Immunohistochemical Algorithms and Gene Expression Profiling in Primary Cutaneous B-cell Lymphoma

Anna Lucia Peluso  
University of Salerno: Universita degli Studi di Salerno

Caterina Picariello  
University of Salerno: Universita degli Studi di Salerno

Pasquale Cretella  
University of Salerno: Universita degli Studi di Salerno

Immacolata Cozzolino  
University of Campania Luigi Vanvitelli: Universita degli Studi della Campania Luigi Vanvitelli

Alessandro Caputo  
Universita degli Studi di Salerno Dipartimento di Medicina e Chirurgia  
https://orcid.org/0000-0001-5139-3869

Massimo Triggiani  
University of Salerno: Universita degli Studi di Salerno

Alessandro Puzziello  
University of Salerno: Universita degli Studi di Salerno

Carmine Selleri  
University of Salerno: Universita degli Studi di Salerno

Stefano Pepe  
University of Salerno: Universita degli Studi di Salerno

Antonio Ieni  
University of Messina: Universita degli Studi di Messina

Carlo Baldi  
University Hospital 'San Giovanni di Dio e Ruggi d'Aragona': Azienda Ospedaliera Universitaria 'San Giovanni di Dio e Ruggi d'Aragona'

Pio Zeppa  
https://orcid.org/0000-0001-7358-5925

Research article

Keywords: Primary cutaneous B-cell lymphoma, nodal diffuse large B-cell lymphoma, Cell of Origin, Gene expression profiling

DOI: https://doi.org/10.21203/rs.3.rs-72812/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

**Objective**: to assess whether immunohistochemical (IHC) algorithms used to classify the cell of origin (COO) of nodal Diffuse Large B-cell lymphoma (nDLBCL) in Germinal Center type (GCB) and non-GCB subtypes may be applied to Primary Cutaneous B-cell lymphoma (PCBCL) too, and which of these algorithms performs better on PCBCL.

**Design**: retrospective case control study.

**Setting**: Pathology Department of the University Hospital “San Giovanni di Dio e Ruggi d’Aragona” Salerno, Italy.

**Participants**: fourteen PCBCL, including Primary Cutaneous follicle centre lymphoma (PCFCL) and primary cutaneous diffuse large B-cell lymphoma, Leg type (PCDLBCL-LT) and 14 nDLBCL were evaluated for 7-year period (January 2011 to December 2017). Primary cutaneous marginal zone cell lymphoma (PCMZL) cases were not included in the present study.

**Intervention**: evaluation of immunohistochemical CD10, BCL6, MUM1/IRF4, BCL2, MYC and Ki-67 expression and classification according to three different algorithms. Gene expression profiling (GEP) was performed on the same series using Lymph2Cx assay (Nanostring). The data obtained were compared and analysed.

**Results**: all the IHC algorithms showed 13 GCB and 15 non-GCB. GEP showed 12 GCB, 12 activated B cell–type and 4 unclassified.

**Conclusions**: the PCBCL were classifiable as GCB and non-GCB like the nDLBCL as IHC algorithms were concordant to GEP and produced the same results.

Summary Box

**What is already known on this topic:**

Primary cutaneous lymphomas (PCBCL) are classified in PCMZL, PCFCL and PCDLBCL-LT. A sub-classification of these entities, like that of nDLBCL, based on the identification of Cell-of-Origin (COO) would be useful for diagnostic and prognostic purposes but no consistent data are available.

**What this study adds:**

Data obtained in our series support the reliability of both GEP and IHC algorithms in the prognostic and predictive evaluation of PCBCL with accuracy comparable to that reported for nDLCL

**Introduction**

Primary cutaneous B-cell lymphoma (PCBCL) is a heterogeneous group of lymphoproliferative disorders, which accounts for 20–25% of all primary cutaneous lymphomas [1]. The World Health Organization (WHO) and the European Organization for Research and Treatment of Cancer (EORTC) classify PCBCL in three main histotypes: 1) primary cutaneous marginal zone B-cell lymphoma (PCMZL); 2) primary cutaneous follicle centre cell lymphoma (PCFCL); 3) primary cutaneous diffuse, large, B-cell lymphoma, leg type (PCDLBCL-LT) [1–3]. Whereas the PCMZL and PCFCL are indolent diseases (with disease-related 10-year survival greater than 90%), diffuse PCFCL and PCDLBCL-LT show an aggressive behaviour with a disease-related 5-year survival of approximately 50%. Despite the difficulties, it is useful to discriminate diffuse PCFCL from PCDLBCL-LT because the latter has a significantly shorter survival, lower response to therapy and requires multiagent chemotherapy and anti-CD20 monoclonal antibodies [4]. Considering nodal diffuse large B-cell lymphoma (nDLBCL) as their possible counterpart, they have been exhaustively investigated by gene expression profiling (GEP) and by immunohistochemistry (IHC) applying different antibodies and algorithms [5–7]. The classification of PCBCL in GCB and non-GCB might have relevant prognostic and predictive implications [8, 9]. Nonetheless, the reproducibility of GEP and IHC classification of PCBCL has not been definitively
assessed [4, 8–10]. Moreover, the available studies on this subject have led to conflicting results [5]. In these studies, the discrimination between PCDLBCL-LT and other PCBCL, particularly PCFCL with diffuse growth pattern and predominance of centroblasts is often investigated [5]. For this purpose, the application of immunohistochemistry (IHC) algorithms used to subclassify nDLBCL in GCB and non-GCB might be useful but has not been validated on PCBCL. For instance, the expression of B-Cell lymphoma-2 (BCL2) antigen and multiple myeloma-1/Interferon regulatory factor-4 (MUM1/IRF4) are common in PCDLBCL-LT, but BCL2 is weakly or not expressed in PCFCL [11], even if about 10–20% of PCFCL are BCL2 positive [12]. In fact, as in the case of cutaneous pseudolymphoma, BCL2 overexpression seems to be associated with chromosomal amplification of BCL2 rather than with the t(14,18)(q32,q21) translocation, which determines BCL2-IGH rearrangement and is common in nDLBCL GCB [12].

The aim of the present study is to assess whether the IHC algorithms utilized to classify the cell of origin (COO) of the nDLBCL may be also applied on PCBCL, and which of these algorithms performs better on PCBCL. For this purpose, a series of diffuse PCFCL and PCDLBCL-LT has been investigated by IHC and their phenotypes have been compared to an additional series of corresponding nDLBCL. The data obtained have been classified using different algorithms and validated by GEP, which is the gold standard for this classification.

**Methods**

**Study group**

Fourteen PCBCL, including PCFCL and PCDLBCL-LT, collected over a 7-year period (January 2011 to December 2017) at the Pathology Department of the University of Salerno (Italy), were retrieved and evaluated together with 14 nDLBCL used as controls. PCMZL cases were not included in the present study.

**Patients**

Cutaneous and lymph nodal biopsies were available for all the samples. All the corresponding patients had received a diagnosis of PCBCL on longstanding single or multiple cutaneous lesions from different sites (Tab. 1).
Table 1

Clinical, histologic, phenotypic and gene expression profiling of 14 PCBCL.

| Patients | 1c   | 2c   | 3c   | 4c   | 5c   | 6c   | 7c   |
|----------|------|------|------|------|------|------|------|
| Sex      | M    | M    | M    | F    | F    | M    | M    |
| Age      | 65   | 54   | 76   | 54   | 56   | 80   | 49   |
| Site     | Trunk | Eyelid | Arm | Leg | Leg | Arm | Trunk |
| Size, mm | 44   | 18   | 23   | 15   | 25   | 10   | 12   |
| Relapse  | Yes (LN cervical) | Yes (Local) | Yes (Local) | Yes (Local) | No | No | No |
| Diagnosis | PCDLBCL-LT | PCFCL | PCFCL | PCDLBCL-LT | PCDLBCL-LT | PCFCL | PCFCL |
| Reactive T-cells | Yes | Yes | Yes | Yes | No | No | Yes |
| Growth pattern | Diffuse | Follicular | Diffuse | Diffuse | Diffuse | Follicular | Follicular |
| Dendritic meshwork | No | Yes | Yes | No | No | No | No |
| Skin ulceration | No | No | No | No | No | Yes | No |
| Necrosis | No | No | Yes | Yes | Yes | Yes | No |
| Nuclear debris | No | No | Yes | Yes | Yes | Yes | No |
| "Starry sky" pattern | No | No | No | No | No | No | No |
| Ki67 positivity (%) | 80% | 20% | 80% | 60% | 90% | 60% | 60% |
| CD2       | -    | -    | -    | -    | -    | -    |
| CD4       | -    | -    | -    | -    | -    | -    |
| CD5       | -    | -    | -    | -    | -    | -    |
| CD8       | -    | -    | -    | -    | -    | -    |
| CD3       | +    | +    | +    | +    | -    | -    | + |
| CD20      | +    | +    | +    | +    | +    | +    | + |
| CD79A     | +    | +    | +    | +    | +    | +    | + |
| PAX5      | -    | -    | -    | -    | -    | -    |
| Alk       | -    | -    | -    | -    | -    |
| CyclinD1  | -    | -    | -    | -    | -    |
| Cytokeratin | -    | -    | -    | -    | -    |
| MYC       | -    | -    | -    | -    | -    |
| CD30      | -    | -    | -    | -    | -    |
| CD68      | -    | -    | -    | -    | -    |
| CD138     | -    | -    | -    | -    | -    |
| Bcl2      | +    | +    | -    | +    | +    | +    |
| Bcl6      | +    | +    | +    | -    | -    | +    | + |
| CD10 | + | + | - | - | + | + | + |
| Mum1/IRF4 | + | - | - | + | + | - | - |
| Hans’ algorithm | Non-GCB | GCB | GCB | Non-GCB | Non-GCB | GCB | GCB |
| Colomo’s algorithm | Non-GCB | GCB | GCB | Non-GCB | Non-GCB | GCB | GCB |
| Muris’ algorithm | Non-GCB | GCB | GCB | Non-GCB | Non-GCB | GCB | GCB |
| t(14;18)-PCR | No | No | No | No | No | No | No |
| GEP | ABC | GCB | GCB | Unclassified | ABC | GCB | GCB |

| Patients | 8c | 9c | 10c | 11c | 12c | 13c | 14c |
|---|---|---|---|---|---|---|---|
| Sex | F | M | F | M | F | F | F |
| Age | 80 | 76 | 45 | 58 | 73 | 40 | 67 |
| Site | Arm | Leg | Head | Trunk | Trunk | Trunk | Arm |
| Size, mm | 30 | 23 | 14 | 28 | 16 | 9 | 21 |
| Relapse | Yes (local) | No | No | Yes (local) | No | No | No |
| Diagnosis | PCFCL | PCDLBCL-LT | PCFCL | PCFCL | PCFCL | PCFCL | PCFCL |
| Reactive T-cells | No | No | Yes | No | No | Yes | No |
| Growth pattern | Diffuse | Diffuse | Mixed | Diffuse | Mixed | Follicular | Mixed |
| Dendritic meshwork | No | No | Yes | Yes | No | No | No |
| Skin ulceration | No | No | No | No | No | No | No |
| Necrosis | Yes | No | No | Yes | No | No | No |
| Nuclear debris | Yes | No | No | Yes | No | No | No |
| “Starry sky” pattern | No | No | No | No | No | No | No |
| Ki67 positivity (%) | 87% | 75% | 30% | 78% | 82% | 65% | 80% |
| CD2 | - | - | - | - | - | - | - |
| CD4 | - | - | - | - | - | - | - |
| CD5 | - | - | - | - | - | - | - |
| CD8 | - | - | - | - | - | - | - |
| CD3 | - | - | + | - | - | + | - |
| CD20 | + | + | + | + | + | + | + |
| CD79A | + | + | + | + | + | + | + |
| PAX5 | - | - | - | - | - | - | - |
| Alk | - | - | - | - | - | - | - |
| CyclinD1 | - | - | - | - | - | - | - |
The pathological history in all the patients was negative for previous lymphoma.

After the histological diagnosis clinical staging was assessed in each of them by flow cytometry of peripheral blood, immunoglobulin gene rearrangements in lesional skin or in peripheral blood, complete and differential blood cell count, routine serum biochemistry analysis with lactate dehydrogenase (LDH), serum protein electrophoresis, liver function tests, computed tomography-fluorodeoxyglucose-positron emission tomography (CT-FDG-PET) scan and bone marrow biopsy. The staging and therapy were assessed according to the European Society for Medical Oncology (ESMO) recommendations for primary cutaneous lymphoma [13]. All the patients, after specific therapies, underwent strict clinical follow-up.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4-µm serial sections of formalin-fixed, paraffin-embedded (FFPE) tissue from each sample using CD20, CD79A, PAX5, CD10, BCL6, MUM1/IRF4, BCL2, CD3, CD68, CD5, CD2, CD4, CD8, CD138, CD30, ALK, CyclinD1, MYC, Cytokeratin and Ki-67.

The sections were stained using the BenchMark XT autostainer (Ventana Medical Systems Inc, Tucson, Arizona) with standard protocols [14-16]. The following pre-diluted monoclonal antibodies were used in all cases: CD20 (1:100; clone L26; cat. N. 760-2531; Ventana); CD79a (1:100; clone SP18; cat. N. 790-4432; Ventana); PAX5 (1:100; clone SP34; cat. N. 790-4464; Ventana); CD10 (1:25; clone SP67; cat. N. 790-4506, Ventana), BCL6 (1:100; clone GI191/A8; cat. N. 790-4464; Ventana); MUM1 (1:100; clone MRQ-43; cat. N. 790-4529; Ventana); BCL2 (1:100; clone 124; cat. N. 790-4464; Ventana); CD3 (1:100; clone 2GV6; cat. N. 790-4341; Ventana); CD68 (1:100; clone KP-1; cat. N. 790-2931; Ventana); CD5 (1:100; clone SP57; cat. N. 790-4506; Ventana); CD2 (1:100; clone MRQ-11; cat. N. 790-4377; Ventana); CD4 (1:100; clone SP57; cat. N. 790-4423; Ventana); CD8 (1:100; clone SP57; cat. N. 790-4460; Ventana); CD138 (1:100; clone B-A38; cat. N. 760-4248; Ventana); CD30 (1:100; clone Ber-H2; cat. N. 790-2926; Ventana); ALK (1:100; clone D5F3; cat. N. 790-4794; Ventana); CyclinD1 (1:100; clone SP4-R; cat. N. 790-4508; Ventana); MYC (1:100; clone Y69; cat. N.
IHC results were independently blindly interpreted by two pathologists (I.C. and P.Z.) to assess intrapersonal and interpersonal reproducibility.

IHC evaluation was performed through the nuclear (PAX5, BCL6, MUM1/IRF4, MYC, CyclinD1) and cytoplasmic membrane (CD10, BCL2, CD5, CD20, CD79a, CD3, CD4, CD8, CD2, CD30, ALK, Cytokeratin) positivity. For this purpose, 10 high-power fields were evaluated for each case and the proportion of cells with positive signals, rather than signal intensity, was quantified and assessed as positive or negative [1,2]. CD20, CD79a, PAX5, CD10, BCL6, MUM1/IRF4, CD3, CD68, CD5, CD2, CD4, CD8, CD138, CD30, ALK, CyclinD1 and Cytokeratin staining were considered positive when 30% or more of the cells showed antigen expression [1,2,17-19]. BCL2 staining was considered positive when expressed in over 50% of tumor cells [1,2,17-19]. MYC staining was considered positive when expressed in over 40% of tumor cells [1,2,17-19] A Ki-67 rate >50% of nuclei was considered a “high proliferation” rate [1,2]. Small or indeterminate cells were not considered in cell counting.

Immunoreactivity was evaluated without any knowledge of the patient’s survival or other clinical data. IHC results were merged with the histological features of PCBCL and nDLBCL.

**Immunohistochemical classification**

Hans’, Colomo’s and Muris’ algorithms, used for nDLBCL groups (GCB and non-GCB), were applied in order to assign the cell of origin (COO) to PCBCL [12,15-17]. In particular, PCBCL and nDLBCL were considered GCB when expressing CD10-/BCL6+/MUM1- or CD10+/BCL6+/MUM1- profiles by using the Hans’ algorithm [17], or MUM1-/CD10±/BCL6+ or MUM1-/CD10+/BCL6+ profiles with the Colomo’s algorithm [18] or BCL2+/CD10±/MUM1- or BCL2-/CD10±/MUM1- with Muris’ algorithm [19]. PCBCL and nDLBCL were considered non-GCB when expressing the CD10-/BCL6+/MUM1+ profiles at Hans’ algorithm, or MUM1+/CD10-/BCL6+ profiles at Colomo’s algorithm or BCL2+/CD10±/MUM1+ profiles at Muris’ algorithm.

**Gene Expression Profiling**

To assign the PCBCL to the GCB or activated B cell (ABC) groups, samples were analyzed with the Lymphoma/Leukemia Molecular Profiling Lymph2Cx assay, a digital gene expression (NanoString) – based test for COO assignment in FFPE tissue (FFPET).

Ten-micrometer scrolls of FFPET were cut with a surface area of ≥ 8 mm² of each case tested by GEP. RNA was extracted using the Qiagen RNeasy FFPET kit (cat. N. 73504, Qiagen, Inc., Valencia, CA, USA), following the manufacturer’s instruction, after treatment with Qiagen Deparanization Solution (cat. N. 19093, Qiagen) and digital GEP was performed on 200 ng RNA using NanoString technology (Seattle, WA) with 23-Gene Signature for COO Classification Lymph2Cx (Nanostring Technologies, Seattle, WA, USA). GEP was performed on Affymetrix U133 plus 2.0 microarrays at Polo Tecnologico Pharmadiagen Srl Laboratory (Pordenone, Italy). The data algorithm nCounter-based Lymph2Cx Assay Misclassification Rate was 2%. GEP analysed cases were blindly classified according to the COO assignment [6,7].

**Gene expression profiling analysis**
The 14 PCBCL and 14 nDLBCL samples were analysed using the RUO version of the GEP NanoString Lymphoma Subtyping Test (LST) algorithm to determine the COO molecular subtype of each sample. The LST algorithm measures the geometric mean of 5 housekeeping genes (HK geomean) to ensure RNA quality based on a pre-defined clinical quality control (QC) threshold of 128. An HK geomean value below 64 is deemed to be insufficient in terms of RNA quality to provide a subtyping result (subtype listed as N/A). A value between 64 and 128 is borderline quality since it meets previously published thresholds for RNA quality within clinical research studies [5,6], but does not meet the clinical QC threshold of 128 for individual patients. Samples that met the QC threshold were marked as “pass” (tab. 3).
| Samples (N.) | IHC Analysis | Hans | Colomo | Muris | GEP |
|------------|--------------|------|--------|-------|-----|
| 1c         | BCL2+/CD10-/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 2c         | BCL2+/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 3c         | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 4c         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | Unclassified |
| 5c         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 6c         | BCL2+/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 7c         | BCL2+/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 8c         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 9c         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 10c        | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 11c        | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 12c        | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 13c        | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 14c        | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 1n         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 2n         | BCL2+/CD10+/BCL6-/MUM1- | GCB | GCB | GCB | GCB |
| 3n         | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | Unclassified |
| 4n         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | Unclassified |
| 5n         | BCL2+/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 6n         | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 7n         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 8n         | BCL2+/CD10+/BCL6-/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 9n         | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 10n        | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 11n        | BCL2+/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | GCB |
| 12n        | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |
| 13n        | BCL2-/CD10+/BCL6+/MUM1- | GCB | GCB | GCB | Unclassified |
| 14n        | BCL2+/CD10+/BCL6+/MUM1+ | non-GCB | non-GCB | non-GCB | ABC |

1c to 14c: primary cutaneous large, B-cell lymphoma cases; 1n to 14n: nodal DLBCL cases; ABC: Activated-B-Cell; GCB: Germinal Center-B-Cell; ABC: Activated-B-Cell; GCB: Germinal Center-B-Cell; IHC: immunohistochemistry; GEP: Gene Expression Profiling.

Each sample meeting the QC threshold is reported as one of the three molecular subtypes GCB, or ABC or Unclassified within the equivocal zone. Subtypes are determined using a Linear Predictor Score (LPS), which is computed by summing the...
products of 15 weighted gene coefficients and the gene expression measurements and applying pre-defined thresholds \([6,7]\).

Subtypes were ABC when LPS was higher or equal to 2433.5 (\(\geq 2433.5\)); GCB when LPS was lower or equal to 1907.8 (\(\leq 1907.8\)) and unclassified when LPS value was in equivocal zone (tab. 3).

(t(14,18)(q32;q21)) Polymerase Chain Reaction

To evaluate the t(14,18)(q32;q21) causing the BCL2-IGH rearrangements, genomic DNA was extracted by 8 sections (5–10 \(\mu\)m thick) using the Qiagen QIAamp DNA FFPE Tissue Kit (Qiagen, cat. n. 56404), following the manufacturer's instructions, after treatment with Qiagen Deparanization Solution (Qiagen, cat. n. 19093). The kit combines the selective binding properties of a silica-based membrane by allowing the genomic DNA purification from FFPE sections without overnight incubation. Once paraffin was dissolved in xylene, samples were lysed under denaturing conditions with proteinase K and incubated at 90°C to reverse formalin cross-linking. The DNA was bound to the membrane and contaminants flowed through by centrifugation at 8000 rpm; DNA was then washed to remove the residual contaminants by centrifugation at 8000 rpm and then eluted from the membrane in 20-100 \(\mu\)l of Buffer ATE by centrifugation at 13200 rpm. In less than 30 minutes, the pure and concentrated DNA was ready for use in amplification reactions or for storage at \(-20°C\).

The PCR analysis of t(14,18)(q32;q21) was carried out to evaluate BCL2-IGH rearrangement, according to Rambaldi A. et al. [20]. Briefly, the first round of amplification was in 50 \(\mu\)l of final volume of 1X GoTaq(R) Green Master Mix (2X; Promega, Madison, WI, USA, cat. n. M7123) using 100 ng of DNA with primers MBR for major breakpoint region, or mcr for minor cluster region and JH consensus region.

Samples were amplified on Veriti® Thermocycler (Applied Biosystems, Carlsbad, California, USA) with an initial denaturation at 95°C for 7 minutes, followed by either 27 cycles of denaturation for MBR or 30 cycles for the mcr. Each cycle was performed with 1 minute of denaturation at 94°C and 1 minute of annealing at 55°C for the MBR and 55°C for mcr amplifications and 1 minute of extension at 72°C. A nested PCR reaction was performed using 5 \(\mu\)l of 1:10 dilution of the first-round amplification product using oligonucleotide primers internal to the original primers, such as MBR nested, mcr nested and JH nested of which nucleotide sequences were described by Rambaldi A. et al. [20]. PCR reactions were performed according the following PCR temperature profile: denaturation for 7 minutes at 94°C, 30 cycles (1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C for each cycle) and final extension at 72°C for 10 minutes. A 25 \(\mu\)l aliquot of PCR product was verified on a 2% agarose gel containing ethidium bromide in Tris-borate electrophoresis buffer and visualized under UV light. Specifically, positive and negative PCR controls were processed, and samples were run in duplicate to ensure PCR reproducibility.

Data comparison

IHC data were compared to the GEP data to validate results and to one or more IHC algorithms to be used as GEP surrogate. Concordance percentage was calculated to determine the reproducibility between the GEP and IHC in PCBCL and nDLBCL. To evaluate the influence of biological, histological, immunohistochemical, or molecular data on the distribution of cases with GEP and IHC, ANOVA analysis was carried out with a significance of 5% and with two tails for independent data. Statistical analysis was performed using Graphpad Prism 8.0®. Different clinical and histological features were evaluated in discordant cases as possible cause of disagreement.

Results

Clinical data

The 14 PCBCL sites of onset were the trunk (5 cases, 36%), arms (4 cases, 29%), legs (3 cases, 21%), eyelid (1 case, 7%) and head (1 case, 7%). nDLBCL were from cervical (5 cases, 36%), inguinal (4 cases, 29%), submandibular (3 cases, 21%) and para-aortic (2 cases, 14%) lymph nodes. The median size of PCBCL was 19.5 mm (range: 9–44 mm). The median diameter of
nDLBCL was 18 mm (range, 8–60 mm). The male: female ratio of PCBCL was 7:7 with median age of 61.5 years (range: 40–80 years); the male: female ratio of nDLBCL was 6:8 with a median age of 64.5 years (range: 43–77 years). The 4 PCDLBCL-LT (29% of the PCBCL cases) concerned 2 females and 2 males, with median age of 60.5 years (range: 54–76 years). Lesions were on the legs (2 females and 1 male) and the trunk (1 male) with a median size of 25 mm (range: 15–44 mm). The 10 PCFCL (71% of the PCBCL cases) were 5 males and 5 females with median age of 62.5 years (range: 40–80 years). Lesions were on the trunk (4 cases), the arms (4 case), the head (1 case) and the eyelid (1 case) with a median size of 17 mm (range: 9–30 mm). Patients follow-up revealed relapse in 2 PCDLBCL-LT (lymph node and local, respectively) and 4 PCFCL (local).

The flow cytometry of peripheral blood, immunoglobulin gene rearrangements in lesional skin or in peripheral blood, complete and differential blood cell count, routine serum biochemistry analysis with LDH, serum protein electrophoresis, liver function tests were negative and CT-FDG-PET scan and bone marrow biopsy did not show systemic diffusion of corresponding PCBCL. As far the staging is concerned, all cases were T3-N0-M0-B0 according to ESMO recommendations [13]. All the patients underwent specific therapies, namely PCFCL with solitary or localized skin lesion received radiotherapy at the dose of 24-30 Gy, PCFCL with multifocal lesions received rituximab and/or steroids, the PCDLBCL-LT received rituximab, cyclophosphamide, doxycycline, vincristine, prednisone (R-CHOP).

All the PCBCL patients were alive at the moment of the study except one (case n. 1c) who died due to disease progression and eventually central nervous system involvement. All the above-reported data are summarized in Tables 1 and 2.

Histological features and classification

Four PCBCL (29%) showed monomorphic, diffuse infiltration of the dermis by large, roundish or irregular, nucleolated cells (monotonous proliferation of centroblasts and immunoblasts), with numerous mitoses. The cells did not show epidermotropism and small lymphocytes were scant or absent (Fig. 1A and Tab. 1); these cases were diagnosed as PCDLBCL-LT. Among these cases, 2 PCDLBCL-LT (14% of PCBCL) showed reactive T cells, whereas in the remaining 2 cases T lymphocytes were few or absent (Tab. 1). Only one (25%) PCDLBCL-LT case showed skin ulceration and 2 (14% of PCBCL) cases showed necrosis and nuclear debris (Tab. 1).

Ten cases (71%) showed follicular, or follicular and diffuse (mixed), or diffuse infiltrate in the dermis of small to large centrocytes with a variable number of centroblasts; these cases were diagnosed as PCFCL (Fig. 2A and Tab. 1). 4 (29% of PCBCL) cases showed follicular growth pattern, 3 (21% of PCBCL) cases diffuse growth pattern and 3 (21% of PCBCL) mixed growth pattern (Fig. 2A and Tab. 1). Five of these cases (36% of PCBCL) showed reactive T cells and 4 (29%) cases showed a dendritic meshwork. Four PCFCL (29% of PCBCL) showed small areas of necrosis with nuclear debris (Tab. 1).

The 14 lymph nodes used as control, had all been diagnosed as nDLBCL with diffuse growth pattern (Figs. 3A and 4A and Tab. 2).
Table 2
Clinical, histologic, phenotypic and gene expression profiling of 14 nDLBCL.

| Patients | 1n | 2n | 3n | 4n | 5n | 6n | 7n |
|----------|----|----|----|----|----|----|----|
| Sex      | F  | F  | F  | M  | F  | M  | M  |
| Age      | 74 | 77 | 64 | 73 | 77 | 72 | 53 |
| Site     | Para-aortic lumbar | Cervical | Para-aortic lumbar | Inguinal | Cervical | Inguinal | Cervical |
| Size, mm | 15 | 33 | 17 | 60 | 20 | 11 | 15 |
| Relapse  | No | No | No | No | No | No | No |
| Diagnosis| DLBCL | DLBCL | DLBCL | DLBCL | DLBCL | DLBCL | DLBCL |
| Reactive T-cells | Yes | No | Yes | Yes | Yes | Yes | No |
| Growth pattern | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse |
| Necrosis | No | Yes | No | Yes | No | No | No |
| Nuclear debris | No | Yes | No | Yes | No | No | No |
| "Starry sky" pattern | No | No | No | Yes | No | No | No |
| Ki67 % positivity | 95% | 90% | 70% | 90% | 90% | 90% | 90% |
| MYC      | -  | -  | -  | -  | -  | -  | -  |
| CD20     | -  | +  | +  | -  | +  | +  | +  |
| CD79A    | +  | +  | +  | +  | +  | +  | +  |
| PAX5     | +  | -  | -  | +  | +  | +  | +  |
| CD138    | -  | -  | -  | -  | -  | -  | -  |
| CD30     | -  | -  | -  | -  | -  | -  | -  |
| CyclinD1 | -  | -  | -  | -  | -  | -  | -  |
| CD5      | -  | -  | -  | -  | -  | -  | -  |
| CD43     | -  | -  | -  | -  | -  | -  | -  |
| Bcl2     | +  | +  | +  | +  | +  | +  | +  |
| Bcl6     | -  | -  | +  | +  | -  | +  | +  |
| CD10     | -  | +  | -  | -  | +  | -  | -  |
| Mum1/IRF4| +  | -  | +  | +  | -  | +  | +  |
| Hans’ algorithm | Non-GCB | GCB | Non-GCB | Non-GCB | GCB | Non-GCB | Non-GCB |
| Colomo’s algorithm | Non-GCB | GCB | Non-GCB | Non-GCB | GCB | Non-GCB | Non-GCB |
| Muris’ algorithm | Non-GCB | GCB | Non-GCB | Non-GCB | GCB | Non-GCB | Non-GCB |
| t(14;18)-PCR | No | Yes | No | Yes | No | No | No | No |
|--------------|----|-----|----|-----|----|----|----|----|
| GEP          | ABC| GCB | Unclassified | Unclassified | GCB | ABC | ABC | ABC |

| Patients | 8n | 9n | 10n | 11n | 12n | 13n | 14n |
|----------|----|----|-----|-----|-----|-----|-----|
| Sex      | F  | F  | F   | F   | M   | M   | M   |
| Age      | 58 | 43 | 48  | 72  | 65  | 59  | 52  |
| Site     | Submandibular | Inguinal | Cervical | Submandibular | Submandibular | Inguinal | Cervical |
| Size, mm | 26 | 9  | 21  | 40  | 10  | 8   | 18  |
| Relapse  | No | No | No  | No  | No  | No  | No  | No  |
| Diagnosis| DLBCL | DLBCL | DLBCL | DLBCL | DLBCL | DLBCL | DLBCL |
| Reactive T-cells | No | No | No  | No  | No  | No  | No  | Yes |
| Growth pattern | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse | Diffuse |
| Necrosis  | No | No | No  | No  | No  | Yes | No  | No  |
| Nuclear debris | No | No | No  | No  | No  | Yes | No  | No  |
| "Starry sky" pattern | No | No | No  | No  | No  | No  | Yes | No  |
| Ki67 % positivity | 95% | 90% | 45% | 90% | 75% | 80% | 30% |
| MYC       | -  | -  | -   | -   | -   | -   | -   |
| CD20      | +  | +  | +   | +   | +   | +   | +   |
| CD79A     | +  | +  | -   | +   | +   | +   | +   |
| PAX5      | +  | +  | +   | +   | +   | -   | +   |
| CD138     | -  | -  | -   | -   | -   | -   | -   |
| CD30      | -  | -  | -   | -   | -   | -   | -   |
| Cyclin1   | -  | -  | -   | -   | -   | -   | -   |
| CD5       | -  | -  | -   | -   | -   | -   | -   |
| CD5       | -  | -  | -   | -   | -   | -   | -   |
| Bcl2      | +  | -  | -   | +   | +   | +   | +   |
| Bcl6      | -  | +  | +   | +   | +   | +   | +   |
| CD10      | -  | +  | +   | +   | -   | +   | -   |
| Mum1/IRF4 | +  | -  | -   | -   | +   | -   | +   |
| Hans' algorithm | Non-GCB | GCB | GCB | GCB | GCB | Non-GCB | GCB | Non-GCB |
| Colomo's algorithm | Non-GCB | GCB | GCB | GCB | GCB | Non-GCB | GCB | Non-GCB |
Six of these (43%) showed reactive T cells intermingled in the tumour, being absent in the remaining 8 (57%) cases (Tab. 2). Necrosis and nuclear debris were observed in 3 out of 14 (21%) nDLBCL and 2 (14%) cases showed also “starry sky” pattern (Tab. 2).

Clinical and histological data of the 14 PCBCL and 14 nDLBCL are summarized in Tables 1 and 2.

### Immunohistochemistry

The immunohistochemical evaluation showed the following results: CD20 positivity was observed in all 14 PCBCL cases and in 12 nDLBCL (86%) being negative in cases 1n and 4n (Tabs. 1 and 2). In all 28 PCBCL and nDLBCL cases CD79A positivity was observed, whereas PAX5 was expressed only by 11 nDLBCL cases, being absent in 2n,3n,13n and in the all 14 PCBCL cases.

CD10 positivity was observed in 13 out of 28 (46%) cases, namely in 7 out of 14 (50%) PCFCL and in 6 out of 14 (43%) nDLBCL (Tabs. 1 and 2, Figs. 2B and 3B). BCL6 positivity was showed in 20 out of 28 (71%) cases: 9 PCFCL (64% of PCBCL), 1 PCDLBCL-LT (7% of PCBCL) and 10 nDLBCL (Tabs. 1 and 2, Figs. 2C, 3C and 4C). MUM1/IRF4 positivity was observed in 15 out of 28 (54%) cases (50% PCBCL and 57% nDLBCL), namely: 4 PCDLBCL-LT (29% of PCBCL), 3 PCFCL (21% of PCBCL) and 8 nDLBCL (Tabs. 1 and 2, Figs 1D and 4D). BCL2 positivity was observed in 21 out of 28 (75%) cases (71% PCBCL and 79% nDLBCL): 4 PCDLBCL-LT (29% of PCBCL), 6 PCFCL (43% of PCBCL) and 11 nDLBCL (Tabs. 1 and 2, Tab. 1 and 2). CD3 positive reactive lymphocytes were observed in 13 out of 28 (46%) cases (50% PCBCL and 43% nDLBCL), namely: 2 PCDLBCL-LT, 5 PCFCL (36% of PCBCL) and 6 nDLBCL (Tabs. 1 and 2). CD2, CD4, CD5, CD8, CD30, CD68, CD138, ALK, MYC, CyclinD1 and Cytokeratin were unexpressed in the neoplastic cells of the all 28 PCBCL and nDLBCL but not in the internal controls (Tabs. 1 and 2).

Ki-67 showed a median value of 76,5% (range: 20-90%) for PCBCL and 80% (range, 30-95%) for nDLBCL (Tabs. 1 and 2). In particular, the Ki-67% median value was 77,5% (range, 60-90%) in PCDLBCL-LT and 71,5% (range, 20-87%) in PCFCL cases. According to the Ki67 rate, 12 PCBCL (86%) and 12 nDLBCL (86%) were defined to have “high proliferative rate” (>50%) namely 4 PCDLBCL-LT and 8 PCFCL (Tabs. 1 and 2). The remaining 2 PCFCL and 2 nDLBCL were classified as “low proliferative rate” (<50%).

### “Cell of Origin” classification by Immunohistochemical Algorithms

The IHC COO classification in both series was performed according to Hans’, Colomo’s and Muris’ algorithms [15,17-19] using CD10, BCL6, MUM1 and BCL2 IHC stains. Applying Hans’ algorithm, 13 out of 28 (46%) samples were classified as GCB [7 out of 14 (50%) PCBCL and 6 out of 14 (43%) nDLBCL] by showing CD10+/BCL6+/MUM1- (6 PCFCL and 4 nDLBCL) and CD10+/BCL6+/MUM1- (2 nDLBCL) profiles; 15 out of 28 (54%) samples were classified as non-GCB [7 out of 14 (50%) PCBCL and 8 out of 14 (57%) nDLBCL] by showing CD10-/BCL6+/MUM1+ (3 PCDLBCL-LT and 2 nDLBCL) profiles (Tabs. 1, 2 and 3). Applying Colomo’s algorithm, 13 out of 28 (46%) samples were classified as GCB [7 out of 14 (50%) PCBCL and 6 out of 14 (43%) nDLBCL] by showing MUM1-/CD10+/BCL6+ (7 PCFCL and 4 nDLBCL) or MUM1-/CD10+/BCL6- (2 nDLBCL) profiles; 15 out of 28 (54%) samples were classified as non-GCB [7 out of 14 (50%) PCBCL and 8 out of 14 (57%) nDLBCL] by showing the MUM1+/CD10-/BCL6- (3
PCDLBCL-LT, 1 PCFCL and 2 nDLBCL) profile or showing the MUM1+/CD10-/BCL6+ (1 PCDLBCL-LT, 2 PCFCL and 6 nDLBCL) (Tabs. 1, 2 and 3). Applying Muris' algorithm, 13 out of 28 (46%) samples were classified as GCB [7 out of 14 (50%) PCBCL and 6 out 14 (43%) nDLBCL] by showing BCL2+/CD10+/MUM1- (3 PCFCL and 3 nDLBCL) or BCL2-/CD10+/MUM1- (4 PCFCL and 3 nDLBCL) profiles; 15 out of 28 (54%) samples were classified as non-GCB [7 out of 14 (50%) PCBCL and 8 out 14 (57%) nDLBCL] by showing the BCL2+/CD10-/MUM1+ (4 PCDLBCL-LT, 3 PCFCL and 8 nDLBCL) profile (Tabs. 1, 2 and 3).

Comparing the three algorithms, 13 out of 28 (46%) samples were GCB (7 PCFCL and 6 nDLBCL), 14 out of 28 (50%) were non-GCB (4 PCDLBCL-LT, 3 PCFCL and 8 nDLBCL). No samples showed discordant results when IHC algorithms were compared to each other (Tabs. 1, 2 and 3). The only case (PCDLBCL-LT, case 5c) with skin ulceration was non-GCB (Tabs. 1, 2 and 3).

**“Cell of Origin” classification by Gene Expression Profiling**

At GEP, all the 28 samples met the QC threshold of 128 and subtypes reported as GCB, ABC or unclassified were provided. The subtype distribution was 12 GCB (43%), 12 ABC (43%) and 4 unclassified (14%) (Tabs. 1, 2, 3 and 4). QC status of each sample and subtype output for all the samples that passed RNA quality are summarized in Table 4.
Table 4
Cell of origin subtype and QC results by GEP.

| Sample ID | LPS  | Subtype   | HK Geomean | QC   |
|-----------|------|-----------|------------|------|
| 1 c       | 3140 | ABC       | 938        | Pass |
| 2 c       | 157  | GCB       | 1198       | Pass |
| 3 c       | 19   | GCB       | 1126       | Pass |
| 4 c       | 2130 | Unclassed | 850        | Pass |
| 5 c       | 2648 | ABC       | 1154       | Pass |
| 6 c       | 314  | GCB       | 2153       | Pass |
| 7 c       | 784  | GCB       | 930        | Pass |
| 8 c       | 2478 | ABC       | 3487       | Pass |
| 9 c       | 3143 | ABC       | 1322       | Pass |
| 10 c      | 1906 | GCB       | 899        | Pass |
| 11 c      | 3254 | ABC       | 2092       | Pass |
| 12 c      | 2578 | ABC       | 1509       | Pass |
| 13 c      | 45   | GCB       | 3216       | Pass |
| 14 c      | 189  | GCB       | 986        | Pass |
| 1 n       | 2924 | ABC       | 1491       | Pass |
| 2 n       | 547  | GCB       | 802        | Pass |
| 3 n       | 2070 | Unclassed | 8425       | Pass |
| 4 n       | 2016 | Unclassed | 823        | Pass |
| 5 n       | 944  | GCB       | 863        | Pass |
| 6 n       | 2418 | ABC       | 1047       | Pass |
| 7 n       | 3340 | ABC       | 1047       | Pass |
| 8 n       | 2864 | ABC       | 863        | Pass |
| 9 n       | 243  | GCB       | 897        | Pass |
| 10 n      | 1452 | GCB       | 1146       | Pass |
| 11 n      | 1402 | GCB       | 1407       | Pass |
| 12 n      | 2507 | ABC       | 2013       | Pass |
| 13 n      | 2238 | Unclassed | 1054       | Pass |
| 14 n      | 3009 | ABC       | 3048       | Pass |

GEP: gene expression profiling; LPS: Linear Predictor Score; HK geomean: housekeeping genes; QC: quality control; ABC: Activated-B-Cell; GCB: Germinal Center-B-Cell; 1c to 14c: primary cutaneous large, B-cell lymphoma cases; 1n to 14n: nodal DLBCL cases. HK geomean was the geometric mean of 5 housekeeping genes; LPS were computed by summing the products of 15 weighted gene coefficients and the gene expression measurements and applying pre-defined thresholds; ABC is sample with LPS $\geq 2433.5$; GCB is sample with LPS $\leq 1907.8$; Unclassed is sample with an equivocal zone; Pass: sample met the pre-defined clinical QC threshold of 128.
In particular, GCB were represented by 7 out of 14 (50%) PCBCL (7 PCFCL) and 5 out of 14 nDLBCL (36%); ABC cases were 6 out of 14 (43%) PCBCL (3 PCDLBCL-LT and 3 PCFCL) and 6 out of 14 nDLBCL (43%); unclassified cases were 1 PCDLBCL-LT and 3 nDLBCL (Tabs. 1, 2, 3 and 4 and Figs. 5 and 6). Among ABC, only one PCDLBCL-LT showed skin ulceration (case 5c); among unclassified only 2 nDLBCL (cases 4n and 13n) showed starry sky pattern (tabs. 1, 2, 3 and 4).

**PCR evaluation of t(14,18)(q32;q21)**

On agarose gel electrophoresis, the BCL2-IGH expected bands were showed by all positive controls and by 6 nDLBCL (43%) (Tab. 2). The reproducible sensitivity level was $10^{-5}$ for MBR and $10^{-4}$ for mcr [20]. Comparing PCR data with IHC, 3 nDLBCL (cases 2n, 4n, 11n) resulted BCL2 positive in both procedures (Tab. 2). The remaining 3 cases showed only PCR positivity (Tab. 2), whereas 8 cases (1n, 3n, 5n, 6n, 7n, 8n, 12n and 14n) showed only IHC BCL2 positivity. With reference to IHC algorithms and GEP, t(14;18) was detected in 5 GCB and 1 non-GCB as defined by IHC' algorithms; and in 4 GCB and 2 unclassified as defined by GEP (Tab. 2).

**Data comparison**

The concordance percentage between IHC algorithms and GEP was calculated. Among PCBCL, concordance was 93% with IHC algorithms and GEP, with identical results in 10 PCFCL (7 GCB and 3 ABC), 3 PCDLBCL-LT (ABC) (Tabs. 1, 2 and 3). Concordance between GEP ABC and IHC non-GCB was 43%, whereas concordance between GEP GCB and IHC GCB was 50%. Moreover, the three IHC algorithms showed a 100% concordance with each other (Tabs. 1, 2 and 3). Among nDLBCL, concordance was 79% with IHC algorithms and GEP, results being identical in 11 nDLBCL (5 GCB and 6 ABC) (Tabs. 1, 2 and 3). Concordance between GEP ABC and IHC non-GCB PCBCL was of 43%, whereas concordance between GEP GCB and IHC GCB was 79%.

ANOVA analysis showed that the different distribution of the PCBCL (p=0.9795) and nDLBCL (p=0.9951) cases among GEP and IHC cases was not influenced by biological, histological, immunological and molecular data. No statistically significant differences were found when ANOVA was carried out for distribution PCBCL in GEP (p=0.7114) and IHC (p=0.8911) groups; similarly when we compared GEP GCB vs IHC GEP (p=0.9999) and GEP ABC vs IHC non-GCB (p=0.4808). Regarding nDLBCL no statistically significant differences were found when ANOVA was carried out only on GEP (p=0.9538) and IHC (p=0.3096) groups and when we compared GEP GCB vs IHC GEP (p=0.5191) and GEP ABC vs IHC non-GCB (p=0.2127).

The 4 out of 28 IHC vs GEP discordant cases were: 1 PCDLBCL-LT (case 4c) that resulted non-GCB at IHC algorithms and unclassified at GEP (Tabs. 1, 2 and 3); 2 nDLBCL (cases 3n and 4n) were non-GCB at IHC and unclassified at GEP, while the other case (13n) was GCB at IHC algorithms and unclassified at GEP (Tabs. 1, 2 and 3). Among discordant cases, 4c PCDLBCL-LT case was a 54-year-old female with a 15 mm lesion on the leg and local relapse, with reactive T cells, diffuse growth pattern, necrosis and nuclear debris, Ki-67 of 60% and IHC BCL2 positivity (Tab. 1). The 2 nDLBCL (cases 3n and 4n) that were non-GCB at IHC algorithms and unclassified at GEP were a female and a male aged 64 and 73 respectively, with 17 mm and 60 mm lesions on para-aortic lumbar and inguinal nodules, with reactive T cells (Tab. 2). The female nDLBCL (case 3n) showed diffuse growth pattern, Ki-67 of 70% and IHC BCL2 positivity, while the male nDLBCL (case 4n) showed diffuse growth pattern, nuclear and necrotic debris and starry sky pattern, Ki-67 of 90%, IHC BCL2 and t(14;18) positivity (Tab. 2). The 13n nDLBCL case GCB at IHC algorithms and unclassified at GEP was a male aged 59 with a 8 mm lesion on inguinal nodules, diffuse growth pattern, nuclear and necrotic debris and starry sky pattern, Ki-67 of 80% and t(14;18) positivity (Tab. 2).

**Discussion**

PCBCL are a rare and heterogeneous sub-group of primary cutaneous lymphoma; the latest WHO classification of lymphoid tumours has classified PCBCL in PCMZL, PCFCL and PCDLBCL-LT [1–3]. PCMZL is a defined entity with specific prognosis and therapeutic indications. The distinction between PCFCL and PCDLBCL-LT may be less defined whereas prognosis and
treatment may be different [13]. Therefore, an accurate sub-classification of these entities, like that of nDLBCL, might be useful. nDLBCL is the largest and most heterogeneous group of non-Hodgkin lymphomas (NHLs); they may arise de novo or from a pre-existing differentiated NHL. Evidence shows that their prognosis and response to therapies are influenced by the corresponding COO; namely, GCB has a better response to therapy and longer disease-free survival than ABC and unclassified cases.

Numerous studies have investigated the COO classification of nDLBCL and its clinical relevance either by IHC or GEP, but little corresponding data on PCBCL are available so far [8, 9, 12, 21–35]. This is probably due to the heterogeneity of PCBCL, their relatively recent classification and their low incidence when compared to nDLBCL. Despite that, a COO sub-classification of PCFCL and PCDLBCL-LT would be desirable for prognostic and predictive evaluations as PCFCL are generally treated with radiotherapy only where PCDLBCL-LT require chemotherapy. A large and comprehensive study on GEP of PCBCL has been performed by Hoefnagel et al. [8]. In this study differences in GEP of PCFCL and PCDLBCL-LT have been detected and considered to be the cutaneous counterpart of nDLBCL GCB and ABC types, respectively. For this purpose, Hoefnagel et al. [8] investigated the whole genome of PCBCL and concluded that the different expression of few genes determines the distinction of PCBCL in 2 subtypes. This distinction was validated by quantitative PCR of seven genes and by the MUM1/IRF4 expression only by IHC. In our study we analysed 14 PCFCL and 14 nDLBCL cases by GEP focusing on a small number of genes (23 Lymphoma/Leukaemia-related and 5 housekeeping genes) of the Lymph2Cx assay of Nanostring Technologies (Seattle, WA, USA) GEP, that are involved in cell cycle apoptosis, B-cell differentiation, B-cell receptor signalling, antigen presentation, chromatin regulation/DNA methylation [6, 7]. IHC and GEP data of two parallel series of PCBCL and nDLBCL were compared to validate IHC data and to analyse the performance of three IHC algorithms frequently used in clinical practice. As previously reported, most PCFCL were CD10+ (70%, 7 out of 10) and BCL6+ (90%, 9 out of 10), but only 6 out of 10 (60%) showed BCL2 positivity. Only 3 PCFCL were MUM1/IRF4+ (80%, 4 out 5) while the all 4 PCDLBCL-LT were MUM1/IRF4+ (100%) and BCL2+ (100%). Therefore, BCL2 expression seems to differentiate PCDLBCL-LT from PCFCL with diffuse growth pattern and predominance of centroblasts, since BCL2 was strongly expressed in PCDLBCL-LT, as already observed elsewhere [39–41]. Moreover, the molecular analysis of t(14;18) confirmed that the BCL2-IGH translocation was negative in PCBCL [23, 42]. Therefore, according to the literature, BCL2 over-expression in PCBCL seems to be associated with BCL2 amplification rather than with a translocation; conversely BCL2 over-expression is rare in PCFCL lacking either amplification or translocation [39]. As for BCL2 expression, in nDLBCL 11 cases (78%) showed BCL2 over-expression at IHC and 6 cases (43%) the t(14;18), with 3 cases showing translocation and over-expression.

The comparison between GEP and IHC data in PCBCL showed concordance in 93% (13 out of 14 cases) with IHC algorithms (Table 3). IHC profiles and GEP were concordant in nDLBCL cohorts at 79% (11 out of 14 cases) with IHC algorithms (Table 3). The percentage of nDLBCL is similar to data provided elsewhere [5, 6, 9, 15, 17–19, 21, 26, 30, 34].

In addition to the GEP, some studies have been conducted to identify PCBCL genetic differences by using whole genome sequencing or comparative genomic hybridization array analysis [29]. Different alterations were found including gains such as 18/18q; 7/7p; 3/3q; 1p; 12/12q; 13/13q or loss such as 5q of different chromosomes. Oncogene gains (SAS/CDK4, MYCL1, MYC, FGFR2, BCL2, CSE1L, PDGFB) or losses (AKT1, IGF1R, JUNB, FGR, ESR, ABL1, TOP2A, ERBB2, CCNE1, BCR) were also detected as demonstrated by Mao et al. by comparative genomic hybridization and microarray-based genomic analysis [29]. Multiple genomic mutations in the NF-kB and B-cell signalling pathways have been characterized in PCDLBCL-LT [26], similarly to MYD88 somatic mutation in PCDLBCL-LT [31]. MicroRNA studies on PCBCL have demonstrated the overexpression of microRNA 17–92 cluster and its paralogs miR-106a-363 in PCDLBCL-LT, that have a causative role in lymphomagenesis, when compared to PCFCL [22]. Conversely miR-9-5p, miR-31-5p, miR-129-2-3p and miR214-3p are overexpressed in PCFCL when compared to PCDLBCL-LT [28]. Nonetheless the same authors assess that microRNAs — reported as overexpressed in the ABC type when compared to the GCB type — in nDLBCL are not differentially expressed between PCFCL and PCDLBCL-LT [28]. Another study has highlighted different profiling of genes of apoptosis and cytotoxic immune response being the first enhanced in PCDLBCL-LT and the second in PCFCL [41]. PCDLBCL-LT and PCFCL may be differentiated for specific chromosomal abnormalities too [24]. According to their study, performed by array-based comparative genomic hybridization, amplification of 2p16.1 and deletion of 14q32.33 chromosomes frequently occur in PCFCL [24]. Conversely amplification of
18q21.31-q21.33 including the BCL-2 and MALT1 genes and deletions of 9p21.3 were specific of PCDLBCL-LT [24]. All these studies produced significant information on PCBCL but do not have clinical implications in terms of prognostic and predictive evaluation.

With reference to the IHC differentiation of the PCFCL and PCDLBCL-LT, Hallerman et al. [25] observed that BCL2, OCT2 and MUM1 positivity was associated with poor prognosis and BCL6 positivity with a better prognosis, whereas the differentiation between PCBCL and PCDLBCL-LT was less straightforward. Similar data were reported by Sundram U et al. [33]: analysing 14 PCBCL, they noted that the patients were divided into two groups: one with BCL6 positivity, MUM1 negativity and overall survival of 176 months; the second with BCL6 negativity, MUM1 positivity and shortest overall survival (26 months). Another study showed that PCFCL and PCDLBCL-LT have a germinal centre B-cell differentiation stage signature when applying gene profiling on 17 PCBCL [9].

Consequently, most PCFCL were GCB when applying the three IHC algorithms, while most PCFCL with diffuse growth pattern and all the PCDLBCL-LT were ABC by GEP. Therefore, PCFCL with the follicular growth pattern might be equivalent to the nDLBCL GCB type and the PCFCL with diffuse growth pattern and PCDLBCL-LT might be referable to the nDLBCL ABC type.

Data obtained in our series support the reliability of IHC algorithms in the prognostic and predictive evaluation of PCBCL with accuracy comparable to that reported for nDLCL. With reference to the different applied algorithms, Hans’ and Muris’ algorithms seem to be the most suitable for PCBCL.

In conclusion, our study confirms that GEP classification of PCBCL reproduces the classification of nDLBCL. A good correlation has been assessed between the GEP classification and the IHC expression of CD10, MUM1/IRF4, BCL6, BCL2 and MYC. These data may be utilized for the prognostic evaluation of PCBCL, but further validation on larger series is required.

**Declarations**

**Contributor and guarantor information:**

P.Z, I.C., A.P, S.P., M.T., C.S., A. I. and A.L.P. conceived and designed the study. A.L.P., P.C, C.P, A.C. and P.Z. equally contributed in writing the text of the article. P.C, A.C, and C.B. collected patient clinical and histological data. P.C and A.C provided photographic material and performed statistical analysis. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

**Copyright/license for publication:**

the Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, a worldwide licence to the Publishers and its licensees in perpetuity, in all forms, formats and media (whether known now or created in the future), to i) publish, reproduce, distribute, display and store the Contribution, ii) translate the Contribution into other languages, create adaptations, reprints, include within collections and create summaries, extracts and/or, abstracts of the Contribution, iii) create any other derivative work(s) based on the Contribution, iv) to exploit all subsidiary rights in the Contribution, v) the inclusion of electronic links from the Contribution to third party material where-ever it may be located; and, vi) licence any third party to do any or all of the above.

**Patient consent:**

written informed consent to perform this study was obtained from all the participants.

**Competing interests declaration:**
all authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Research involving Human Participants and/or Animals:

The study was approved by the IRCCS Pascale Ethical Committee on the basis of the Institute's ethical regulations for research on human tissues. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Transparency statement:

the lead author (the manuscript's guarantor), P.Z. affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned (and, if relevant, registered) have been explained.

Funding:

This work received funding from the Italian Ministry for Education, University and Research (MIUR – Ministero dell’Istruzione, Università e Ricerca), “Fondo per gli Investimenti della Ricerca di Base (FIRB)”.

Patients and public involvement:

Patients and public have been not involved in no one of the various phases of this research.

References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Arber DA, Hasserjian RP, Le Beau MM, Orazi A and Siebert R. “WHO Classification of tumours of haematopoietic and lymphoid tissues”. WHO Press. IARC. 2017;280-284;291-297;303-304.
2. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, Advani R, Ghielmini M, Salles GA, Zelenetz AD and Jaffe ES. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016;127(20):2375-2390.
3. La Selva R, Alberti Violett S, Delfino C, Grandi V, Cicchelli S, Tomasini C, Fierro MT, Berti E, Pimpinelli N, and Quaglino P. A Literature Revision in Primary Cutaneous B-cell Lymphoma. Indian J Dermatol. 2017;62(2):146–157.
4. Sokołowska-Wojdyłło M, Olek-Hrab K, Ruckemann-Dziurdzińska K. Primary cutaneous lymphomas: diagnosis and treatment. Postep Derm Alergol. 2015;32(5):368–383.
5. Gutierrez-Garcia G, Cardesa-Salzmann T, Climent F, Gonzalez-Barca E, Mercadal S, Mate JL, Sancho JM, Arenillas L, Serrano S, Escoda L, Martinez S, Valera A, Martinez A, Jares P, Pinyol M, Garcia-Herrera A, Martinez-Trillos A, Gine’E, Villamor N, Campo E, Colomo L, and Lopez-Guillermo A. Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. Blood. 2011;117(18):4836-4843.
6. Scott DW, Wright GW, Williams PM, Lih CJ, Walsh W, Jaffe ES, Rosenwald A, Campo E, Chan WC, Connors JM, Smeland EB, Mottok A, Braziel RM, Ott G, Delabie J, Tubbs RR, Cook JR, Weisenburger DD, Greiner TC, Glinsmann-Gibson BJ, Fu K,
Staudt LM, Gascoyne RD, and Rimsza LM. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. Blood. 2014;123(8):1214-1217.

7. Yoon N, Ahn S, Yoo HY, Kim SJ, Kim WS, Ko YH. Cell-of-origin of diffuse large B-cell lymphomas determined by the Lymph2Cx assay: better prognostic indicator than Hans algorithm. Oncotarget. 2017;8(13):22014-22022.

8. Hoefnagel JJ, Dijkman R, Basso K, Jansen PM, Hallermann C, Willemze R, Tensen CP, Vermeer MH. Distinct types of primary cutaneous large B-cell lymphoma identified by gene expression profiling. Blood. 2005;105(9):3671-3678.

9. Storz MN, van de Rijn M, Kim YH, Mraz-Gernhard S, Hoppe RT, Kohler S. Gene expression profiles of cutaneous B cell lymphoma. J Invest Dermatol. 2003;120(5):865-870.

10. Subramaniyam S, Magro CM, Gogineni S, Tam W. and Mathew S. Primary Cutaneous Follicle Center Lymphoma Associated With an Extracutaneous Dissemination A Cytogenetic Finding of Potential Prognostic Value. Am J Clin Pathol. 2015;144;805-810.

11. Haverkos B, Tyler K, Gru AA, Winardi FK, Frederickson J, Hastings J, Elkins C, Zhang X, Xu-Welliver M, Wong HK, Porcu P. Primary Cutaneous B-Cell Lymphoma: Management and Patterns of Recurrence at the Multimodality Cutaneous Lymphoma Clinic of The Ohio State University. Oncologist. 2015;20(10):1161-1166.

12. Lima M. Cutaneous primary B-cell lymphomas: from diagnosis to treatment. An Bras Dermatol. 2015;90(5):687-706.

13. Willemze R, Hodak E, Zinzani PL, Spech L, Ladetto M. Primary cutaneous Lymphomas: ESMO Clinical Practice Guidelines. Ann. Oncol. 2018;29(Suppl. 4): iv30-iv40

14. Zeppa P, Cozzolino I. Fine-Needle Cytology: Technical Procedures and Ancillary Techniques. Monogr Clin Cytol. 2018;23:4-13.

15. Cozzolino I, Varone V, Picardi M, Baldi C, Memoli D, Ciancia G, Selleri C, De Rosa G, Vetranì A and Zeppa P. CD10, BCL6, and MUM1 Expression in Diffuse Large B-Cell Lymphoma on FNA Samples. Cancer (Cancer Cytopathol). 2016;124:135-143.

16. Vigliar E, Cozzolino I, Picardi M, Peluso AL, Fernandez LV, Vetranì A, Botti G, Pane F, Selleri C, Zeppa P. Lymph node fine needle cytology in the staging and follow-up of cutaneous lymphomas. BMC Cancer. 2014;14:18.

17. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Müller-Hermelink HK, Campo E, Brazel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO, Chan WC. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103(1):275-282.

18. Colomo L, Lopez-Guillermo A, Perales M, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. Blood. 2003;101(1):78-84.

19. Muris JJ, Meijer CJ, Vos W, van Krieken JH, Jiwa NM, Ossenkoppele GJ, Oudejans JJ. Immunohistochemical profiling based on Bcl-2, CD10 and MUM1 expression improves risk stratification in patients with primary nodal diffuse large B cell lymphoma. J Pathol. 2006;208(5):714-723.

20. Rambaldi A, Lazzari M, Manzoni C, Carlotti E, Arcaini L, Baccarani M, et al. Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. Blood. 2002;99:856–862.

21. Batlle-López A, González de Villambrosia S, Mazorra F, Malatexberria S, Sáez A, Montalban C, Sánchez L, Garcia JF, González-Barca E, López-Hernández A, Ruiz-Marcellan MC, Mollejo M, Grande C, Richards KL, Hsi ED, Tzankov A, Visco C, Xu-Monette ZY, Cao X, Young KH, Piiris MA, Conde E, Montes-Moreno S. Stratifying diffuse large B-cell lymphoma patients treated with chemoimmunotherapy: GCB/non-GCB by immunohistochemistry is still a robust and feasible marker. Oncotarget. 2016;7,(14):18036 – 18049.

22. Battistella M, Romero M, Castro-Vega LJ, Gapihan G, Bouhidel F, Bagot M, Feugeas JP, Janin A. The High Expression of the microRNA 17-92 Cluster and its Paralogs, and the Downregulation of the Target Gene PTEN, Is Associated with Primary Cutaneous B-Cell Lymphoma Progression. J Invest Dermatol. 2015;135(6):1659-1667.

23. Berthold Streubel; Brigitte Scheucher; Julia Valencak; Daniela Huber; Peter Petzelbauer; Franz Trautinger; Felix Weihsgenruber; Christine Mannhalter; Lorenzo Cerroni; Andreas Chott et al.. Molecular Cytogenetic Evidence of t(14;18)
24. Dijkman R(1), Tensen CP, Jordanova ES, Knijnenburg J, Hoefnagel JJ, Mulder AA, Rosenberg C, Raap AK, Willemze R, Szuhaí K, Vermeer MH. Array-based comparative genomic hybridization analysis reveals recurrent chromosomal alterations and prognostic parameters in primary cutaneous large B-cell lymphoma. J Clin Oncol. 2006;24(2):296-305.

25. Hallermann C, Niermann C, Fischer RJ, Schulze HJ. New prognostic relevant factors in primary cutaneous diffuse large B-cell lymphomas. J Am Acad Dermatol. 2007;56(4):588-597.

26. Hallermann C, Niermann C, Fischer RJ, Schulze HJ. New prognostic relevant factors in primary cutaneous diffuse large B-cell lymphomas. J Am Acad Dermatol. 2007;56(4):588-597.

27. Jabłonska J, Jesionek-Kupnicka D, Potemski P, Kowalik A, Sygut J, Kordek R. Comparison of two different immunohistochemical algorithms identifying prognostic subgroups of DLBCL. Pol J Pathol. 2010;61(3):124-132.

28. Kim EJ, Lewis DJ, Duvic M. Novel Mutations Involving NF-κB and B-Cell Signaling Pathways in Primary Cutaneous Large B-Cell Lymphoma, Leg-Type and Comparison with Sézary Syndrome. J Invest Dermatol. 2017;137(9):1831-1833.

29. Koens L, Qin Y, Leung WY, Corver WE, Jansen PM, Willemze R, Vermeer MH, Tensen CP. MicroRNA profiling of primary cutaneous large B-cell lymphomas. PLoS One. 2013;8(12):e82471.

30. Mao X, Lillington D, Child F, Russell-Jones R, Young B, Whittaker S. Comparative genomic hybridization analysis of primary cutaneous B-cell lymphomas: identification of common genomic alterations in disease pathogenesis. Genes Chromosomes Cancer. 2002;35(3):144-155.

31. Meyer PN, Fu K, Greiner TC, Smith LM, Delabie J, Gascoyne RD, Ott G, Rosenwald A, Braziel RM, Campo E, Vose JM, Lenz G, Staadt LM, Chan WC, Weisenburger DD. Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. J Clin Oncol. 2011;29(2):200-207.

32. Pham-Ledard A, Cappellen D, Martinez F, Vergier B, Beylot-Barry M, Merljo JP. MYD88 somatic mutation is a genetic feature of primary cutaneous diffuse large B-cell lymphoma, leg type. J Invest Dermatol. 2012;132(8):2118-2120.

33. Zhao S, Dong X, Shen W, Ye Z & Xiang R. Machine learning-based classification of diffuse large B-cell lymphoma patients by eight gene expression profiles. Cancer Medicine. 2016;5(5):837–852.

34. Bita Fakhri and Brad Kahl. The 2016 WHO Classification of Lymphoid Malignancies: An Overview of the Major Changes From the 2008 Classification. ASCO Annual Meeting, ASCO Daily News. 2007.

35. Lucioni M, Berti E, Arcaini L, Croci GA, Maffi A, Klersy C, Goteri G, Tomasini C, Quaglino P, Riboni R, Arra M, Dallera E, Grandi V, Alaibac M, Ramponi A, Rattotti S, Cabras AG, Franceschetti S, Fraternali-Orcioni G, Zerbinati N, Onida F, Ascani S, Fierro MT, Rupoli S, Gambacorta M, Zinzani PL, Pimpinelli N, Santucci M and Pauli M. Primary cutaneous B-cell lymphoma other than marginal zone: clinicopathologic analysis of 161 cases: Comparison with current classification and definition of prognostic markers. Cancer Medicine. 2016;5(10):2740-2755.

36. Paulli M, Lucioni M, Maffi A, Croci GA, Nicola M, Berti E. Primary cutaneous diffuse large B-cell lymphoma (PCLLBC), leg-type and other: an update on morphology and treatment. G Ital Dermatol Venereol. 2012;147(6):589-602.

37. Akyurek N, Uner A, Benekli M and Barista I. Prognostic Significance of MYC, BCL2, and BCL6 Rearrangements in Patients With Diffuse Large B-Cell Lymphoma Treated With Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone Plus Rituximab. Cancer. 2012;118:4173-4183.

38. Szczepanowski M, Lange J, Kohler CW, Masque-Soler N, Zimmermann M, Aukema SM, Altenbuchinger M, Rehberg T, Mahn F, Siebert R, Spang R, Burkhardt B, Klapper W. Cell-of-origin classification by gene expression and MYC-rearrangements in diffuse large B-cell lymphomas of children and adolescents. Br J Haematol. 2017;179(1):116-119.
41. van Galen JC, Hoefnagel JJ, Vermeer MH, Willemze R, Dijkman R, Tensen CP, de Boer WP, Meijer CJ, Oudejans JJ. Profiling of apoptosis genes identifies distinct types of primary cutaneous large B cell lymphoma. J Pathol. 2008;215(3):340-346.

42. Fiona J Child, R. Russell-Jones, Search for more papers by this author J. Woolford, Search for more papers by this author E. Calonje, Search for more papers by this author A. Photiou, Search for more papers by this author G. Orchard, Search for more papers by this author S.J. Whittaker. Absence of the t(14;18) chromosomal translocation in primary cutaneous B-cell lymphoma. British Journal Dermatology. 2001;144(4):735-744.

**Figures**

**Figure 1**

Immunohistochemistry of a cutaneous diffuse large B-cell lymphoma, leg-type; A: A polymorphous population of large, irregular, nucleolated lymphoid cells infiltrating the papillary and reticular dermis (Haematoxylin-Eosin); B: CD10 negativity; C: BCL6 nuclear negativity; D: MUM1/IRF4 nuclear positivity; E: BCL2 cytoplasmic positivity.

**Figure 2**

Immunohistochemistry of a cutaneous follicle centre lymphoma; A: A polymorphous population of centrocytes and centroblasts occupying the papillary and reticular dermis (Haematoxylin-Eosin); B: CD10 diffuse, cytoplasmic positivity; C:
BCL6 diffuse nuclear positivity; D: MUM1/IRF4 negativity; E: BCL2 negativity.

Figure 3
Immunohistochemistry of a nodal diffuse large B-cell lymphoma, Germinal Center B-cell type (GCB). A: A polymorphous population of large lymphomatous cells (Haematoxylin-Eosin); B: CD10 diffuse, cytoplasmic positivity; C: BCL6 diffuse nuclear positivity; D: MUM1/IRF4 negativity with occasional positive cells; E: BCL2 negativity with positivity in the residual uninvolved lymph node.

Figure 4
Immunohistochemistry of a nodal diffuse large B-cell lymphoma, non-Germinal Center B-cell type (non-GCB). A: A polymorphous population of large cells with irregular nuclei, dispersed cromatin and evident nucleoli (Haematoxylin-Eosin); B: CD10 negativity; C: BCL6 diffuse nuclear positivity; D: MUM1/IRF4 diffuse nuclear positivity; E: BCL2 negativity with occasional positive cells.