## Clinical relevance of silent red blood cell autoantibodies

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*doi:10.3324/haematol.2017.177675*

**SUPPLEMENTAL MATERIAL**

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## PATIENTS AND METHODS

### Patient assessment

Data on the initial clinical, hematologic and serologic evaluation at baseline were recorded and included: complete blood count, immunohematologic characteristics of the RBC autoantibodies, reticulocyte count, haptoglobin, bilirubin, LDH, serum electrophoresis and clinical examination.

Examinations made to exclude or identify associated disorders were also considered, and included: 1) The autoantibody profile: antinuclear (ANA), anti-cardiolipin (aCL) IgG/IgM, lupus anticoagulant (LAC), anti-β2glycoprotein1 (aβ2GPI) IgG/IgM, anti-thyroid peroxidase antibody (TPO) and thyroglobulin antibody (TGAb). 2) B and C hepatitis serology (HBsAg; HBeAg; HBsAb; HBcAb; HBeAb, HCVAb.; 3) Total body CT scan or an abdomen ultrasonography combined with a chest X-ray. 4) Bone marrow (BM) biopsies or aspirates with flow cytometry analysis to detect clonal B-lymphocytes. Radiologic exams and BM investigations were not performed in pregnant females. BM biopsy was not performed in patients with a known cancer diagnosis.

Patients were followed on a regular basis over time at our Hematologic unit. Data on blood counts, bilirubin levels and immunohematologic tests carried out during the follow-up were also recorded. In the absence of clinical and laboratory signs of hemolysis, blood counts and bilirubin levels were monitored every 4-6 months.

### Immunohematologic assessment

Direct antiglobulin test (DAT) was performed with a broad-spectrum antiserum and with monospecific anti-IgG, -IgA, -IgM, -C3d and -C3b antisera, in liquid phase and by column agglutination (reagents from Ortho Clinical Diagnostics, Raritan, New Jersey, USA and Diamed, Cressier sur Morat, Switzerland). Eluate testing was performed by Rubin's method and with low pH glycine buffer using a commercial kit (ELU-KIT™ II, Immucor, Norcross, Georgia, USA). Eluates incubated with O RBCs screening panel, untreated and treated (ficin/papain) were also checked for antibodies not revealed by DAT. An indirect antiglobulin test (IAT) with untreated and treated (ficin/papain) homologous red blood cells (Resolve C - Ortho Clinical Diagnostics and ID-Diamed Panel- DiaMed) was also performed. After the first positive test, RBC autoantibodies were checked again 6 months later, and thereafter, less frequently, about every 12-24 months. No further tests were done after two consecutive negative tests.

**Statistical analysis**

Statistical comparisons for categorical variables, were performed using two-way tables for the Fisher's exact test. Overall survival (OS) was calculated from the date of the first detection of the RBC autoantibodies to the date of death or the last follow-up. Survival analyses were performed using the Kaplan-Meier method and differences in terms of OS were evaluated by means of the Log-Rank test.

**Ethics**

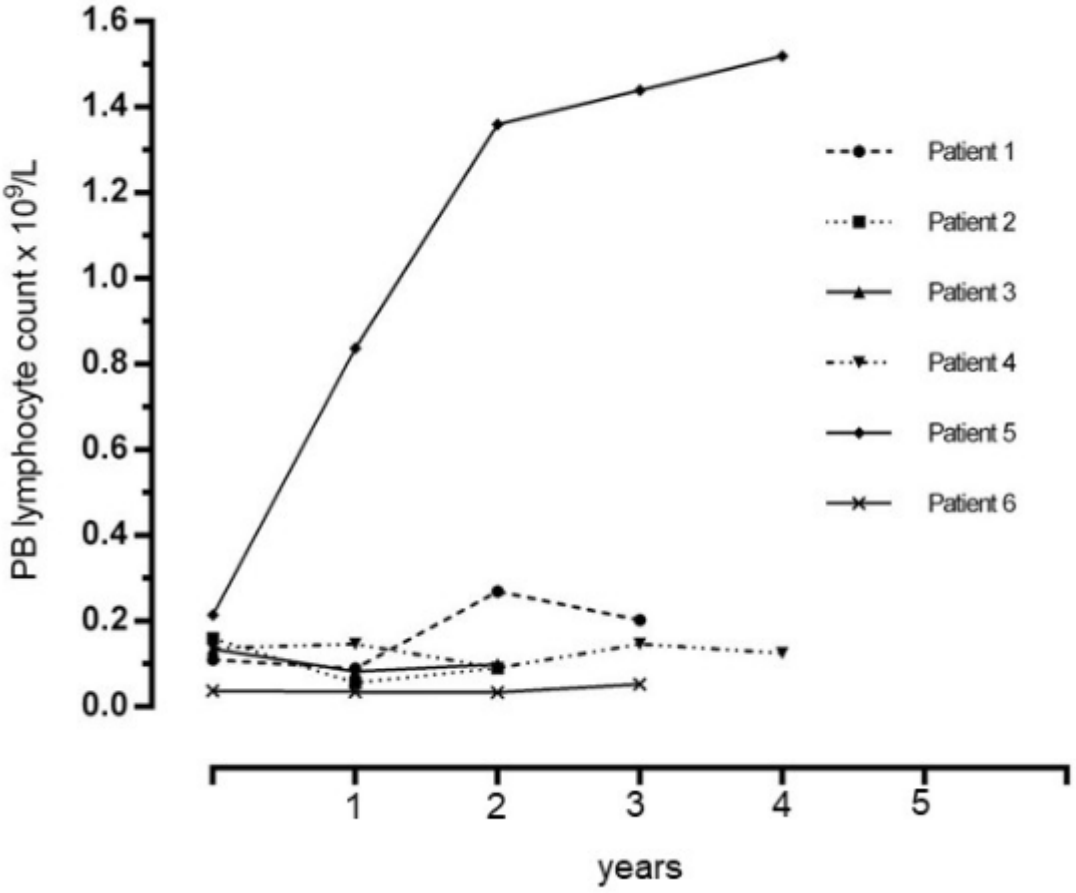
This observational retrospective study was approved by the Institutional Review Board, by the Ethics Committee (OssAEA0l study; protocol no. 533/12; ref.#2501/14.06.2012) and performed in accordance with the Declaration of Helsinki.

**Supplemental Table 1. Clinical characteristics of lymphoproliferative disorders (LDs).**

| | N (%) |
|---|---|
| No of patients with LPD | 7/50 (14) |
| Marginal NHL[(1)] | 1/7(14) |
| MBL | 6/7(86) |
| CLL-like, CD20+/CD5+/CD23+ | 3 |
| NHL like, CD20+/CD5- | 3 |
| Median MBL count x $10/^{9}$ L (range) | 0.135 (0.56-0.215) |

Abbreviations: LD, Lymphoproliferative disorder; NHL, Non Hodgkin Lymphoma; CLL, chronic lymphocytic leukaemia; MBL, Monoclonal B-cell lymphocytosis
[1)] Bone marrow infiltration associated with abdominal enlarged lymph-nodes revealed by CT scan and peripheral blood monoclonal B-cell lymphocytosis.

**Supplemental Figure 1. Monoclonal B-lymphocytes (MBLs) count during the follow-up.**



**Supplemental Figure 2. Survival of subjects with silent red blood cell autoantibodies: IgG versus IgM autoantibodies.**