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All other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Musa Yilmaz, Hagop Kantarjian, Naval Daver, Farhad Ravandi designed the study, analyzed the data, and wrote the paper. Musa Yilmaz, Sherry Pierce, Naval Daver, collected and analyzed the data. Sanam Loghavi, Keyur Patel, Rashmi Kanagal-Shamanna performed the molecular analysis. Courtney DiNardo, Gautam Borthakur, Tapan Kadia, Elias Jabbour, Betul Oran, Uday R. Popat, Ghayas Issa, Nicholas J. Short, Maro Ohanian, Marina Konopleva, Hagop Kantarjian, Farhad Ravandi enrolled patients. All authors contributed to data collection, reviewed and approved the manuscript, and shared final responsibility for the decision to submit.

DATA AVAILABILITY STATEMENT
Research data are not shared.

REFERENCES
1. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-1759.
2. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-447.
3. Levis MJ, Perl AE, Martinelli G, et al. Effect of gilteritinib on survival in patients with FLT3-mutated (FLT3mut+) relapsed/refractory (R/R) AML who have common AML co-mutations or a high FLT3-ITD allelic ratio. J Clin Oncol. 2019;37(15 suppl):7000-7000.
4. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. N Engl J Med. 2017;377(5):454-464.
5. Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. N Engl J Med. 2019;381(18):1728-1740.
6. Dohner K, Thiede C, Jahn N, et al. Impact of NPM1/FLT3-ITD genotypes defined by the 2017 European LeukemiaNet in patients with acute myeloid leukemia. Blood. 2020;135(5):371-380.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

The mutational landscape of cold agglutinin disease: CARD11 and CXCR4 mutations are correlated with lower hemoglobin levels

To the Editor:
Primary cold agglutinin disease (CAD) is a rare autoimmune hemolytic anemia mediated by monoclonal IgM autoantibodies that bind to the blood group I antigen resulting in erythrocyte agglutination and complement activation. Primary cold agglutinin disease is caused by a unique indolent B-cell lymphoproliferative disorder of the bone marrow. We have previously reported recurrent KMT2D and CARD11 mutations in a small series of CAD patients by whole exome
sequencing (WES) of six cases and targeted sequencing of 10 cases.1 Recently we reported the presence of trisomy 3, 12, or 18 in CAD using cytogenetic microarray and WES. Our group has evaluated the use of bendamustine-rituximab therapy for CAD.2 Unfortunately, present B-cell directed therapies are not always well tolerated by unfit patients. Therefore, a search for new, low-toxic treatment options is warranted. The goal of this study was to explore genetic changes in CAD that may serve as more specific targets for treatment. In this study, we present comprehensive data on the mutational landscape of CAD in the context of clinical data.

We have analyzed the bone marrow from 18 patients with CAD, included in a recent clinical trial,2 by WES (Supplementary Information S1). The total number of nonsynonymous mutations detected in each CAD sample ranged from 13 to 62, while the number of all mutations in the sample cohort in the exome regions ranged from 36 to 163 (Figure 1(A)).

Four genes showed nonsynonymous mutations in more than 20% of patients (Table S1): KMT2D (12/18; 67%; including splice region mutations), IGLL5 (8/18, 44% and 11/18, 61% including non-coding region mutations), CARD11 (6/18, 33%), and CXCR4 (4/18, 22% and 5/18, 28% including non-coding region mutations). Additionally, several genes showed recurrent nonsynonymous mutations in two or three cases each (11%–17%): HIST1H1E, TMPRSS7, CSMD3, FAT1, FAT4, PHLDB1, CFTR, EP300, GRIK2, LPPN3, LTB, MACF1, NBEA, NEFH, PCNX2, PKHD1L1, RRAGC, SLC30A9, TMEM132E and ZNF618 (Figure 1(B),(C); Table S3). Many of these genes are also mutated in lymphomas. Several other unique gene mutations were also found (Table S3).

All patients with either CARD11 or CXCR4 mutations, or both, had concurrent KMT2D mutations (Figure 1(C)). Almost all patients with KMT2D mutations (10/12, 83%) had at least four other recurrent mutations. Patients with CARD11 (six cases) or CXCR4 (five cases) mutations had at least four other recurrent mutations. All CARD11 mutations are projected to be activating mutations disrupting the coiled-coil domain of CARD11.3 The CXCR4 functional mutations were detected in four patients (Table S1). These mutations are frameshift mutations located very close to the C-terminal end of the protein at positions 316–330, and are projected to prevent receptor internalization creating a state of prolonged activation. The IGLL5 gene mutations were located only in the five prime UTR comprising exon 1 and intron 1, with the region affected being less than 600 bp long.

A difference in hemoglobin levels prior to treatment was found between patients with and those without mutations in either CARD11 or CXCR4, or both (Figure 1(C), Figure S3, Table S2). The median difference between the lower limit of normal and actual hemoglobin level (adjusted for sex) for patients with CARD11, CXCR4, or both, mutations was 4.5 g/dl (mean difference 4.7 g/dl) and for patients without CARD11 or CXCR4 mutations 1.6 g/dl (mean difference 2.2 g/dl). The difference between the groups was statistically significant (p = 0.026; Mann–Whitney 2-tailed U test).

Pathway analysis showed that chromatin modification and chromatin organization were the most affected pathways in CAD patients. Most of the patients (15/18; 83%) had mutations of at least one gene involved in chromatin modification or organization (Figure 1(C) and Figure S3). In addition to KMT2D there were multiple other genes mutated in different samples: ASH1L, ARID1B, BRD1, CACNA1D, CREBBP, DNMT3A, EP300, HDAC1, HIST1H2BO, HIST1H3G, JADE3, KAT8, KDM6A, NFkB1, PPARD, PRDM16, SAP18, SETD1B, SMARCC1 and TBL1XR1. Additionally, 14/18 (78%) samples showed mutations in genes involved in regulation of the nuclear factor kappa B (NF-κB) pathway (Figure 1(C),(D); Figure S3). In addition to CARD11 and CXCR4, other genes included were CASP3, COL1A1, CREBBP, DLL1, EP300, ERBB3, FBXW7, FGF1, HDAC1, IRAK2, LRRC7, LTB, MTO1, NFkB1, RBCK1, TBL1XR1 and TLR3. Only 2/18 patients did not show mutations affecting either chromatin modification or organization, or affecting the NF-κB pathway (CAD-1.34 and CAD-7). Of interest, these two patients had close to normal hemoglobin levels (Figure S3).

Somatic KMT2D mutations are found in many lymphoma types, while constitutional KMT2D mutations give rise to Kabuki syndrome. It is suggested that KMT2D is a tumor suppressor gene, and KMT2D mutations might act as driver mutations in lymphoma. Interestingly, some of the Kabuki patients develop autoimmune hemolytic anemia (AIHA).3 Eight of the CAD patients in our study had inactivating KMT2D mutations. Three patients had missense mutations in the C-terminal SET domain, and one patient (CAD-13) had mutation affecting a splice site (Supplementary Information S1). These mutations are very likely to inactivate or impair KMT2D activity. Therefore, it is most likely that all detected mutations (12/18; 67%) in the CAD patients significantly reduce KMT2D activity. The presence of KMT2D mutations might be exploited for targeted treatment.

Five of six CARD11 mutations were located in coiled-coil region in exon 6, and therefore are predicted to result in NF-κB activation.5 The exon 5 mutation in a sixth sample is also located in the coiled-coil region and may be an activating mutation. Both nonsense and frame-shift somatic mutations in the C-terminal domain of CXCR4 have been reported in 27% of Waldenström macroglobulinemia (WM) patients.4 The location of mutations in WM is very close to the region of mutations detected in CAD samples. Based on the biochemical structure of CXCR4 and the previous functional studies (Supplementary Information S1), CXCR4 mutations in CAD patients may also prevent receptor internalization creating a state of prolonged activation. In our series, a CXCR4 mutation always occurred in combination with KMT2D mutation. CXCR4 gene mutations are of potential therapeutic interest as target for CXCR4 inhibitors. CAD patients with KMT2D and CARD11 or CXCR4 mutations, have all low hemoglobin levels. Further functional studies are required to explain the effect of these mutations upon disease severity.

Note, NF-κB signaling is important for lymphoma development, due to its role in lymphocyte survival and proliferation. In normal B cells, the NF-κB pathway is transiently activated in response to antigen stimulation. However, during lymphoma development acquired genetic mutations might cause constitutive activation of the NF-κB pathway.5 In CAD, we found activating mutations that are expected to cause constitutive activation of this pathway. Note, CARD11 plays an important role in NF-κB activation through the B cell receptor (BCR), and CXCR4 signaling intercrosses with BCR signaling (Figure 1(D)).6 Therefore, activating mutations in CARD11 and CXCR4, as the ones we found in CAD
The mutational landscape of CAD detected by whole exome sequencing. (A) Number of somatic mutations in individual CAD patients. Mutations are color-coded as nonsynonymous (blue) or synonymous (orange). (B) Recurrent somatic mutations in CAD patients. The y-axis shows the percentage of patients with mutations in genes indicated on x-axis. (C) Overview of recurrently mutated genes and hemoglobin levels for individual CAD patients. The hemoglobin levels are calculated as percent (%) of the lowest normal value adjusted for sex, and are color-coded to indicate the level of anemia: up to 70% (red), between 71%–90% (yellow) or above 90% (white). Two separate columns indicate whether nonsynonymous mutations were found in genes involved in either chromatin modification/organization or in NF-κB pathway. (B) and (C) Only genes with nonsynonymous mutation found in more than 10% of samples are shown. (D) Schematic representation of signaling cascades leading to activation of nuclear factor kappa B (NF-κB). Indicated are mutations in C-terminal end of CXCR4 (22% cases) and CARD11 (33% cases) that likely result in abnormal activation of NF-κB pathway (overall 44% cases). A more detailed description of NF-κB pathway activation is given in Supplementary Information S1.
samples, might cause constitutional NF-κB activation. The latter might enable cold-agglutinin producing B cells to survive and proliferate.

The IGLL5 mutations are seen in lymphomas (Supplementary Information S1) however, the function of IGLL5 in CAD requires more study. The MYD88 L265P mutation was absent in 27 CAD patients in our previous study, but was found in a minority of patients by another group. In this series, one unique patient (CAD-1.32) displayed the MYD88 L265P mutation. This patient did not have any other recurrent somatic gene mutations that are common in CAD, except for IGLL5 mutations (Figure 1(C)). Trisomy 3, 12 and 18, frequently seen in CAD, was also absent in this case. Further, IGHV3-7 clonal rearrangement was found with an additional minor IGHV4-34 clone (0.2% of reads; unpublished data). Flow cytometry assessment revealed two IGK-restricted B cell populations, one CD5 positive and the other CD5 negative. Therefore, it seems likely that this patient may have a composite lymphoproliferative disorder, possibly lymphoplasmytic lymphoma and CAD, the latter represented by the minor clone. This is supported by the observation of absence of hemolytic anemia at relapse of the lymphoplasmytic lymphoma in this patient; relapse occurred 7 years after initial treatment with bendamustine and rituximab2 had resulted in complete remission with absence of hemolytic anemia. The clonal B cell population was CD5 negative.

In conclusion, CAD showed a relatively low mutational burden, but with recurrent gene mutations. Current study confirms and expands our previous findings1 in a larger series of CAD patients. The most common recurrent mutations were in genes known to be involved in lymphoma development. The CAD patients with a KMT2D mutation associated with CARD11 or CXCR4 mutations, or both, had lower hemoglobin levels at diagnosis compared to patients with absence of KMT2D mutation or patients with KMT2D mutation without CARD11 or CXCR4 mutations. Both CARD11 and CXCR4 mutations in CAD are expected to be activating mutations, likely activating the NF-κB pathway. Gene mutations observed affect the NF-κB pathway as well as chromatin modification or organization. This is the most comprehensive study of gene mutations in CAD as of yet, and identifies possible new avenues for targeted treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Agnieszka Malecka, Gunhild Tøren, Jan Delabie, Anne Tierens, Sigbjørn Berentsen and Geir E. Tjønnfjord designed the study. Agnieszka Malecka, Gunhild Tøren, Ingunn Østlie and Jędrzej Malecki performed the analyses. Gunhild Tøren, Jan Delabie, Anne Tierens, Sigbjørn Berentsen and Geir E. Tjønnfjord supervised the study. Jan Delabie, Anne Tierens, Ulla Randen, Sigbjørn Berentsen and Geir E. Tjønnfjord reviewed the diagnostic patient samples and collected the clinical data. Agnieszka Malecka, Gunhild Tøren, Jan Delabie and Jędrzej Malecki prepared the manuscript. All authors have critically read the manuscript.

ETHICS STATEMENT

The patients included in this study were enrolled in a clinical trial (NCT02689986).2 The study was approved by the Regional Committee for Medical and Health Research Ethics of Southeast Norway (2012/131/REK).

PATIENT CONSENT STATEMENT

Written informed consent was procured by using consent forms approved by the Regional Committee for Medical and Health Research Ethics of Southeast Norway.

DATA AVAILABILITY STATEMENT

In accordance with Norwegian legislation and the ethical approval of the study, all sensitive data are stored in protected databases at Oslo University Hospital. On request, the data will be made available for other institutions. However additional ethical approval might be required before sharing.

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REFERENCES

1. Malecka A, Tøren G, Tierens A, et al. Frequent somatic mutations of KMT2D (MLL2) and CARD11 genes in primary cold agglutinin disease. Br J Haematol. 2018;183(5):838-842.
Refractory primary autoimmune myelofibrosis treated with ruxolitinib

To the Editor:
Autoimmune myelofibrosis (AIMF) is a rare non-clonal entity characterized by bone marrow fibrosis and autoimmune phenomena.1,2 Cytokines elaborated by bone marrow-lymphocytes appear to be the cause of marrow fibrosis in AIMF.3 Autoimmune myelofibrosis can be classified as primary (P-AIMF) when it occurs without an established autoimmune disease, or secondary (S-AIMF) when it occurs in the setting of an established autoimmune disease like systemic lupus erythematosus, rheumatoid arthritis or Sjögren disease).1,3 The lymphoid aggregates in the marrow characteristic of AIMF are implicated as the source of the inflammatory cytokines— including interferon-gamma, interleukin-8, IL-2, IL-17, transforming growth factor beta, and lipocalin-2 that lead to marrow fibrosis.

Pullarkat et al. initially defined P-AIMF by the following criteria: (1) grade 3–4 fibrosis in the marrow, (2) lack of clustered or atypical megakaryocytes, (3) lack of myeloid, erythroid dysplasia, eosinophilia, or basophilia, (4) lymphocyte infiltration of the bone marrow, (5) lack of osteosclerosis, (6) absent or mild splenomegaly, (7) presence of autoantibodies, and (8) absence of disorder known to cause MF.1 A lack of mutations associated with myeloproliferative neoplasms can be added to these criteria.

Since the underlying pathophysiology of AIMF is immune mediated, it is crucial to differentiate it from primary myelofibrosis, a myeloproliferative neoplasm. Note, AIMF is generally very responsive to steroids and immunosuppression. Some cases can be steroid refractory and pose a challenge due to severe cytopenias. We report a case of transfusion dependent P-AIMF, refractory to multiple lines of treatment who was treated successfully with the JAK inhibitor ruxolitinib with regression of fibrosis in the marrow.

A previously healthy 61-year-old Latina female presented with severe fatigue and was found to be profoundly anemic with hemoglobin of 7 g/dl. Complete blood counts (CBC) were otherwise normal: WBC of 6.5 × 10³/l (neutrophils 3.5 × 10³/l, lymphocytes 2.2 × 10³/l), MCV of 86.3 fl, and platelet count of 149 × 10³/l. Laboratory studies were consistent with hypoproliferative anemia. Further laboratory studies showed kappa light chain of 44.7 mg/l (normal range: 3.3–19.4 mg/l), lambda light chain of 27.5 mg/l (normal range: 5.7–26.3 mg/l), kappa/lambda ratio at 1.63 (normal range: 0.26–1.65), Serum protein electrophoresis showed no abnormal M-spike. Serum Immunoglobulin levels were normal and T-cell receptor gamma gene rearrangement was not detected. Antinuclear antibodies were not detected. Direct antiglobulin test was positive for IgG and negative for C3. There was no clinical/laboratory evidence of hemolysis. Physical examination was unremarkable except for anemia.

Bone marrow biopsy in July 2018 showed a hypercellular marrow with left shifted erythroid precursors and marked myelofibrosis (MF 3/3), interstitial lymphoid aggregates, and adequate stainable iron. There was no dysplasia to support diagnosis of myelodysplastic syndrome, nor any megakaryocytic atypia or clustering suggestive of primary MF. Cytogenetics was normal. Peripheral blood smear showed decreased red blood cells without tear-drop erythrocytes or leukoerythroblastosis. A diagnosis of AIMF was made based on established criteria of reticulin fibrosis with no clustering of atypical megakaryocytes, lack of dysplasia in erythroid or myeloid series, no eosinophilia or basophilia, presence of reactive lymphoid aggregates in the bone marrow, lack of osteosclerosis, absence of splenomegaly, presence of autoantibodies (positive direct antiglobulin test), and absence of another underlying disorder causing marrow fibrosis.6

Patient was red cell transfusion dependent at presentation. She was initially treated with prednisone at 1 mg/kg daily for 3 months with no clinical response in terms of red cell transfusion dependency. Her treatment was then changed to weekly rituximab, again with no clinical response after 4 weeks of therapy. She then received danazol followed by cyclosporine for 3 months without a clinical response and remained transfusion dependent with resultant iron overload for which she was placed on deferasirox.

Follow up BM biopsy on March 2019 showed a hypercellular bone marrow (80% cellularity) (Figure 1(A)) with marked reticulin fibrosis (MF 3/3) (Figure 1(B)), focal collagen fibrosis (Figure 1(B), inset), myeloid hyperplasia with decreased megakaryocytes. Next generation sequencing (NGS) using a 73 gene panel (including CALR, JAK1/2/3, MPL and other common myeloid mutations) did not detect any genetic alterations.