Primary cold agglutinin-associated lymphoproliferative disease: a B-cell lymphoma of the bone marrow distinct from lymphoplasmacytic lymphoma

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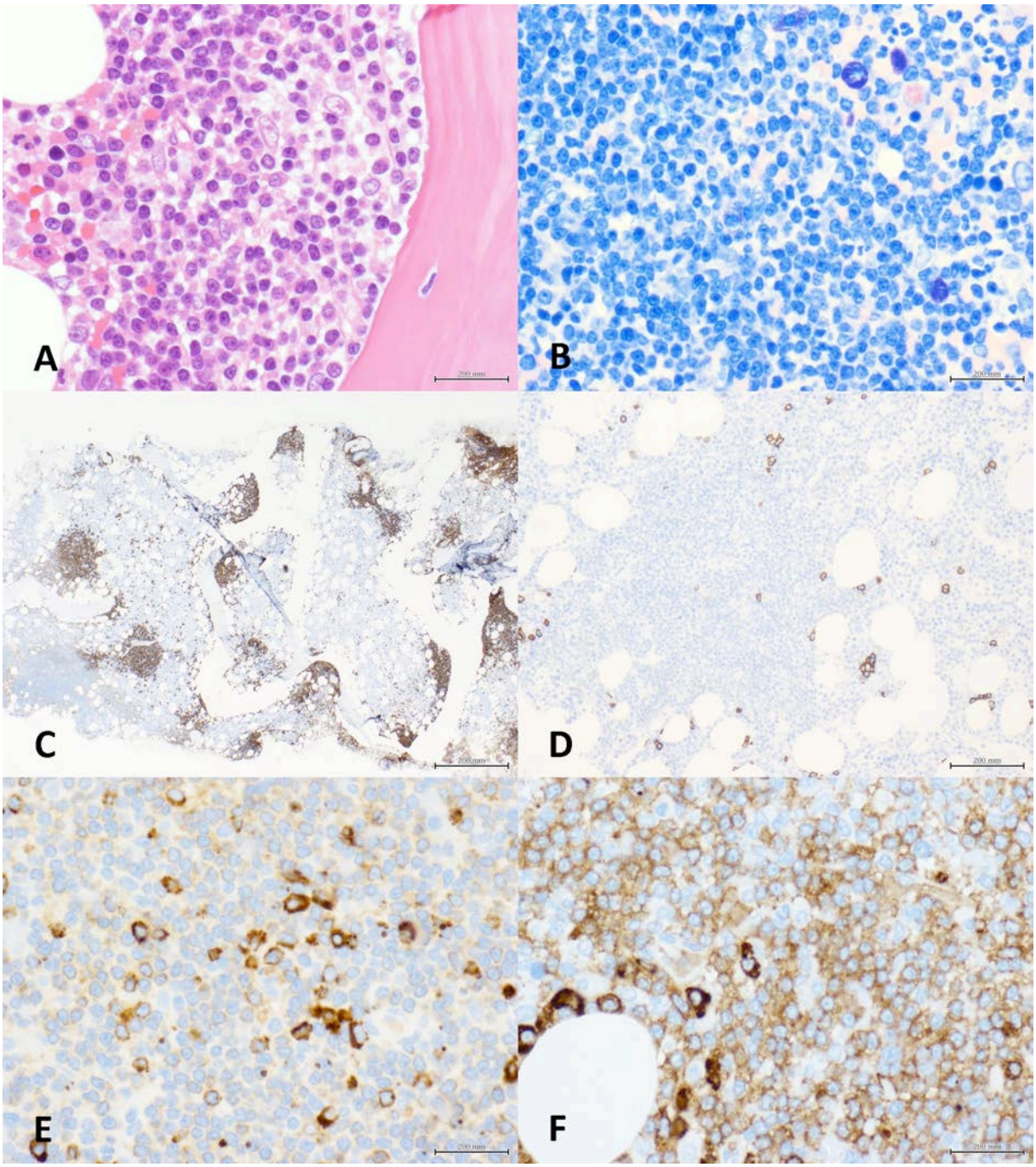
Supplementary table 1. Hemoglobin level and number of bone marrow lymphoid nodules

Patient	Hb-level	Number of lymphoid nodules
1	8,7	10
2	6,1	5
3	15,6	1
5	7,9	8
7	9,6	4
8	9,2	6
9	4,5	1
10	13,1	7
11	9	10
12	8,6	12
13	7,7	3
14	11,9	4
15	8,3	8
16	7,4	4
18	9,4	1
19	10,4	5
20	8,2	5
22	9,8	7
26	7	4
28	8,8	4
30	7,4	16
31	10,2	4
34	8,9	2
36	8,6	13
53	8,4	12

Supplementary Figure 1



Legend to Supplementary figure 1

The figure illustrates histological and immunophenotypic findings in lymphoplasmacytic lymphoma. Lymphoma cells have a predilection for the paratrabecular area (panel A, H&E staining, 400X). Typically mast cells are seen admixed with lymphoma cells or surrounding the lymphoma infiltrations (panel B, Giemsa staining, 400X). The paratrabecular infiltration of lymphoplasmacytic lymphoma is highlighted by staining for CD20 (panel C, anti-CD20 immunoperoxidase staining, 20X). A variable number of lymphoma cells, usually the more plasmacytic differentiated ones, express CD138 (panel D, anti-CD138 immunoperoxidase staining, 10X). In addition to the plasmacytic cells, lymphoplasmacytoid cells of lymphoplasmacytic lymphoma express intracytoplasmic monotypic immunoglobulin rather than membranous immunoglobulin (panels E and F, immunoperoxidase staining for IgK and IgM, respectively, 400X).

Supplementary methods

Immunohistochemistry:

The antibodies used were: anti-CD45, anti-CD20, anti-BCL6, anti-MUM1, anti-IgA, anti-IgD, anti-IgG, anti-IgM, anti-Ki67, anti-BCL10 (all from Dako, Glostrup, Denmark); anti-CD10, anti-BCL2, anti-CD21, anti-CD23, anti-CD5, anti-CD43, anti-cyclin D1 (all from Novocastra Laboratories, Newcastle upon Tyne, U.K.); anti-PAX5, anti-CD27 (BD Biosciences, San José, CA); anti-BLIMP1 (Affinity BioReagents, Golden, CO) and anti-CD3 (Thermo Fisher Scientific, Fremont, CA). For all antibodies, heat-induced epitope retrieval was performed in a microwave oven by heating the slides for 5 min at 750W and subsequently for 15 minutes at 500W in retrieval buffer. The Envision detection system (Dako) was used. The color reaction was developed with 3,3'-diaminobenzidine and H_2O_2 and the slides were counterstained with haematoxylin. The immunohistochemical staining was performed in a Dako Autostainer (Dako) according to the instructions of the manufacturer.

Flow cytometry:

The four-color analysis was performed with the following antibody combinations labeled with fluorescein isothyocyanate (FITC)/ Phycoerithrin (Pe)/ peridininchlorophyll cyanine 5.5 (PercPCY5.5)/allophycocyanine (APC): (1) CD20/CD5/CD19/CD43; (2) FMC7/CD23/CD19/CD5; (3) Igκ/Igλ/CD20/CD19; (4) CD22/CD24/CD19/CD34; (5) cyBCL2/CD10/CD19/CD38. From 2011, an eight-color flow cytometry analysis was used with the following antibody combinations labeled with Pacific Blue/ e450 (PB/e450), Krome Orange (KO), FITC/ Pe / PercPCy5.5/ Phycoerithrin cyanine 7(PeCy7)/APC/ APC Hilite7 or APC/cyanine7 (APCH7/cy7): (1) CD20+CD4/CD45/CD8+Igλ/CD56+ Igκ/CD5/CD19+TCRγδ/CD38; (2) CD20/CD45/CD23/CD10/CD79b/CD19/CD200/CD43. All antibodies for the four-color panels were purchased from Becton-Dickinson (San José, CA, USA) except anti-FMC7, anti-CD22, anti-CD23 and anti-BCL2, which were purchased from Dako. For the eight-color panels, anti-CD56, anti-CD5, anti-CD3 and anti-CD79b were purchased from Becton-Dickinson; anti-CD23 from Dako; anti-CD200 from eBioscience (San Diego, CA); anti-CD8, anti-Igκ and anti-Igλ from Cytognos (Salamanca, Spain) and the remaining of the antibodies from Beckman Coulter (Brea, CA). Flow cytometry analysis was performed on a FACSCalibur or LSRII instrument (Becton-Dickinson), using CellQuest Pro and FACSDiva software (Becton-Dickinson), respectively.

Immunoglobulin heavy chain gene sequencing, BCL6 and MYD88 mutation analyses:

The immunoglobulin heavy chain variable regions were amplified using the IgH Somatic Hypermutation Assay kit according to the instructions of the manufacturer (InVivoScribe Technologies, France) and products were separated using the ABI 3100 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). PCR products were sequenced from both ends with the Big Dye Terminator v 1.1 sequencing kit (Applied Biosystems) using the ABI 3100 Genetic Analyzer (Applied Biosystems). All sequences proved to be *IGHV4-34* sequences. However a number of sequences were of suboptimal quality for detailed analysis due to background immunoglobulin sequences resulting from the consensus PCR technique. Therefore, some of the samples were re-amplified using *IGHV4-34* specific primers (5'-CAG GTG CAG CTA CAG CAG T-3' or 5'-CAG GTG CAG CTA CAA CAG T-3') and AmpliTaq Gold polymerase (Applied Biosystems) and sequencing was repeated. The final nucleotide sequences obtained were analyzed using BLAST (www.ncbi.nlm.nih.gov) and IMGT/V-QVEST (www.imgt.org) databases.

BCL6 intron 1 analysis:

The primer pairs were: 5'- CCG CCG CTG CTC ATG ATC ATT ATT T-3' and 5'- ACC AAA ACA ACA CAA GGG AGG GTG G-3'; 5'-GGC CGG TTT GGG GAG GCT TTT-3' and 5'-GAG CGG GCA GCC TCC CTT TT-3'; 5'-CCC TTC CCC TGT CCT TCT GGG T-3' and 5'-GGC TCT CTT CCA TCG GCC TCG-3', respectively. PCR amplification using AmpliTaq Gold polymerase (Applied Biosystems) consisted of an initial denaturation step at 95°C for 7 min., followed by 35 cycles at 95°C for 30 sec, 58°C or 60°C for 30 sec and 72°C for 45 sec and a final extension step at 72 °C for 8 min.

MYD88 L265P mutation analysis:

PCR was carried out using Phusion hot start DNA polymerase (Thermo Fisher Scientific) according to the supplier's instructions with the following PCR primers: 5'-TGC AGG TGC CCA TCA GAA GCG-3' and 5'-CAG ACA GTG ATG AAC CTC AGG ATG C-3'. A single nucleotide extension reaction was subsequently performed, according to the instructions of the manufacturer (Applied Biosystems). The extension primers were as follows: 5'-CCC CCC CCC CAG GTG CCC ATC AGA AGC GAC-3' and 5'-CCT TGT ACT TGA TGG GGA TC-3'. PCR products were fractionated by capillary electrophoresis using a 3100 Genetic Analyzer and GeneMapper v.4.1 Software (Applied Biosystems). The sensitivity of the

MYD88 L265P mutation analysis was 3%, as determined by testing a dilution series of DNA extracted from the ABC DLBCL cell line OCILy10, with a known MYD88 L265P mutation, in DNA from normal donor blood white cells.