# Low-count monoclonal B-cell lymphocytosis persists after seven years of follow-up and is associated with a poorer outcome

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 doi:10.3324/haematol.2017.183954

Received: November 3, 2017.

Accepted: March 15, 2018.

Pre-published: March 22, 2018.

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# SUPPLEMENTARY INFORMATION

## Supplementary methods

**Flow cytometry immunophenotypic studies.** A total amount of between 1 and 4 mL of EDTA-anticoagulated PB per case and follow-up time point was immunophenotyped, using either a direct immunofluorescence stain-and-then-lyse technique[1,2] or following a "Bulk Lysis Protocol" (www.EuroFlow.org) (Supplementary Table 1); per sample, cells were stained with both a *"screening tube*" for the analysis of the distribution of the different lymphocyte subsets, and a *"characterization tube"*, aimed at the identification and characterization of the B-cell clone(s). Panels A and B in Supplementary Table 1 detail the composition of each "tube" for samples stained at baseline, while Panels C and D in Supplementary Table 1 show the antibody combinations for staining samples collected during follow-up.

For detection of clonal B-cell populations, ≥5x$10^6$ cells were measured in a FACSCanto II flow cytometer -Becton/Dickinson Biosciences (BD), San José, CA-. Instrument setup, calibration and daily quality control were performed according to well-established protocols.[3,4] All cases defined as $MBL^{lo}$ showed ≥1 clonal -imbalanced surface membrane (sm) Ig kappa/smIg lambda ratio of >3:1 or <1:3- and/or aberrant B-cell population, showing either a CLL-like phenotype ($CD19^+$ $CD20^{lo}$ $CD5^{+/++}$ $smIg^{lo}$) or any other non-CLL-like aberrant B-cell phenotype.[1,2] The minimum number of clustered events required to define a population was of ≥50 cells.[1,2]

**Statistical analyses.** Conventional descriptive and comparative statistics –Mann-Whitney U and Kruskal-Wallis tests, as well as Wilcoxon and Friedman tests, and the Spearman correlation test– were performed for all relevant variables. For objective evaluation of sequential changes in the number of clonal B-cells per subject, a 99% confidence interval (CI) was estimated per sample, using a resampling bootstrap method that takes into account random variation in the size of each clonal B-cell population;[5,6] values above or below the baseline 99% CI were considered to be real changes in the size of the B-cell clones studied during follow-up. Overall survival (OS) curves were plotted according to the method of Kaplan-Meier and the (one-sided) log-rank test was used to compare OS curves of $MBL^{lo}$ subjects *vs*. (age- and sex-matched) non-$MBL^{lo}$ controls from the general population studied in parallel at baseline.

Information regarding the status (alive vs. death) of both groups was collected from the Spanish Index of Deaths (INDEF, *Ministerio de Sanidad, Servicios Sociales e Igualdad*, Madrid, Spain). Multivariate analyses using the Cox regression model was performed to predict the variables independently associated with a greater/lower risk of death. Mortality data -including causes of death- for the general population from the same geographical area for the 2008-2016 period was obtained from the Spanish Statistical Office (www.ine.es). A predictive linear regression model was built to estimate the time CLL-like $MBL^{lo}$ clones might potentially take to progress to $MBL^{hi}$ and CLL. Statistical significance was set at P-values ≤0.05. All statistical analyses were performed with SPSS 19.0 software (SPSS-IBM, Armonk, NY), except for the bootstrap calculations and the linear regression model, that were accomplished with MATLAB R2015a (Mathworks, Natick, MA).

# Supplementary Tables

## SUPPLEMENTARY TABLE 1.- Combination of fluorochrome-conjugated antibodies used for the immunophenotypic analysis of peripheral blood samples by flow cytometry

### A. Screening tube used at baseline ("stain-and-then-lyse" SOP ≥5x10$^6$ cells analyzed)[1,2]

| Fluorochrome | PacB | PacO | FITC | PE | PerCPCy5.5 | PECy7 | APC | AF700 |
|---|---|---|---|---|---|---|---|---|
| **Marker** | CD20 | CD45 | CD8+anti-λ | CD56+anti-κ | CD4 | CD19 | CD3 | CD38 |
| **Clone** | 2H7 | HI30 | UCHT4+ polyclonal | C5.9+ polyclonal | L200 | J3-119 | SK7 | HIT2 |
| **Source** | eBioscience | Invitrogen | BD+Dako | BD+Dako | BD | Beckman Coulter | BD | Exbio |

### B. MBL$^{lo}$ characterization tube used at baseline ("stain-and-then-lyse" SOP with ≥5x10$^6$ cells analyzed)[1,2]

| Fluorochrome | PacB | PacO | FITC | PE | PerCPCy5.5 | PECy7 | APC | AF700 |
|---|---|---|---|---|---|---|---|---|
| **Marker** | CD20 | CD45 | Anti-κ | Anti-λ | CD19 | CD10 | CD5 | CD38 |
| **Clone** | 2H7 | HI30 | Polyclonal | Polyclonal | HIB19 | HI10a | L17F12 | HIT2 |
| **Source** | eBioscience | Invitrogen | Dako | Dako | BD | BD | BD | Exbio |

### C. LST (EuroFlow® Lymphocyte Screening Tube): used at follow-up (EuroFlow "Bulk Lysis" SOP with ≥5x10$^6$ cells analyzed)

| Fluorochrome | PacB | OC515 | FITC | PE | PerCPCy5.5 | PECy7 | APC | APCH7 |
|---|---|---|---|---|---|---|---|---|
| **Marker** | CD20+ CD4 | CD45 | CD8+ Anti-κ | CD56+ Anti-λ | CD5 | CD19+ anti-TCRγδ | CD3 | CD38 |
| **Clone** | 2H7+ RPA-T4 | HI30 | UCHT4+ polyclonal | C5.9+ polyclonal | HIB19 | HI10a+ 11F2 | UCHT2 | HB7 |
| **Source** | eBioscience +BD | Cytognos | Cytognos | Cytognos | BD | Beckman Coulter+BD | BD | BD |

### D. MBL$^{lo}$ characterization tube used at follow-up (EuroFlow "Bulk Lysis" SOP with ≥5x10$^6$ cells analyzed)

| Fluorochrome | PacB | PacO | FITC | PE | PerCPCy5.5 | PECy7 | APC | AF700 |
|---|---|---|---|---|---|---|---|---|
| **Marker** | CD20 | CD27 | CD5 | CD305 | CD79b | CD19 | CD3+ anti-κ | anti-λ |
| **Clone** | 2H7 | L128 | UCHT2 | DX26 | 3A2-2E7 | J3-119 | SK7+ Polyclonal | Polyclonal |
| **Source** | eBioscience | BD | BD | BD | BD | Beckman Coulter | BD+ Cytognos | Cytognos |

"Stain-and-then-lyse" and "Bulk Lysis" protocols were compared in parallel and no statistical differences were found regarding the detection of normal B-cells, clonal B-cells or any of the major cell populations in the peripheral blood.[3,4] For more detailed protocols please see **www.EuroFlow.org**. Abbreviations (alphabetical order): **AF700**: alexa fluor® 700; **APC**: allophycocyanine; **APCH7**: allophycocyanine-hilite®7; **BV510**: brilliant violet™ 510; **FITC**: fluorescein isothiocyanate; **MBL$^{lo}$:** low-count monoclonal B-cell lymphocytosis; **OC515:** orange Cytognos 515; **PacB:** pacific blue™; **PE**: phycoerythrin; **PECy7**: phycoerythrin-cyanine7; **PerCPCy5.5**: peridinin chlorophyll protein-complex cyanine 5.5. Manufacturers (alphabetical order): Becton Dickinson Biosciences (BD), San José, CA; Beckman Coulter, Brea, CA; Cytognos, Salamanca, Spain; Affymetrix eBioscience (eBioscience), San Diego, CA; ExBio, Vestec, Czech Republic; Invitrogen (ThermoFisher Scientific), Waltham, MA.

**SUPPLEMENTARY TABLE 2.- Panel of probes and fluorochromes used for interphase fluorescence *in situ* hybridization (iFISH) studies and the corresponding chromosomal regions targeted**

| Probe name | Fluorophore | Targeted chromosome band/ region | Probe size (Kb)[#] |
|---|---|---|---|
| *Custom Kit for CLL-like clones* | | | |
| *ATM* | SG | 11q22.3 | 180 |
| *D12Z3* | SA | 12p11.1-q11 | NA |
| *D13S25* | SGo | 13q14.3 | 306 |
| *P53* | SR | 17p13.1 | 159 |
| *LSI IGH DC BA* | SO/SG | 14q32 | 250/900 |
| *Probes for other non-CLL B-CLPD* | | | |
| *LSI IGH/CCND1 DC DF* | SO/SR | 11q13/14q32 | 942/1500 |
| *LSI IGH/BCL2 DC DF* | SG/SO | 14q32.3/18q21.3 | 1600/870 |
| *ON 7q32/SE7 DC* | SR/SG | 7q32.1/7p11.1-q11 | 682/NA |
| *LSI BCL6 DC BA* | SO/SG | 3q27 | 349/816 |
| *LSI MALT DC BA* | SO/SG | 18q21 | 600/765 |

[#]Information obtained from Kreatech Diagnostics, CytoCell Ltd. and Vysis Inc. Customized probes kits from Kreatech Diagnostics (Amsterdam, The Netherlands), CytoCell Ltd. (Cambridge, UK) and Vysis Inc. (Abbott Park, IL, USA). Abbreviations (alphabetical order): **BA:** break-apart probe; **B-CLPD:** B-cell chronic lymphoproliferative disorder; **CLL:** chronic lymphocytic leukemia; **DC:** dual color; **DF:** dual fusion; **IGH:** heavy chain immunoglobulin; **LSI**: locus specific identifier; **NA**: not applicable; **SA**: spectrum aqua; **SE**: α-satellite centromeric probe; **SG**: spectrum green; **SGo**: spectrum gold; **SO**: spectrum orange; **SR**: spectrum red.

**SUPPLEMENTARY TABLE 3.- Clinical and biological characteristics of non CLL-like MBL$^{lo}$ subjects distributed by phenotypic category at baseline and after 7y follow-up.**

| | MZL-like (n=6) | | HCL-like (n=1) | | MCL-like (n=2) | | Unclassifiable non-CLL B-CLPD (n=2) | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Follow-up | Baseline | Follow-up | Baseline | Follow-up | Baseline | Follow-up |
| Follow-up time (months) | NA | 84 (66-87) | NA | 84 | NA | 63 (63-63) | NA | 85 (83-86) |
| Male/Female* | 2/4 (33%/67%) | | 1/0 (100%/0%) | | 2/0 (100%/0%) | | 2/0 (100%/0%) | |
| % Leukocytosis (>10x10$^9$/L)* | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| % Lymphocytosis (>4x10$^9$/L)* | 0 (0%) | 1 (17%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| % Cytopenias* | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| N. total T cells/µL | 1086 (796-1965) | 1459 (755-2908) | 808 | 792 | 1101 (1091-1111) | 614 (276-953) | 1161 (1655-1667) | 1662 (1448-1877) |
| N. CD4$^+$ T cells/µL | 611 (351-1395) | 857 (447-1995) | 457 | 570 | 645 (467-824) | 238 (184-293) | 784 (732-836) | 717 (629-805) |
| N. CD8$^+$ T cells/µL | 445 (307-522) | 628 (273-848) | 315 | 203 | 423 (237-609) | 349 (66-633) | 661 (572-750) | 619 (404-833) |
| N. CD4$^+$/CD8$^+$ T cells/µL | 4.9 (1.8-27) | 8.2 (1.1-29) | 0.51 | 2.0 | 2.2 (0.19-4.3) | 9.5 (8.7-10) | 5.6 (4.9-6.2) | 12 (7.6-16) |
| N. CD4$^-$/CD8$^-$ T cells/µL | 15 (11-107) | 35 (8.1-141) | 36 | 16 | 31 (11-50) | 17 (15-19) | 211 (167-254) | 315 (222-407) |
| N. NK cells/µL | 426 (150-668) | 412 (252-914) | 799 | 938 | 571 (173-848) | 285 (178-392) | 263 (248-279) | 313 (276-361) |
| N. total B cells/µL | 173 (45-1066) | 335 (49-1208) | 26 | 73 | 611 (47-1173) | 80 (22-138) | 149 (137-160) | 196 (110-203) |
| N. normal B cells/µL | 77 (35-125) | 43 (25-104) | 23 | 22 | 59 (46-72) | 35 (21-49) | 114 (92-136) | 124 (58-190) |
| N. clonal B cells/µL | 87 (4.7-980) | 288 (12-1149) | 3.1 | 52 | 551 (1.4-1101) | 45 (1.3-90) | 34 (0.62-68) | 74 (2.7-144) |
| % cases with ≥2 MBL clones* | 3 (50%) | 3 (50%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| % Lymphadenopathies* | 0 (0%) | 1 # (17%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| % Progression to B-CLPD* | NA | 0 (0%) | NA | 0 (0%) | NA | 0 (0%) | NA | 0 (0%) |
| % Deaths* | NA | 0 (0%) | NA | 0 (0%) | NA | 1 (50%) | NA | 0 (0%) |

Results expressed as median (range) or as * number of cases (percentage).

# A single axillar adenopathy (size 1x1cm).

Abbreviations (alphabetical order): **B-CLPD:** B-cell chronic lymphoproliferative disorder; **HCL:** hairy cell leukemia; **MBL$^{lo}$:** low-count monoclonal B-cell lymphocytosis; **MCL:** mantle cell lymphoma; **MZL:** marginal zone lymphoma; **N.:** number, **NA:** not applicable.

**SUPPLEMENTARY TABLE 4.- Frequency of CLL-associated cytogenetic alterations and percentage of cells affected by each genetic abnormality in the 21 individuals evaluated both at baseline and at follow-up.**

| | **All MBL$^{lo}$ cases (n=21)** | | **CLL-like MBL$^{lo}$ cases (n=18)** | | **Non-CLL-like MBL$^{lo}$ cases (n=3)** | | P-value |
|---|---|---|---|---|---|---|---|
| | Baseline | Follow-up | Baseline | Follow-up | Baseline | Follow-up | |
| ***Chromosomal region*** | | | | | | | |
| del(13q14)(*D13S25*)<br>% altered cells | 4/15 (27%)<br>50±40% | 8/15 (53%)<br>55±36% | 4/14 (29%)<br>(50±40%) | 8/14 (57%)<br>55±36% | 0/1 (0%)<br>NA | 0/1 (0%)<br>NA | <0.04[a,b] |
| Trisomy 12<br>% altered cells | 2/14 (14%)<br>34±35% | 2/14 (14%)<br>45±35% | 1/13 (7.7%)<br>59% | 1/13 (7.7%)<br>70% | 1/1 (100%)<br>9% | 1/1 (100%)<br>20% | NS |
| del(11q)(*ATM*)<br>% altered cells | 1/9 (11%)<br>8% | 1/9 (11%)<br>50% | 0/8 (0%)<br>NA | 0/8 (0%)<br>NA | 1/1 (100%)<br>8% | 1/1 (100%)<br>50% | NS |
| del(17p)(*TP53*)<br>% altered cells | 0/8 (0%)<br>NA | 0/8 (0%)<br>NA | 0/7 (0%)<br>NA | 0/7 (0%)<br>NA | 0/1 (0%)<br>NA | 0/1 (0%)<br>NA | NS |

Results expressed as number of cases (percentage of cases) and mean ± SD of percentage of cells affected by each specific genetic alteration. [a] Baseline vs. follow-up (year +7) for all cases, [b] Baseline vs. follow-up (year +7) for CLL-like MBL cases, [c] Baseline vs. follow-up (year +7) for non CLL-like MBL cases. Abbreviations (alphabetical order): **CLL:** chronic lymphocytic leukemia; **MBL$^{lo}$:** low-count monoclonal B-cell lymphocytosis; **NA:** not applicable; **NS:** not statistically significantly different (P>0.05).

**SUPPLEMENTARY TABLE 5.- Absolute number of distinct circulating peripheral blood lymphocyte subsets in CLL-like MBL$^{lo}$ subjects and age- and gender-matched non-MBL healthy donors**

| | Non-MBL HD (n=250) | #CLL-like MBL$^{lo}$ subjects (n=56) | P-value |
|---|---|---|---|
| Gender (Male/Female)* | 114/136 (46%/54%) | 22/34 (39%/61%) | NS |
| Age (years) | 73 (49-97) | 75 (49-91) | NS |
| N. of Total T cells/µL | 1092 (435-2951) | 1508 (460-3753) | <0.001 |
| N. of CD4$^+$ T cells/µL | 643 (125-1659) | 898 (227-2045) | <0.001 |
| N. of CD8$^+$ T cells/µL | 362 (13-1939) | 479 (96-1742) | <0.01 |
| N. of CD4$^+$/CD8$^+$ T cells/µL | 7.1 (0.71-74) | 8.1 (1.3-147) | NS |
| N. of CD4$^-$/CD8$^-$ T cells/µL | 35 (2.7-212) | 65 (1.9-338) | <0.001 |
| N. of normal B cells/µL | 119 (16-776) | 138 (26-536) | NS |
| N. of NK cells/µL | 291 (39-1215) | 373 (89-3415) | <0.01 |

Results expressed as median (range) or as * number of cases (percentage). #2/56 individuals simultaneously carried at least one CLL-like clone and a non-CLL-like clone. Abbreviations (alphabetical order): **CLL:** chronic lymphocytic leukemia; **HD:** healthy donors; **MBL$^{lo}$:** low-count monoclonal B lymphocytosis; **N.:** number; **NA:** not applicable; **NK:** natural killer; **NS:** no statistically significant differences (P>0.05).

**SUPPLEMENTARY TABLE 6.- List of variables studied in the Cox Regression model and their corresponding hazard ratios (HR) (95% confidence intervals; CI) and P-values for their association with OS for the whole MBL$^{lo}$ plus non-MBL cohort.**

| **Variables**<br>Whole cohort | **HR (95%CI)** | **P-value** |
|---|---|---|
| **Cardiovascular disease** | **2.65 (1.30 - 5.41)** | **0.007** |
| **Age (<65y vs. ≥65y)** | **5.08 (1.48 - 17.49)** | **0.01** |
| **Solid tumor** | **2.86 (1.26 - 6.46)** | **0.01** |
| **MBL$^{lo}$ clones** | **2.14 (0.97 - 4.72)** | **0.06** |
| N. of PB neutrophils (/µL) | 1.00 (1.00 - 1.001) | 0.12 |
| N. of PB CD4$^{+}$/CD8$^{+}$ T cells (/µL) | 1.02 (0.99 - 1.04) | 0.20 |
| Diabetes | 0.56 (0.22 - 1.45) | 0.23 |
| N. of PB CD4$^{+}$ T cells (/µL) | 0.99 (0.99 - 1.00) | 0.23 |
| N. of PB monocytes (/µL) | 1.001 (0.99 - 1.00) | 0.30 |
| Severe infections | 0.99 (0.99 - 1.00) | 0.30 |
| Hypertension | 1.45 (0.68 - 3.11) | 0.34 |
| Exposure to toxics | 1.34 (0.65 - 2.76) | 0.43 |
| N. of PB CD4$^{-}$/CD8$^{-}$ T cells (/µL) | 1.25 (0.52 – 3.00) | 0.62 |
| N. of PB eosinophils (/µL) | 0.99 (0.99 - 1.00) | 0.69 |
| Psychiatric disorders | 0.99 (0.99 - 1.00) | 0.69 |
| Gender | 1.09 (0.39 - 3.07) | 0.86 |
| Autoimmune diseases | 0.94 (0.43 - 2.04) | 0.87 |
| N. of PB CD8$^{+}$ T cells (/µL) | 0.90 (0.10 - 8.56) | 0.93 |

Abbreviations (alphabetical order): **CI:** confidence interval; **HR:** hazard ratio; **MBL$^{lo}$:** low-count monoclonal B-cell lymphocytosis; **N.:** number; **OS:** overall survival; **PB:** peripheral blood.

## REFERENCES TO SUPPLEMENTARY METHODS AND TABLES:


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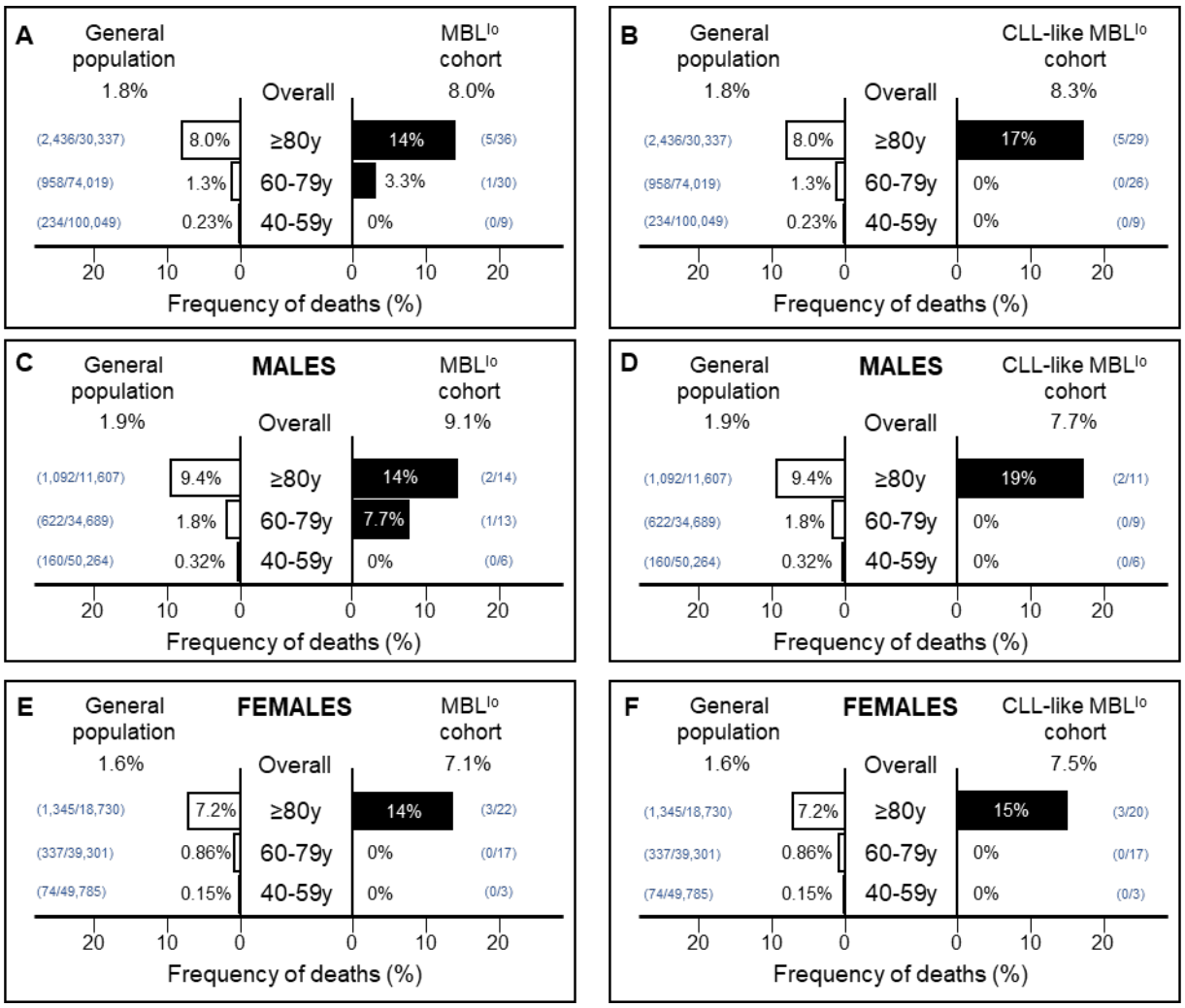
# Supplementary Figures

## SUPPLEMENTARY FIGURE 1.




**SUPPLEMENTARY FIGURE 1. Death frequencies among MBL$^{lo}$ individuals vs. the general population of Salamanca region occurring between January 2015 and December 2016. Panels A** and **B** represent the frequency of deaths by age group for the general population of Salamanca (white) vs. that of MBL$^{lo}$ and CLL-like MBL$^{lo}$ individuals (black), respectively. **Panels C** and **D** illustrate the frequency of deaths for males from the general population vs. the whole MBL$^{lo}$ cohort and CLL-like MBL$^{lo}$ individuals, respectively, whereas **Panels E** and **F** show the same comparisons for females.

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