# Autoimmune conditions and chronic infections in chronic lymphocytic leukemia patients at diagnosis are associated with unmutated IgV$_H$ genes

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## Supplementary methods

### Determination of IgV$_H$ gene usage and mutation status

Genomic DNA from MNC and BM samples was isolated using the MagNA Pure LC DNA isolation system (Roche). PCR amplification of clonal VDJ rearrangements was performed using oligonucleotide primers VH1/7-VH6 and OL4 as forward and reverse primers respectively (VH1/7: 5'-ATG GAC TGG ACC TGG AGG-3'; VH2: 5'-CAC (AG)CT CCT GCT GCT GAC CA-3'; VH3a: 5'-GCT GGG TTT TCC TTG TTG C-3'; VH3b: 5'-ATG GAG TT(GT) GG(AG) CTG AGC TG-3'; VH4: 5'-ATG AAA CAC CTG TGG TTC TT-3'; VH5: 5'-CTC CTC CTG GCT GTT CTC C-3'; VH6: 5'-CTG TCT CCT TCC TCA TCT TCC-3'; OL4: 5'-ACC TGA GGA GAC GGT GAC C-3'). PCR products were separated on agarose gels. PCR products of primer combinations showing amplification of clonal rearrangements were selected for purification and subsequent sequencing.

Sequencing reactions were performed using the BigDye version 3.1 cycle sequencing kit (Applied Biosystems), and corresponding forward and reverse PCR primers. Products were analyzed using the ABIPRISM 3100 Genetic Analyzer (Applied Biosystems). Forward and reverse sequence reads were aligned, and resulting VDJ-fragments submitted for detailed analysis at IMGT/V-QUEST (*http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg*) to identify most closely related germline fragments.[1] Homology to the nearest germline gene was calculated by counting nucleotide exchanges in relation to the entire most closely related V$_H$ germline fragment or to the length of the sequenced fragment available as previously described.[2]

### Patient selection

The charts of CLL patients seen between 1991 and 2006 at the Division of Hematology and Hemostaseology at the Vienna General Hospital were reviewed for information on chronic stimulation at or before diagnosis of CLL. Selection was based on availability of well kept chart reviews and known IgV$_H$ mutation status. The majority of patients (75%) were first seen between 2001 and 2006 (median observation period: 53 months). Written informed consent was obtained for collection of blood cells for the determination of molecular parameters (Ethics approval numbers 38/1998, 505/2002, 495/2003). A diagnosis of autoimmune disease (AI) was made based on clinical manifestations. Chronic infections (CI) were defined as chronic or latent infection or recurring history of an infection. For patient selection refer to algorithm in Figure 1. Based on patient chart reviews, 69 of 270 patients had a history of chronic stimulation. Of these, IgV$_H$ data were available in 186 patients comprising 39 patients with chronic stimulation (CS group) and 147 patients without chronic stimulation (non-CS group). These 147 included patients who had had tumors at some point in their life or suffered from metabolic diseases like diabetes mellitus type II (DM) or non-chronic infections. Only patients with no concomitant disease (n=82) were included in the final analysis in the non-CS group. In the CS group, however, we considered long-term chronic antigenic stimulation to outweigh concomitant disease and did not exclude such patients. However, we did not include patients with immune thrombocytopenia (ITP) in this subgroup, a disease mainly, but not exclusively, occurring as a complication in the course of CLL. Thus, the final number of patients included in the analysis was 121. Four patients had known familial CLL (1 CI, 1 AI, 2 non-CS).

The median observation time is currently 53 months. Data collected included age, sex, Binet stage, selected laboratory tests (Coombs test, CD38 expression, C-reactive protein), IgV$_H$ gene usage and mutation status, cytogenetics, interval between CLL diagnosis and first treatment, and overall survival.

## References


1. Giudicelli V, Chaume D, Lefranc MP. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. Nucleic Acids Res 2004;32(WEB SERVER ISSUE):W435-40.
2. Kröber A, Seiler T, Benner A, Bullinger L, Brückle E, Lichter P, et al. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood 2002; 100:1410-6.


**Supplementary Table 1.** Baseline characteristics of the analyzed CLL patient cohort (CS + non-CS; n=121).

| *Median age/range* | *61* | *25-83* |
|---|---|---|
| Gender: male (%) / female (%) | 62.0 | 38.0 |
| Binet stage (n=121) | | |
| A (n/%) | 105 | 86.8 |
| B (n/%) | 12 | 9.9 |
| C (n/%) | 4 | 3.3 |
| Coombs test (n=108) | | |
| Positive (n/%) | 6 | 5.6 |
| Negative (n/%) | 102 | 94.4 |
| CRP (n=117) | | |
| < 0.5 (n/%) | 91 | 77.8 |
| ≥0.5 (n/%) | 26 | 22.2 |
| Median/range | 0.5 | 0.04-14.14 |
| CD38 expression (n=96) | | |
| Median/range | 5.5 | 0-85 |
| Mutation status (clones: n=140) | | |
| UM (%)/M (%) | 36.4 | 63.6 |
| Genetic abnormalities (n=100) | | |
| Normal (n/%) | 23 | 23.0 |
| 13q- (n/%) | 52 | 52.0 |
| 13q- sole (n/%) | 25 | 25.0 |
| 11q- (n/%) | 19 | 19.0 |
| +12 (n/%) | 16 | 16.0 |
| 17p- (n/%) | 8 | 8.0 |
| 14q32 rearr. (n/%) | 20 | 20.0 |
| Other (6q- etc) (n/%) | 5 | 5.0 |

**Supplementary Table 2.** Comparison of mutation status of CS and non-CS patients with respect to sex of the patient.

| *CS patients* | *UM (%)* | *M (%)* | *p* |
|---|---|---|---|
| Men (Clones: n=26) | 32.6 | 23.9 | |
| Women (Clones: n=20) | 21.7 | 21.7 | |
| Non-CS patients | | | |
| Men (Clones: n=61) | 23.4 | 41.5 | |
| Women (Clones: n=32) | 4.3 | 30.8 | |
| Women<br>CS UM *vs.* non-CS UM | | 0.002 | |
| Non-CS UM<br>Men *vs.* women | | 0.01 | |

**Supplementary Table 3.** Most frequently used $IgV_H$ genes in CS and non-CS patients.

| $IgV_H$ gene | CS (%) | non-CS (%) |
|---|---|---|
| **UM** | | |
| 1-69 | 20.0 | 34.6 |
| 3-7 | 8.0 | 0.0 |
| 3-30 | 8.0 | 3.8 |
| 4-34 | 16.0 | 7.7 |
| 5-51 | 12.0 | 11.5 |
| **M** | | |
| 2-5 | 0.0 | 8.8 |
| 3-15 | 9.5 | 0.0 |
| 3-21 | 9.5 | 4.4 |
| 3-23 | 9.5 | 8.8 |
| 3-30 | 14.3 | 7.4 |
| 3-49 | 9.5 | 0.5 |
| 3-7 | 0.0 | 10.3 |
| 4-34 | 9.5 | 5.9 |
| 6-1 | 9.5 | 5.9 |

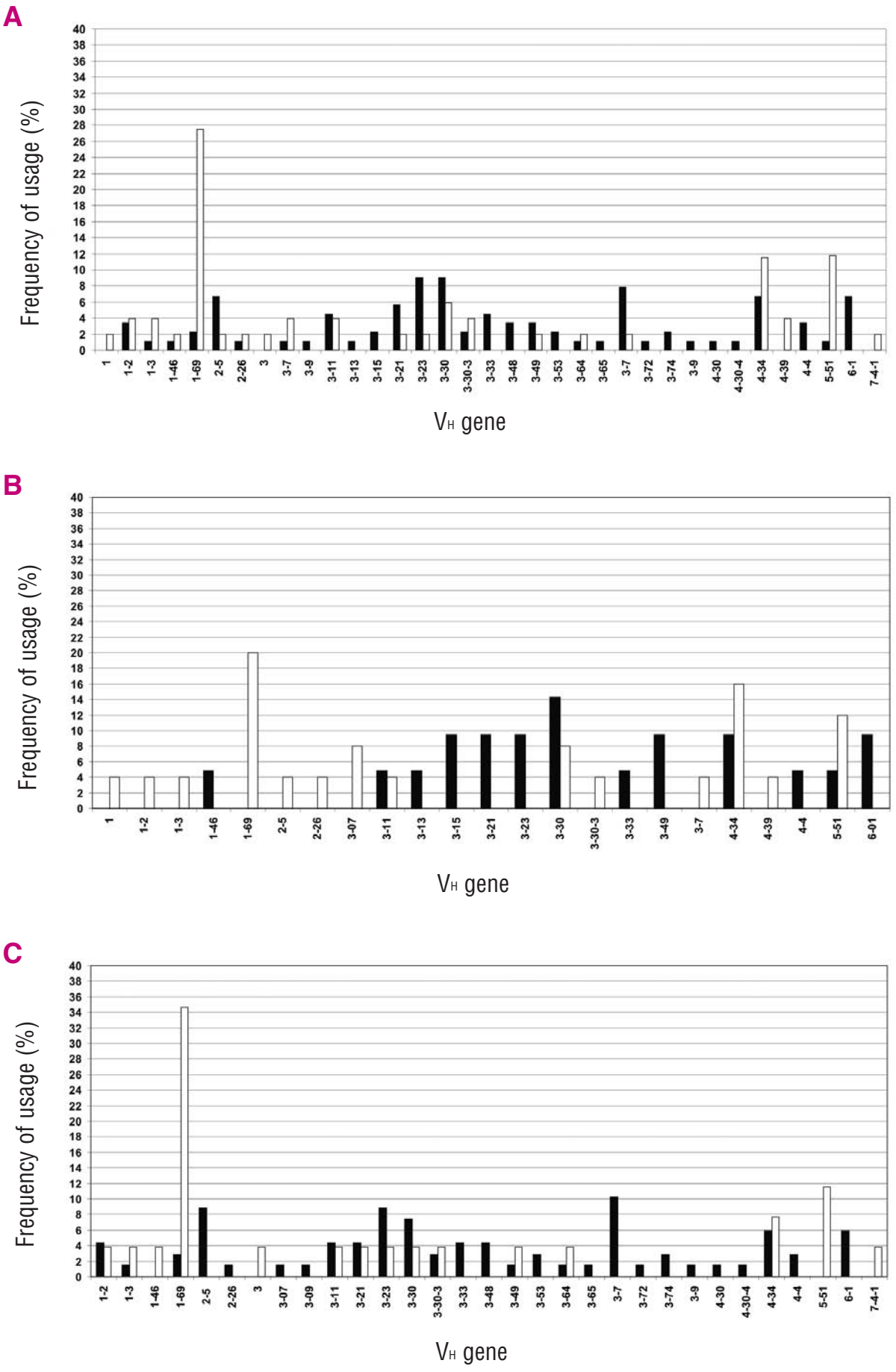
**Supplementary Table 4.** Stereotype subsets of CLL cases described previously that were found in our patient cohort.†

| N | $IgV_H$ gene | $IgD_H$ gene | $IgJ_H$ gene | CS subgroup | set number |
|---|---|---|---|---|---|
| *CS patients (8 of 43 rearrangements; 18,6%)* | | | | | |
| 1 | 1-03 | 6-19 | 4 | CI | 1[1]; Vb[2]; 5[3]; |
| 1 | 1-69 | 2-2 | 6 | CI | 3[1]; II[2]; |
| 1 | 1-69 | 3-10 | 6 | CI | 5[1]; III[2]; |
| 2 | 1-69 | 3-3 | 6 | 1 CI + 1 AI | 7, 9[1]; 3[3]; |
| 1 | 4-39 | 6-13 | 5 | CI | 8[1]; |
| 1 | 2-05 | 2-2 | 6 | AI | 10[1]; |
| 1 | 3-30 | 1-26 | 4 | AI | 40[1]; |
| *Non-CS patients (18 of 76 rearrangements ; 23.7%)* | | | | | |
| N | $IgV_H$ gene | $IgD_H$ gene | $IgJ_H$ gene | set number | |
| 1 | 1-02 | 6-19 | 4 | 1[1]; Va[2]; | |
| 1 | 1-03 | 6-19 | 4 | 1[1]; Vb[2];5[3]; | |
| 5 | 3-21/3-11 | 3-03/5-14/2-2/3-3 | 6 | 2, 9, 21, 22, 25, 41[1]; 1[3]; | |
| 1 | 1-69 | 2-02 | 6 | 3[1]; II[2]; | |
| 1 | 1-69 | 3-16 | 3 | 6[1]; 23; | |
| 3 | 1-69 | 3-03 | 6 | 7, 9[1]; III[2];3[3]; | |
| 1 | 3-23 | 3-03 | 6 | 9, 22[1]; | |
| 1 | 4-34 | ND | 4 | 11[1]; | |
| 1 | 1-69 | 5-24 | 3 | 15[1]; | |
| 1 | 4-34 | 2-15 | 6 | 16[1]; | |
| 1 | 1-69 | 3-09 | 4 | 19[1]; | |
| 1 | 3-53 | 6-25 | 4 | 20[1]; | |

*1. Stamatopoulos K, Belessi C, Moreno C, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. Blood 2007;109:259-70. 2. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med 2004;200:519-25. 3.Tobin G, Thunberg U, Karlsson K, et al. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood 2004;104:2879-85.* *†Only Ig heavy chain gene rearrangements were taken into account, therefore assignment to more than 1 subset may occur.*

**Supplementary Table 5.** Mutation status of non-CS patients with diabetes mellitus, tumors, or non-chronic infections compared to mutation status of CS patients.

| | | |
|---|---|---|
| *Diabetes mellitus type II* | | |
| Mutation status (Clones: n=22 ; Patients: n=15) | | |
| UM (%)/M (%) | 31.8 | 68.2 |
| *Tumors* | | |
| Mutation status (Clones: n=19; Patients: n=16) | | |
| UM (%)/M (%) | 26.3 | 73.7 |
| Prostate cancer, breast cancer, colon cancer, ovarial cancer, renal cell carcinoma, melanoma, M. Waldenström, lymph node tumor of unclear histology, myoma, prostate hyperplasia | | |
| *Non-chronic infections and other illnesses* | | |
| Mutation status (Clones: n=40; Patients: n=34) | | |
| UM (%)/M (%) | 47.5 | 52.5 |
| Hepatitis A, borreliosis, polyarthrosis, arthrosis, gastritis, hypothyroidism, hepathopathy, viral hemorrhagic fever, duodenitis, jaundice | | |
| *Autoimmune diseases* | | |
| Mutation status (Clones: n=26; Patients: n=22) | | |
| UM (%)/M(%) | 53.8 | 46.2 |
| *Chronic infections* | | |
| Mutation status (Clones: n=20; Patients: n=17) | | |
| UM (%)/M (%) | 55.0 | 45.0 |




**Online Supplementary Figure S1.** IgV$_H$ gene usage. (**A**) IgV$_H$ gene usage of entire CLL patient cohort (121patients/140 clones). (**B**) IgV$_H$ gene usage of CS patients (39 patients/46 clones). (**C**) IgV$_H$ gene usage of non-CS patients (82 patients/94 clones). Black bars: mutated genes; white bars: unmutated genes.