *BMI1* and *EZH2*, and the deubiquitinase *USP22* were measured on RNAs extracted from lymph nodes of 56 DLBCL patients. Our study is certainly innovative, because we present here for the first time a new multiplex ddPCR test able to measure in the same reaction the expression of 4 genes, with high sensitivity, accuracy, and reproducibility.

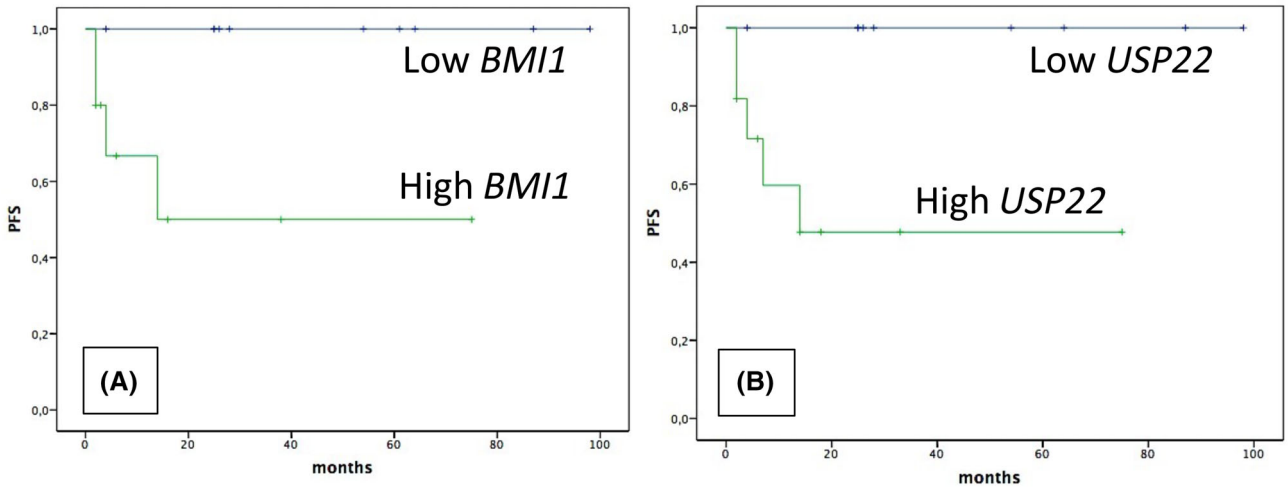

FIGURE 3 The Figure 3 depicts the progression-free survival (PFS) of patients already classified at "high risk" according to no-GCB COO, high IPI, and high BCL2 expression. The 4-year PFS halved in these patients when they also presented high levels of *BMI1* or of *USPP22* ($p = 0.016$, $p = 0.017$) (Figure 3A,B).

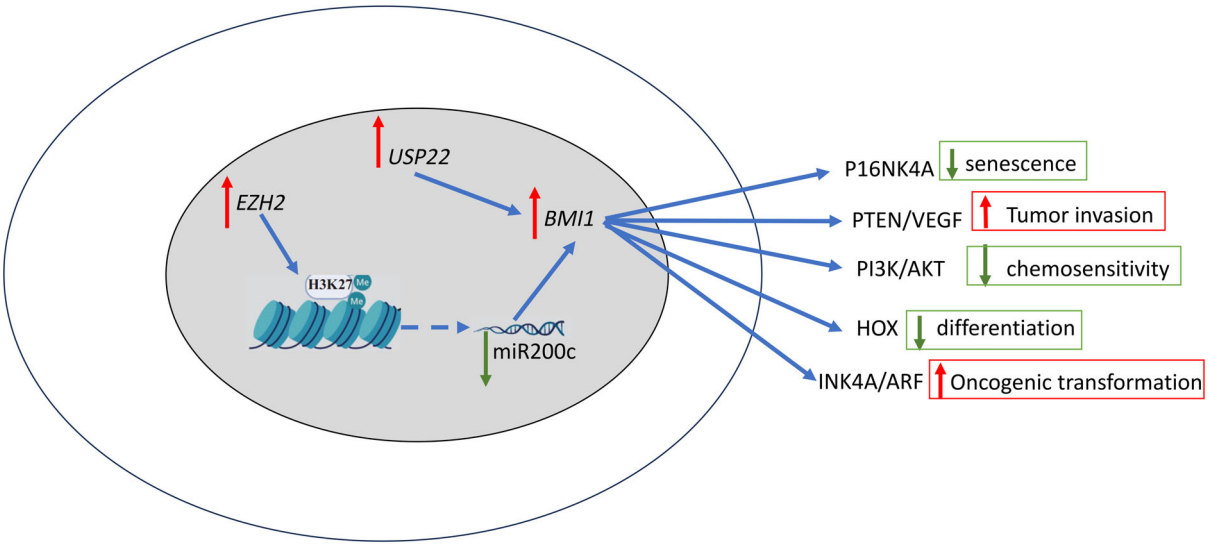

FIGURE 4 The Figure 4 represents a possible pathogenetic mechanism linking Polycomb genes to DLBCL. *EZH2*, when expressed at high levels, could increase the *BMI1* expression by down-regulating miR-200c (that has *BMI1* as target), while *USP22* could increase *BMI1* by a stabilizing action. Consequently, increased levels of *BMI1* could increase oncogenesis through the INK4/ARF pathway, and tumor invasion via PTEN/VEGF. Moreover, increased levels of *BMI1* could also reduce cellular senescence via P16NK4A, cellular differentiation by negatively acting on the *HOX* genes, and chemosensitivity by up regulating the PI3K/AKT pathway. The overall result would be a more aggressive phenotype of diffuse large B-cell lymphoma.

We must consider that ddPCR, differently from real-time PCR, is an absolute quantitative technique that does not require standard reference curves, a great advantage when commercial assays are not available. Moreover, this technique is exportable to most of the molecular laboratories worldwide. Our new method showed high specificity, as shown by aligning the sequences of each gene on the BLAST data source, and high accuracy, because the duplicates were perfectly superimposable. Unfortunately, a direct comparison with the real-time PCR to test eventual different sensitivities was not possible because the standards specific for our target genes are not commercially available.

Moreover, our research offers novel suggestions about the pathogenesis of DLBCL: a significant correlation between *BMI1* and *EZH2* has been previously described in solid tumors[34,35]; we observed high levels of these genes in the ABC and extra-nodal subtypes, thus supporting the hypothesis that also in DLBCL the Polycomb genes can be detrimental. In the Figure 4 we suggest a possible pathogenetic model for DLBCL: high levels of *EZH2* would increase the *BMI1* expression

perhaps by down-regulating some miRNAs, such as miR-200c (that controls *BMI1*), as already reported in other diseases.[36,37] On the other hand, *USP22* might sustain *BMI1* expression by stabilizing its protein.[18] The final output might be the oncogenic action exerted by *BMI1* via p16NK4A and PI3K/AKT.[38] A further intriguing finding is that the epigenetics in DLBCL seems to be independent from other clinical features, including the risk scores; this might explain the poorer outcome sometimes observed in GCB subtypes (that would be at better prognosis). In our series, epigenetics did not condition the quality of response but impaired the PFS of patients already classified at "high risk" based on high IPI, no-GCB COO and high B Cell Lymphoma 2 (BCL2). Indeed, in these cases, high *BMI1* and *USP22* levels duplicated the probability of relapsing/progressing; on this basis, we could offer to these patients different treatments, including CAR-T cells.

Finally, we must consider that Polycomb genes could also represent some possible therapeutic targets; indeed, *BMI1* and *EZH2* inhibitors already exist (PTC596 and tazemetostat); *USP22* inhibitors have not been still reported, even if trichostatin A, a histone deacetylase inhibitor, seems to reduce expression of this gene.[39]

A limit of this study is that we did not measure the 3 chosen genes in normal lymph nodes; this is because we used only RNA still available from samples included in our previous study.[22] This might represent an obvious limitation, but our work was principally focused on setting of a novel ddPCR. This method, that we demonstrated to be accurate and sensitive, could be exported from the bench to the bedside for discriminating patients at "very high" risk—at high IPI, no-GCB, with high expression of BCL2 and *BMI1* or *USP22*. For these patients we could imagine a "patient-tailored" therapy through *BMI1*, *EZH2* or *USP22* inhibitors; before that, further in vitro or animal models would be useful for proving our hypothesis.

AUTHOR CONTRIBUTIONS

Lusci Gemignani A., Sacchi S., and Galimberti S. designed the study. Galimberti S., Lusci Gemignani A. and Morganti R. performed the statistical analyses. Papotti R., Bomben R., Gattei V., Bettelli S., Forti E., Bono C., Guerrini F., and Volpe G. performed molecular tests. Donati V., Di Napoli A. analyzed the tissue samples. Pozzi S., Cox M. C., Flenghi L., Rossi P., Dardanis D., Mansueto G. treated patients.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

INFORMED CONSENT

In the informed consent, patients gave the RNA from samples of diagnosis for sustaining further research on DLBCL.

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