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

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**Running title:** \textit{CAD-associated lymphoproliferative disease}

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Abstract

Primary chronic cold agglutinin disease is a rare hemolytic disease mediated by monoclonal *IGHV4-34*-encoded cold agglutinins with a predominant specificity for the blood group antigen I. The bone marrow of 54 patients was studied to better type the underlying lymphoproliferative disorder. Bone marrow biopsies showed circumscribed intraparenchymatous nodules with small monotonous monoclonal B-cells in 40/54 patients (median infiltration: 10% of marrow cells) with a CD20+, IgM*, IgD*, CD27+, CD5/-, CD11c-, CD23-, CD38- immunophenotype. Neither plasmacytoid cytological features nor expression of plasma cell differentiation-associated transcription factors MUM1, XBP1 and BLIMP1 were noted in these B-cells. However, a limited number of mature monoclonal IgM+, IgD- plasma cells were present outside the lymphoid nodules and were diffusely scattered throughout the marrow. Of interest, the MYD88 L265P mutation, typical of lymphoplasmacytic lymphoma, was not detected (17/17 cases). Somatically mutated monoclonal *IGHV4-34* gene rearrangement was demonstrated in eight patients with frozen samples (mean sequence homology 95.4%). However, mutations of *BCL6* intron 1 were not demonstrated, except in one patient, suggesting that the lymphoma cells have not matured in the germinal center.

In conclusion, cold agglutinin-associated lymphoproliferative disease displays homogeneous histological and immunophenotypic features. The absence of plasmacytoid cells, the presence of plasma cells predominantly outside the nodular lymphoid infiltrates, *IGHV4-34* restriction and absence of MYD88 L265P mutation strongly suggests that cold agglutinin-associated lymphoproliferative disease is a distinct entity that is different from lymphoplasmacytic lymphoma.
Introduction

Primary chronic cold agglutinin disease (CAD) accounts for about 15% of all cases of autoimmune hemolytic anemia.\textsuperscript{1-5} The incidence has been estimated to 1/10\textsuperscript{6} pr. year.\textsuperscript{6,7} Anemia results from binding of monoclonal cold agglutinins, most often IgM with κ light chains, to the I antigen on the erythrocyte surface. Bound immunoglobulins cause red blood cell agglutination and complement activation, leading to phagocytosis of complement-coated red blood cells by the reticulo-endothelial system.\textsuperscript{4,8} About 50% of patients become transfusion dependent. The diagnosis of CAD requires a cold agglutinin titer of \( \geq 64 \), a positive polyspecific as well as a C3d complement protein monospecific direct antiglobulin test.\textsuperscript{5} The agglutinin titer varies greatly among patients with values as low as 64 to over 500 000.\textsuperscript{9} However, the thermal amplitude, defined as the highest temperature at which the antibody binds to red blood cells, is more directly associated with clinical hemolysis than the titer is.\textsuperscript{2,9} Also, the ratio of IgM antibodies that occur as pentamers or hexamers, the latter of which activate complement more easily, determines the severity of the anemia.\textsuperscript{10,11} The immunoglobulin heavy chain of anti-I/i agglutinins is typically encoded by the \textit{IGHV4-34} gene segment. The latter is required for binding to I/i antigen on red blood cells.\textsuperscript{12} More specifically, the \textit{IGHV4-34} framework region 1 (FR1) is mainly responsible for I/i-antigen binding.\textsuperscript{13}

CAD has previously been associated with underlying B-cell lymphoproliferative disease in up to 75% of the patients with lymphoplasmacytic lymphoma being the most common diagnosis.\textsuperscript{6} The demonstration of underlying B-cell lymphoproliferative disease provided the rationale for treatment with rituximab, either as monotherapy or, with better response, in combination with fludarabine therapy.\textsuperscript{14,15}

We reviewed morphological and immunophenotypic findings in bone marrow biopsies and aspirates of 54 patients with CAD to critically reappraise the underlying lymphoproliferative disease. To further characterize the cell of origin we analyzed somatic hypermutations of the rearranged immunoglobulin heavy chain gene as well as the \textit{BCL6} gene. Additionally, we screened for the MYD88 L265P mutation, known to be highly associated with lymphoplasmacytic lymphoma.\textsuperscript{16,17}
Methods

1. Patients

Fifty-four patients with clinically well-documented primary CAD diagnosed in the time period between 1995 and 2012 were studied. There were 36 women and 18 men with an age range of 40-92 (median 73) years. All patients had a clinical history of CAD with a variable degree of anemia, a positive C3d-specific direct antiglobulin test and a cold agglutinin titer in excess of 64. Monoclonal IgM had been detected in serum of all patients by agarose electrophoresis and immunofixation. None of the patients had lymphadenopathy or splenomegaly. Clinical follow-up ranged from 3 to 152 months, with a median follow-up of 72 months.

The study was approved by the institutional and regional ethical committees.

2. Biopsy materials

Archival hematoxylin and eosin-stained sections of bone marrow trephine biopsies from the 54 patients, obtained at diagnosis, were reviewed. Fourteen biopsies were fixed in 4% formaldehyde, 18 in Bplus fixative and 22 in B5 fixative. In addition, part of the diagnostic trephine biopsy of eight patients had been snap-frozen in liquid nitrogen. Two of the patients had undergone splenectomy in an attempt to reduce hemolysis. Hematoxylin and eosin-stained archival sections of formalin-fixed splenic tissue of these patients were reviewed.

3. Immunohistochemistry

Immunohistochemical analysis was extended or repeated whenever archival sections were not available or of bad quality. The primary antibodies and the method used for immunohistochemical analysis of the bone marrow trephine biopsies are described in the supplementary methods.

4. Flow cytometry

Flow cytometry was performed on samples of 25 patients, including a total of 46 bone marrow and 10 paired peripheral blood samples, anticoagulated with heparin and EDTA, respectively. On samples analyzed before 2011, a four-color analysis and from 2011 on, an
eight-color analysis\textsuperscript{19} was performed with antibody combinations as described in the supplementary methods.

5. Immunoglobulin heavy chain gene sequencing, BCL6 and MYD88 mutation analyses

DNA was extracted from frozen bone marrow trephine samples, obtained from eight patients, using the EZ1 tissue kit (Qiagen, Hilden, Germany) and from formaldehyde-fixed paraffin embedded-tissue sections from another 9 patients using the QIAamp DNA FFPE tissue kit (Qiagen). Immunoglobulin heavy chain gene analysis and \textit{BCL6} intron 1 mutation analysis was only performed on DNA from frozen tissue samples whereas \textit{MYD88} mutation analysis, requiring less intact DNA, was performed on frozen and formaldehyde-fixed tissue samples.

\textit{BCL6} intron 1 was amplified using three sets of overlapping PCR primers covering nucleotides 24 to 790 in GenBank sequence AF191831. The primer pairs and PCR conditions are described in the supplementary methods. PCR products were sequenced and analyzed using the BLAST database.

The MYD88 L265P mutation (NM\textunderscore 002468) was analyzed using PCR and a SNaPshot multiplex kit (Applied Biosystems). PCR primers and conditions are described in the supplementary method section. A series of 47 fixed bone marrow trephine biopsies of patients with well-documented lymphoplasmacytic lymphoma were used for control purposes.
Results

Patient follow-up

Eighteen patients are alive as of December 31 2012, without relevant co-morbidity, whereas 12 patients are dead. Twenty four patients were eventually treated at other hospitals and no clinical follow-up information is available. None of the patients were diagnosed with extra-medullary lymphoma or developed extra-medullary lymphoma in the course of their disease. Of the 12 patients who died, 3 died with malignant disease (melanoma, ovarian cancer and acute lymphoblastic leukemia, respectively), one patient died with sepsis whereas eight patients died of unknown cause.

Bone marrow pathology

Lymphoid infiltration consisting of nodular B cell aggregates was seen in the marrow of 40 patients. In contrast, 14 patients showed only few and scattered B cells. In the former patients, lymphoid infiltration varied between 5% and 80% of the intertrabecular surface, with a median of 10%. Infiltration was nodular (Fig. 1A-1D) and consisted of small-sized cells with round to slightly oval nuclei without discernable chromatin pattern (Fig. 1E and 1F). The cells showed a small amount of clear cytoplasm. A few scattered histiocytes were admixed with the lymphoid cells. Mature plasma cells were seen surrounding the lymphoid aggregates but were invariably also seen throughout the marrow between the nodular lymphoid aggregates (Fig. 1G and 1H). Of interest, only few plasma cells were seen within the nodular lymphoid aggregates (Fig. 1G). Plasma cells were not abundant and represented less than 5% (range 2-10) of nucleated cells in the marrow. Of note, features associated with lymphoplasmacytic lymphoma such as paratrabeicular growth, fibrosis, lymphoplasmacytoid cell morphology or an increased number of mast cells surrounding the lymphoid aggregates were not seen (Suppl. Fig. 1).

To investigate whether a correlation existed between the number of lymphoid aggregates in the bone marrow and hemoglobin concentration, Spearman rank order correlation coefficient ($r_s$) was calculated. The $r_s$ was -0.24, indicating only a very weak negative correlation between the extent of marrow lymphoid infiltration and hemoglobin concentration (Suppl. Table 1).
Spleen histology

Review of the records revealed only a partial description of the splenectomy specimens obtained in two patients. The spleen of one patient measured 13 x 11 x 7 cm, but no weight was recorded. The spleen of the second patient weighed 540 grams without a further description of the specimen in the record. Upon review, the histology showed a similar morphology. The red pulp was histologically unremarkable. Scattered plasma cells were noted, but these were not exceptionally numerous. Of note, no abnormal lymphoid infiltration was noted (Fig 2A). The white pulp was hypoplastic and showed periarteriolar sheets and marginal zones with small lymphoid cells (Fig 2A and 2B). Lymphoid follicles were absent.

Immunophenotypic analysis

The lymphoid cells within the lymphoid aggregates in the bone marrow trephine biopsies were mainly B cells with few scattered T cells in-between (Fig 3A and 3B). These lymphoid aggregates were surrounded by IgM- and IgK-expressing plasma cells (Fig 3C-E) that were also observed scattered throughout the marrow. B cells expressed nuclear CD20, PAX5, BCL10 (weakly), but not BCL6, XBP1, MUM1 and BLIMP1 (Fig 3F-3H). B cells showed the following extended immunophenotype: CD19+, CD20 bright+, CD79b bright+, CD22+, FMC7+, IgM dim+, immunoglobulin kappa light chain + (90% of cases), immunoglobulin lambda light chain (10% of cases), CD5+ (11/26 cases), CD27+, CD43 dim+, CD200+, CD10-, CD11c-, CD21-, CD23-, CD25-, cyclin D1-. Only a few cases were CD11c dim+ and CD25+. Of interest, all four of the 14 patients without abnormal lymphoid infiltration on histological examination showed monoclonal B cells by flow cytometry of the bone marrow. In the ten patients of whom flow cytometry of blood was also performed, monoclonal B cells with an identical immunophenotype as seen in the bone marrow were detected.

Plasma cells seen in the red pulp of the spleen in two of the patients expressed predominantly, but not exclusively, IgM and IgK (Fig 2C and Fig 2D). Marginal zone cells in the white pulp showed no obvious restricted immunoglobulin light chain expression (Fig 2C and Fig 2D). Infiltrating T-cells were mainly CD4 positive (Fig 3I and Fig 3J).
**Immunoglobulin heavy chain gene analysis**

Full length rearranged *IgH* variable region analysis was only possible using snap-frozen bone marrow biopsies obtained before treatment in eight patients since these samples contained non-degraded DNA. Monoclonal *IGHV4-34* gene rearrangement was demonstrated in samples of all eight patients. Sequence analysis revealed 92.5 to 98.3% homology to the closest germ line *IGHV4-34-1* sequence (Table 1, GenBank sequence numbers KC581946 to KC581953). Nonpolar hydrophobic amino acid residue in positions 7 (tryptophan), 23 (alanine) and 24 (valine) as well as the polar hydrophilic amino acid in position 25 (tyrosine) of FR1 were conserved and not affected by somatic hypermutation. IgH CDR3 region length varied between 13 and 29 amino-acids (median 15). Two of the patients showed an identical CDR3 amino-acid sequence, which was confirmed by repeated DNA extraction and sequence analysis. The CDR3 regions were characterized by several nonpolar amino acids such as proline, glycine and isoleucine and polar amino acids such as serine (Table 1). Few positively charged amino acids were seen, as reported previously.\(^{12,20}\)

**BCL6 and MYD88 mutation analysis**

*BCL6* gene intron 1 mutation analysis was performed in the eight cases with frozen tissue samples. One of eight cases showed three somatic mutations of the *BCL6* gene (Table 1, GenBank sequence number KC581954). Mutations were not detected in the other seven cases.

The MYD88 L265P mutation analysis was performed on DNA from the eight cases with frozen as well as nine cases with only formalin-fixed paraffin embedded trephine biopsies that showed more than 5% lymphoid infiltration. None of the samples showed the MYD88 L265P mutation. By contrast, MYD88 L265P mutation was demonstrated in fixed and paraffin-embedded bone marrow trephine biopsies in 45 out of 47 cases with lymphoplasmacytic lymphoma.

**Discussion**

The 54 patients in the study fulfilled the clinical criteria for primary CAD. Patients did not have lymphadenopathy, splenomegaly or any other clinical signs of lymphoma at the time of diagnosis. Notwithstanding, 40 out of the 54 patients showed abnormal lymphoid infiltration in the bone marrow that consisted of a variable number of well-circumscribed
nodules of small lymphoid cells with a small amount of clear cytoplasm. These cells expressed CD19, CD20, CD22, CD79b, FMC7, mostly immunoglobulin kappa light chain, IgM, IgD, CD27 and only occasionally CD11c and CD25 but not CD23. Expression of CD5 was seen in less than half of the cases. These histological and immunophenotypic findings are reminiscent of those seen in bone marrow infiltration by extranodal or nodal marginal zone lymphoma. However, none of the patients show evidence of extramedullary disease, excluding the diagnosis of marginal zone lymphoma. Intrasinusoidal infiltration, frequently present in splenic marginal zone lymphoma, was not seen in patients with CAD. In addition, scattered monotypic IgM-expressing plasma cells were diffusely spread in the bone marrow parenchyma and were slightly more numerous in the area surrounding the nodular lymphoid aggregates, findings not typical of marginal zone lymphoma. The presence of plasma cells is reminiscent of lymphoplasmacytic lymphoma and explains why previous studies reported a high incidence of this lymphoma in CAD. However, the histological and immunophenotypic as well as genetic features of CAD differ from those associated with lymphoplasmacytic lymphoma (Table 2). Lymphoplasmacytic lymphoma may show paratrabecular infiltration and may show intrasinusoidal infiltration, features not seen in any of the CAD-associated lymphoid lesions. Importantly, cells with plasma cell differentiation or with cytoplasmic immunoglobulin, so-called lymphoplasmacytoid cells, are typically admixed with the lymphoid proliferation in lymphoplasmacytic lymphoma (Suppl. fig 1). This is not seen in CAD. Instead, bone marrow in CAD is characterized by intraparenchymatous small lymphoid cell aggregates and scattered diffuse infiltration with mature plasma cells that are typically not admixed with the former. Accordingly, the small lymphoid cells in CAD do not express transcription factors that are important for plasma cell differentiation such as MUM1 (IRF4), XBP1 or BLIMP1. By contrast, these transcription factors and surface markers are, although variably, expressed in lymphoplasmacytoid cells of lymphoplasmacytic lymphoma. Furthermore, the marker CD27 is strongly positive in CAD-associated lymphoproliferative disease but has been variably reported to be positive in lymphoplasmacytic lymphoma. By contrast, CD25 is mostly positive in lymphoplasmacytic lymphoma complicated by Waldenström disease, while this marker is only variably expressed in CAD. Lymphoplasmacytic lymphoma mostly uses IGVH3 family genes and shows >5% somatic hypermutation, whereas the mutation rate in CAD-associated lymphoproliferative disease is lower. Importantly, the MYD88 L265P mutation, present
in over 90% of lymphoplasmacytic lymphoma, has not been demonstrated in any of the CAD samples that were tested in our series.16,17

Furthermore, the bone marrow disease in CAD patients is not typical of that seen in other small cell variants of B-cell non-Hodgkin lymphomas such as follicular lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma because cytological and immunophenotypic characteristics typical of these lymphomas are lacking.22 Follicular lymphoma shows most often paratrabecular infiltration with cleaved cells that express BCL6 and CD10, features not present in CAD-associated lymphoproliferative disease. Chronic lymphocytic leukemia and mantle cell lymphoma may show nodular as well as diffuse infiltration with small round cells. However, larger paraimmunoblasts are variably seen in the former and cells with slightly cleaved nuclei in the latter, features not seen in CAD patients. Furthermore, chronic lymphocytic leukemia typically co-expresses CD5 and CD23 and weakly CD20 whereas mantle cell lymphoma co-expresses CD5 and cyclin D1, findings not demonstrated in CAD-associated lymphoproliferative disease. In conclusion, CAD-associated lymphoproliferative disease does not have features of hitherto well-characterized B-cell non-Hodgkin lymphoma types as recognized by the WHO classification of tumors of lymphoid tissues.22

Splenic histology, available in two patients with CAD, showed rather limited changes. The white pulp was hypoplastic and showed small B-cells without plasma cell differentiation. B-cells in the white pulp did not show immunoglobulin light chain restriction. Few scattered monotypic IgM-expressing plasma cells were seen in the red pulp. Apart from the latter, the splenic red pulp did not show other evidence of aberrant lymphoid cell infiltration. Thus, the histological findings in CAD differ from those found in splenic involvement with known B-cell non-Hodgkin lymphoma types that typically show involvement and expansion of the white pulp, or less often of the red pulp. Of interest, monotypic plasma cells were rather few in the spleen indicating that the bone marrow is the predominant source of monoclonal IgM production in CAD.

The extent of bone marrow infiltration does not correlate well with the severity of anemia as indicated by a non-significant Spearman rank correlation coefficient ($r_s = -0.24$). This is not surprising since the thermal amplitude of the secreted IgM has previously been shown to be the most important factor determining severity of anemia.29 In addition, the amount of hexameric versus pentameric IgM produced by CAD-associated lymphoproliferative disease might also determine severity of anemia.10 Hexameric IgM lacks
J chain and is highly complement activating and results therefore in increased red blood cell lysis. Of interest, no abnormal bone marrow infiltration with B cells was detected in the biopsy of 14/54 patients, despite a clinically established diagnosis of CAD and a monoclonal B cell population proven by flow cytometry in the four patients analyzed. Sampling error or minimal bone marrow disease with secretion of highly lytic antibodies might explain this finding.

Waldenström macroglobulinemia is a clinical entity defined by lymphoplasmacytic lymphoma in the bone marrow and monoclonal IgM in serum. Therefore, many CAD patients have previously been classified as having Waldenström macroglobulinemia. Based on the findings presented in the current study, patients with CAD should not be included with true Waldenström macroglobulinemia. A minority of CAD patients show no obvious bone marrow lymphoproliferative disease. We suggest that the latter patients be diagnosed clinically as having an IgM-related disorder. The latter is defined as a clinical condition mediated by monoclonal IgM without evidence of lymphoma. However, patients without obvious bone marrow disease probably represent one end of the spectrum seen in CAD rather than having a distinct disease. This is supported by our previous findings that the extent of bone marrow involvement does not correlate with response to B-cell directed therapy.

Monoclonal IGHV4-34 gene rearrangement is a well-described finding in CAD and is also detected in our patients. The framework region 1 (FR1) of the IGHV4-34 gene segment determines anti-I/i specificity. Especially, amino acid residues 7, 23-25 determine this specificity and are conserved in CAD despite that most rearranged immunoglobulin genes show somatic hypermutation. The latter has also been shown in our cases. In addition to FR1, crystallography has indicated that the exposed C-terminal flanking region but not the antigen-binding pocket site of CDR3 is also important for binding to I/i-antigen. The antigen-binding pocket site of the antibody may therefore bind to another additional antigen. The latter may be heterogeneous in CAD as indicated by variable CDR3 regions without obvious sequence motifs, except for the absence of positively charged amino acids, as shown in our study and in other studies. Of interest, two patients in our series show identical and exceptionally long CDR3 regions. The significance of this is not clear. These CDR3 sequences have not been shown in earlier series and it remains therefore unclear whether this particular sequence is of more general importance for clonal selection by a specific antigen in CAD.
The intermediate level of IgH gene mutation associated with the low level or absence of BCL6 mutation is similar to what has been reported for marginal zone B cell lymphoma.\textsuperscript{36} The absence of mutations in BCL6, a gene highly expressed in germinal center B cells and hence also affected by somatic hypermutation in those cells, is an argument in favor of an origin of the clonal B cells in CAD from B cells that have acquired somatic hypermutation without passing through the germinal center.\textsuperscript{37} Of interest, normal IGHV4-34 expressing B cells home with preference to the splenic marginal zone and are not normally recruited to the germinal center, except in auto-immune disease such as lupus erythematosus.\textsuperscript{38} An origin from marginal zone B cells is further supported by the CD27+, IgM+ and IgD+ immunophenotype of CAD-associated lymphoproliferative disease. Since secondary lymphoid tissue involvement is not a feature of CAD-associated lymphoproliferative disorder, an origin from immature or transitional B-cell precursors of IGHV4-34-expressing marginal zone B cells in the bone marrow might be postulated. Such an origin might not be in contradiction with presence of immunoglobulin somatic hypermutation in CAD-associated lymphoproliferative disease. Somatic mutations in immature or transitional B-cells may be part of a pre-immune diversification of B cells as indicated by data from patients with hyper-IgM syndrome who lack germinal centers.\textsuperscript{39,40} Alternatively, CAD-associated lymphoproliferative disease may arise from IGHV4-34-expressing circulating marginal zone B cells that, for unknown reasons, have homed to the bone marrow.

In conclusion, by studying the bone marrow of a large series of patients with primary CAD, we have provided arguments for an underlying primary bone marrow B-cell lymphoproliferative disease with unique pathologic and genetic features that distinguish the disease from well-characterized B-cell lymphoma types. Of interest, trisomy of chromosome 3q11-q29 is a common feature of the four cases hitherto described\textsuperscript{41,42}, although all cases show additional unique changes. These recurrent genetic changes support the notion that CAD-associated lymphoproliferative disease is a distinct entity. A more extensive genetic analysis is needed to clarify the molecular basis of the disease.
Authorship and Disclosures

Contributions
U.R. and J.D. designed the study. U.R., J.D. and K.B. examined the bone marrow biopsy samples, U.R. and A.T. did the flow cytometry analysis and G.T., A.W. and C.S. performed the molecular analyses. G.E.T. and S.B. did clinical research and collected data. U.R and J.D analyzed the data and wrote the paper. All authors discussed the findings, reviewed the manuscript critically and approved the submitted version.

Disclosure of Conflicts of Interest
The authors declare no conflicts of interest.
References


4. Issitt PD. I blood group system and its relationship to disease.


Table 1. Characteristics of the rearranged immunoglobulin heavy chain genes and \textit{BCL6} mutational status.

<table>
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<tr>
<th>Patient number</th>
<th>\textit{IGVH}4-34 homology (%)</th>
<th>\textit{J}</th>
<th>\textit{D}</th>
<th>CDR3 length</th>
<th>CDR3 amino acid sequences</th>
<th>\textit{BCL6} intron 1 mutations</th>
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<tr>
<td>12</td>
<td>98.3</td>
<td>\textit{J6-02}</td>
<td>\textit{D6-13}</td>
<td>29</td>
<td>AR GCPPP PISAAD TFVESLSCGGL YYGMDV</td>
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<tr>
<td>16</td>
<td>94.1</td>
<td>\textit{J4-02}</td>
<td>\textit{D6-19}</td>
<td>15</td>
<td>AR PFGKD SSGWY VP Y</td>
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<tr>
<td>19</td>
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<td>\textit{D3-03}</td>
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<td>AR GSSG FSRG L FDH</td>
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<td>24</td>
<td>98.1</td>
<td>\textit{J3-01}</td>
<td>\textit{D3-03}</td>
<td>19</td>
<td>AR YPPA ITIFGVV I DAFDV 122 G/T 423 C/G 741 T/A</td>
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<td>92.5</td>
<td>\textit{J5-01}</td>
<td>\textit{D2-21}</td>
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\textit{–}: No mutations detected
Table 2. Comparative characteristics of CAD-associated lymphoproliferative disease, lymphoplasmacytic lymphoma and bone marrow infiltration by marginal zone lymphoma

<table>
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<th>CAD-associated lymphoproliferative disease</th>
<th>Lymphoplasmacytic lymphoma</th>
<th>Secondary infiltration by marginal zone lymphoma</th>
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<tbody>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>architecture</td>
<td>intraparenchymal nodules</td>
<td>varying degree of interstitial, intraparenchymal nodular, paratrabeular and intra sinusoidal infiltration</td>
<td>Intraparenchymal nodules and/or intra sinusoidal infiltration</td>
</tr>
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<td>cytological features</td>
<td>small lymphoid cells with rounded nuclei, occasional larger cells; limited diffuse marrow infiltration with plasma cells that typically are not admixed with the lymphoid cells</td>
<td>mixed infiltrate with small lymphocytes, plasmacytoid cells with occasional Dutcher inclusions and plasma cells</td>
<td>small to medium sized lymphoid cells with more abundant, pale cytoplasm; mostly only few admixed plasma cells</td>
</tr>
<tr>
<td><strong>Immunophenotype</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>lymphoid cells</td>
<td>CD20+, IgKs+ (predominantly), IgMs+, IgDs+, CD27+, CD5-/+</td>
<td>CD20+, IgMs+, IgDs+, CD27+, CD5-/+</td>
<td>CD20+, IgMs+, IgDs+/-, CD5-</td>
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<tr>
<td>plasmacytoid cells</td>
<td>-</td>
<td>CD20+, CD138-, IgMc+, IgKc+(mostly)</td>
<td>-</td>
</tr>
<tr>
<td>plasma cells</td>
<td>CD20-, CD138+, IgMc+, IgKc+(predominantly)</td>
<td>CD20+, CD138-, CD38+, IgMc+, IgKc+(predominantly)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Genetics</strong></td>
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<td></td>
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</tr>
<tr>
<td>IGVH gene usage</td>
<td>IGVH4-34 (100%)</td>
<td>IGVH3 (77%) with IGVH3-23 (35%) and IGVH3-7 (29%)</td>
<td>IGVH1-2 (splenic), IGVH3-4 (nodal), variable according to site (extranodal)</td>
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<td>IG somatic mutation rate</td>
<td>medium (5%)</td>
<td>high (&gt;5%)</td>
<td>variable according to type and site (5-10%)</td>
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<tr>
<td>MYD88 L265P mutation</td>
<td>absent</td>
<td>present (&gt;90% of cases)</td>
<td>present (~10% of cases)</td>
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<td>post-germinal center B cell</td>
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<td><strong>Histological transformation to large cell lymphoma</strong></td>
<td>no</td>
<td>yes (5-13%)</td>
<td>yes (30%)</td>
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s: surface; c: cytoplasmic
Figure Legends

Figure 1

The figure illustrates the bone marrow histology in CAD-associated lymphoproliferative disease. A bone marrow trephine biopsy showing intraparenchymatous nodular lymphoid lesions (panels A and B, H&E-staining, 40X and 200X, respectively). Immunoperoxidase staining for CD20 highlights intraparenchymatous nodular B-cell infiltration (panel C, 200X). Mast cells are not usually discerned around the nodular lymphoid lesions (panel D, Giemsa-staining, 200X). High magnification shows small lymphoid cells with rounded nuclei without prominent nucleoli (panels E and F, H&E staining, 400X and Giemsa staining, 600X, respectively). Scarce plasma cells surround the lymphoid nodules but are also found diffusely within the parenchyma (panel G, anti-CD138 peroxidase staining, 100X). The lymphoid cells express membranous IgM and plasma cells express cytoplasmic IgM (panel H, anti-IgM peroxidase staining, 400X).

Figure 2

The spleen of patient 4 shows a normal red pulp and a rather hypoplastic white pulp (panel A, H&E-staining, 40x). Staining for CD20 highlights the white pulp B lymphocytes. Few B lymphocytes are seen in the red pulp (panel B, anti-CD20 immunoperoxidase staining, 100X). Immunoperoxidase staining for IgK (panel C, 100X) and for IgL (panel D, 100X) does not show an obvious immunoglobulin light chain restriction in B lymphocytes or plasma cells.

Figure 3

The figure illustrates the immunophenotypic findings in CAD-associated lymphoproliferative disease. The lymphoid nodules consist mainly of B lymphocytes (panel A, anti-CD20 immunoperoxidase staining, 100X) and a moderate amount of T lymphocytes (panel B, anti-CD3 immunoperoxidase staining, 100X). Plasma cells are mainly found in the periphery of the nodular infiltrate and the parenchyma and express IgM and IgK (panels C-E, anti-IgM,
anti-IgK and anti-IgL immunoperoxidase staining, respectively, 100X). Lymphoid cells within the nodular lesions do not express MUM1, but do variably express nuclear BCL10 and strongly express PAX5 (panels F-H, immunoperoxidase staining, 400X). T lymphocytes in the lymphoid nodules are mainly CD4 helper cells whereas few CD8 cytotoxic cells are seen (panels I-J, immunoperoxidase staining for CD4 and CD8, respectively, 100X).
**Supplementary table 1.** Hemoglobin level and number of bone marrow lymphoid nodules

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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 H2O2 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 isothiocyanate (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’-GCC 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.