(UPN 23) and three patients did not show additional alterations (UPNs 24–26). However, one patient of the latter group had already presented an additional *NRAS* codon 12 mutation at initial MDS diagnosis. One patient developed a second *RUNX1* mutation along with trisomy 13 (UPN 22). Interestingly, both patients who gained a chromosome 13 (UPNs 1 and 22) had antecedent mutations in *RUNX1*. According to current literature, *RUNX1* mutations occur at a frequency of about 15% in MDS,[7] thus the rate of *RUNX1* mutations is increased in our patient cohort, which is defined by leukemic evolution. Thus, our results suggest that the detection of *RUNX1* mutations in MDS comes along with a propensity to leukemic transformation. *FLT3*-ITDs occurred only after progression to s-AML in our cohort. Both patients who gained *FLT3*-ITDs in the progression from MDS to s-AML presented antecedent mutations in *RUNX1*. These two patients fit nicely into the two-hit hypothesis for the pathogenesis of AML with *RUNX1* mutations altering gene expression (type I mutation) and *FLT3*-ITDs leading to enhanced proliferation (type II mutation).[8] In the present study, *RUNX1*, a known cancer gene, is located in a region affected by UPD. In this patient, a heterozygous RUNX1 mutation has been found at MDS diagnosis. LOH of the remaining healthy *RUNX1* allele due to UPD was accompanied by progression to s-AML within less than 6 months after initial MDS diagnosis. The contribution of UPD to s-AML evolution sheds light on a novel aspect regarding the progression from MDS to s-AML. The second genetic hit is neither an additional mutation nor a cytogenetic alteration but rather the duplication of a *RUNX1*-mutated allele leading to LOH of the unaffected allele.

From a genetic point of view, MDS and AML show an overlapping pattern of cytogenetic and molecular genetic abnormalities. With the exception of AML-specific recurrent balanced rearrangement, mostly unbalanced abnormalities occur both in MDS and AML.

In conclusion, our data demonstrate that 60% (23/38) of patients acquired additional genetic abnormalities during progression from MDS to s-AML. One group (24%) preferentially gained additional chromosome abnormalities while the other group (26%) was characterized by additional molecular mutations. Four patients (11%) presented both additional cytogenetic and molecular genetic changes, one of which acquired UPD(21q) with *RUNX1*-LOH in the course of s-AML development. While *RUNX1* mutations already occurred at a high frequency at MDS stage, mutations in *FLT3* were seen only after progression to s-AML.

Conflict of interest

S Schnittger, TH, WK and CH in part own the MLL Munich Leukemia Laboratory, which offers comprehensive leukemia diagnostics. JF, S Schindela, FD and AK are employed at MLL Munich Leukemia Laboratory and declare no conflict of interest.

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CLL-like B-lymphocytes are systematically present at very low numbers in peripheral blood of healthy adults

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world. The disease is typically diagnosed in adults >40 years old, who show an expansion ($>5 \times 10^9$ cells per l) of clonal B-cells with a unique CD5$^+$, CD23$^+$, B-cell receptor (BCR)^{low} immunophenotype in peripheral blood (PB) and bone marrow, frequently in association with

involvement of other lymphoid tissues, disease symptoms and a heterogeneous clinical outcome. In parallel, monoclonal B-cell lymphocytosis (MBL), typically characterized by an expansion of clonal CLL-like B-cells ($<5 \times 10^9$ cells per l), has been also frequently reported in otherwise asymptomatic subjects.[1,2] Increasing evidence suggests that this could represent a pre-leukemic condition, as CLL frequently develops in individuals with previous history of MBL, and MBL cases progress to CLL at a rate of 1% per year.[2,3]

Interestingly, in addition to MBL cases presenting with lymphocytosis, very low numbers of clonal B-cells with a CLL-like immunophenotype and cytogenetic profile, are also present in healthy subjects with normal lymphocyte counts.[2] Initially, a frequency of CLL-like clones among healthy adults of around 3–6% was reported. Despite this relatively high incidence, controversial results have been reported about the precise frequency of CLL-like MBL in the general population.[2,4,5] Such variability has been related to genetic and microenvironmental differences, as well as to technical variables associated with different sensitivities of the distinct methods applied.[4,5] Accordingly, we have recently shown that the actual frequency of MBL cases presenting with normal lymphocyte counts could increase to as high as 12% of the general adult population >40 years,[4] depending on the sensitivity of the method and the volume of blood screened, in addition to age. Altogether, these observations raise the question about the actual frequency and significance of such small B-cell clones in normal subjects, as regards their pathological vs physiological nature.

Here, we provide further evidence about the great prevalence of small numbers of CLL-like B-cells among adults of increasing age, supporting the notion that they might be present among virtually every subject older than 70 years. Our results are based on the analysis of the frequency of CLL-like MBL in 639 healthy adults (>40 years old) with normal lymphocyte counts, representative of the population living in the area of Salamanca (Western Spain), in whom we investigated the association between the frequency of CLL-like MBL and the volume of sample analyzed. These subjects included a series of 608 previously reported individuals plus 31 new subjects recruited according to the same criteria and who were investigated for the presence of CLL-like MBL in between 0.9 and 1.2 ml of PB, using previously described flow cytometry approaches;[4] in a subgroup of nine donors aged >70 years, who did not show evidence of CLL-like B-cells in the screening phase, a second 50 ml PB sample was obtained and further investigated for the presence of CLL-like B-cell clones (see Supplementary text for more detailed information).

Overall, 80/639 subjects studied (12.5%) showed CLL-like clones defined by the presence of >50 cellular events with a CLL-like aberrant phenotype (CD19^{+}, CD5^{+}, CD20[+dim] CD23^{+}, CD79b[+dim] FMC7[±dim] Cybcl2^{hi} and restricted expression of immunoglobulin ligh-chain[+dim] in the absence of CD10) in the overall sample analyzed (median PB volume: 1200 µl; range: 900–1200 µl). Among these 80 MBL cases, the absolute count (median of 0.47 clonal B-cells per µl; interquartile range: 0.13–1.60 clonal B-cells per µl) and the percentage of CLL-like B-cells from all PB B-cells (median of 0.42; interquartile range: 0.13–1.42), lymphocytes (median of 0.027; interquartile range:
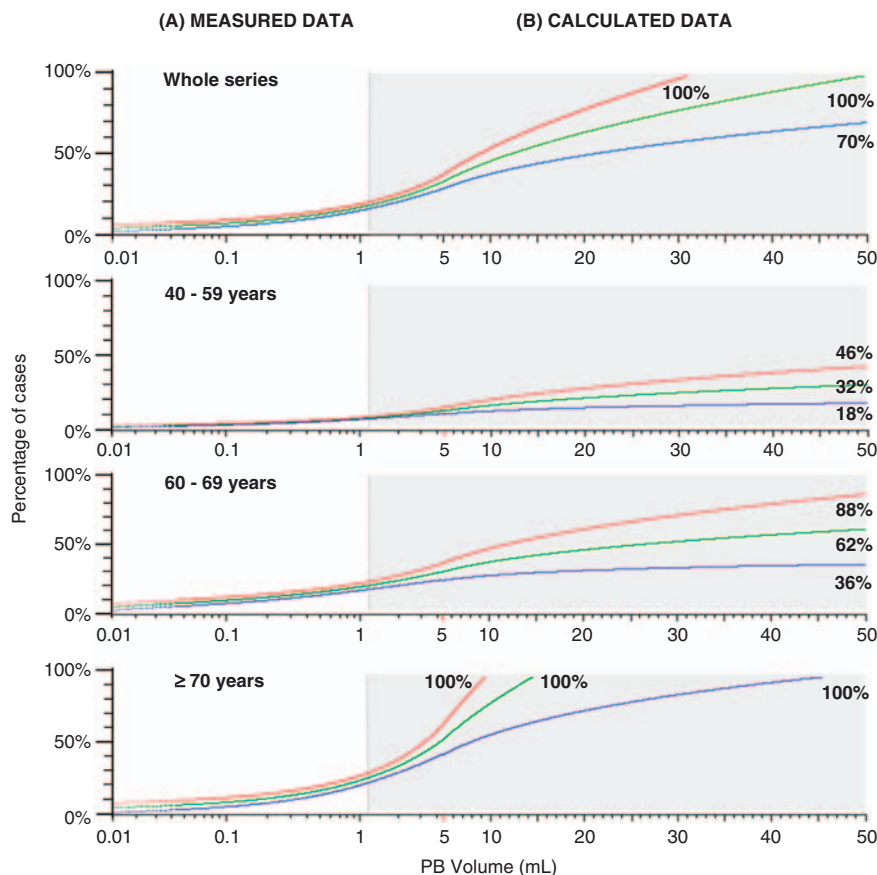

Figure 1 Real and calculated frequency of healthy subjects with detectable CLL-like B-cells on the basis of PB measurements and the statistical prediction model applied, respectively. Curves in the left (column A) correspond to the percentage of cases actually carrying CLL-like B-cells, after increasingly higher volumes of PB (up to 1200 µl of sample) were analyzed for each subject (MEASURED DATA); in turn, curves in the right (column B) correspond to the calculated percentage of cases with CLL-like B-cells, if increasingly higher volumes of PB had been stained (CALCULATED DATA). Both measured and calculated data are shown for the whole series (n = 639; upper curves) and for three groups of the same subjects stratified according to age (40–59 years, 60–69 years and ≥70 years are shown in intermediate upper, intermediate lower and lower panels, respectively). Green lines represent the (measured and calculated) percentage of cases with CLL-B-cells, whereas red and blue lines represent the higher and lower 95% confidence interval bounds.

0.010–0.101) and leucocytes (median of 0.008; interquartile range: 0.002–0.028) was similar to that reported for the first 608 cases.[4] As for the initial 608 cases,[4] a progressively higher frequency of individuals carrying CLL-like B-cell clones was detected in parallel to increasing age: 5.4% of subjects aged 40–49 years, 15.5% in the 60–69 age range and 19.2% in those older than 70 years.

Based on the sequence of acquisition of data in the flow cytometer for single PB leucocytes, then we determined for each MBL case the specific number of cells that were measured (and the corresponding volume of PB) to reach the pre-established threshold of 50 CLL-like B-cells to consider that case as MBL. As shown in Figure 1 (Column A), a progressive increase in the frequency of cases with CLL-like B-cell clones was detected, according to the volume of sample interrogated (from 1 to 1200 µl) from 0 to 15.8–95% confidence interval (CI): 13.8–17.9%—, respectively. Upon grouping the subjects according to age, similar profiles were observed, but with significantly higher frequencies for adults aged ≥60 years, and particularly those aged ≥70 years vs subjects aged 40–59 years: 18.6% (95% CI: 15.8–21.4%) for cases ≥60 and <70 years, and 24.8% (95% CI: 21.2–28.4%) for those aged ≥70 years vs 6.7% (95% CI: 5.9–7.4%) for subjects <60 years (Figure 1, Column A).

According to the overall pattern of increase in the frequency of positive cases observed when progressively higher volumes of sample were measured, a mathematical model was then built to predict whether PB CLL-like B-cells could be detected in virtually every case, if larger volumes of PB had been screened for each subject (Figure 1, Column B). Based on the 95% lower CI bound, this model predicted that CLL-like B-cells could be detected in every subject >70 years if around 50 ml of their PB would had been analyzed (the lower limit of the 95% CI reached 100% for PB volumes of around 46 ml). According to this model, in the hypothetical case that the same volume of PB (46 ml) would be stained and analyzed for each subject in the other age groups, the estimated frequency of cases carrying CLL-like clones for subjects studied at 40–59 years of age was of 32% (95% CI: 18–46%) and of 62% for cases with 60–69 years (95% CI: 36–88%), with an estimated rate of 100% for the whole series (lower limit of the 95% CI of 70%) (Figure 1, Column B).

Based on this prediction, AutoMACS-enriched B-cell preparations were obtained from another ~50 ml of PB from nine subjects aged >70 years (median of 77 years, from 72 to 88 years) who did not show detectable CLL-like B-cells when between 0.9 and 1.2 ml of their PB had been analyzed, and were further investigated (Supplementary Material). Our results confirmed the presence of CLL-like B-cells in all but one of them (Table 1 and Supplementary Figure 1). Noteworthy, both sIgκ+ and sIgλ+ CLL-like B-cell populations were detected in 4/9 of these MBL cases, even with a normally balanced sIgκ/sIgλ ratio in one of them (Case no. 8 in Table 1). Based on these results it could be speculated that the systematic presence of CLL-like B-cells at very low numbers in adults with advanced age (for example, >70 years), more likely represents the normal counterpart of CLL malignant cells than a leukemic B-cell precursor.

In the last decades, an increased degree of genetic predisposition with familial cases has been reported for some CLL patients. However, this does not explain the occurrence of most (sporadic) CLL and/or MBL cases. In this regard, recent epidemiological data,[6] together with studies showing that CLL and MBL B-cells frequently display stereotyped B-cell receptors,[3] suggest that antigen-driven immunological responses

Table 1 Characteristics of CLL-like B-cells identified in 50 ml of PB from nine healthy subjects older than 70 years, who did not show MBL when 0.9–1.2 ml of PB had been screened

Case No.	Age (y)/Sex	Volume PB stained (ml)	No. of lymphocytes ×10^9 per l	No. of total B-cells stained	No. of CLL-like B-cell populations[a]	sIg light chain restriction	Ratio κ/λ[b]	No. of CLL-like events	% of CLL-like B-cells from WBC (×10^{-3})	% of CLL-like B cells from all PB B-cells	No. of CLL-like B cells per µl
Monoclonal cases											
1	78/M	55	3.00	1 042 628	One	λ	NA	124	0.8	0.041	0.066
2	73/M	49	1.61	2 440 204	One	κ	NA	997	0.73	0.05	0.036
3	77/M	46	3.02	2 167 683	One	κ	NA	1483	1.3	0.08	0.112
4	88/M	42	2.37	966 381	One	κ	NA	114	0.14	0.013	0.009
Multiclonal cases											
5	77/F	55	2.38	145 465	Two	κ λ	20	51	0.28 0.014	0.002 0.001	0.0018 0.0009
6	72/F	55	1.22	936 185	Two	κ λ	0.72	73 57	0.08 0.11	0.006 0.008	0.007 0.009
7	82/F	30	1.33	610 711	Two	κ λ	0.18	134 112	0.18 0.95	0.02 0.01	0.009 0.004
8	73/M	48	2.92	2 321 116	Two	κ λ	1.8	374 217	0.49 0.27	0.016 0.009	0.032 0.018
9	87/M	55	2.19	900 792	ND	ND	NA	NA	<0.0006	<1.1×10^{-6}	<1×10^{-6}

Abbreviations: F, female; M, male; NA, not applicable; ND, not detected.
[a]CLL-like B-cells were identified as those cellular events expressing CD19^{+}, CD5^{+}, CD20[+dim], CD79b[+dim] and surface immunoglobulin light-chain Ig[+dim].
[b]Restricted to CLL-like B-cells (considered to be altered when ratio κ/λ >3.1 or <1:3).[14]

could contribute to trigger the expansion (and potentially also the malignant transformation) of small CLL-like B-cell clones. In contrast to CLL, information currently available about the immunoglobulin heavy chain variable repertoire of CLL-like MBL B-cell clones in the general population is limited and does not allow any definitive conclusion.[7] However, preliminary data about the immunoglobulin heavy chain variable repertoire of CLL-like clones from subjects with MBL and normal B-cell counts, point out a relatively low frequency of usage of those immunoglobulin heavy chain variable segments, which are more frequently observed in CLL and MBL associated with absolute B-cell lymphocytosis.[7] The potential association in these latter cases of CLL-like clones with a physiological process related to chronic immune stimulation would be further supported by the reported increase in the frequency of CLL-like clones with age.[4] Previous studies have shown that marked age-related changes occur in the PB B-cell compartment, particularly among individuals >60 years, with a significant decrease in both memory B-cells and circulating plasma cells. Such changes cannot be attributable to a decreased B-cell production, but more likely they reflect either a decreased recruitment of naïve B lymphocytes or a low ability of the memory B-cell compartment to expand. In such a case, immunosurveillance against chronic immune stimuli could only be effective if long-living cells are formed, that is, antigen-specific B-cells could enter into a long-living 'senescent-like' status. Therefore, a crucial question remains about whether these changes are actually found under physiological conditions in every subject, at least once a certain age is reached. Based on the increasing frequencies of CLL-like clones detected for individual subjects through the measurement of increasing volumes of PB, we have built a mathematical algorithm that would allow prediction of the frequency of MBL cases carrying a small CLL-like clone, if larger volumes of blood from individual subjects would have been screened. Based on this model, our results suggest that the vast majority of healthy adults would have a detectable clone of CLL-like B-cells if larger blood volumes had been screened. In fact, the frequency of expected MBL cases among healthy subjects would raise up to 100% for subjects older than 70 years. Confirmation of the presence of CLL-like B-cell populations in 8/9 adults who were initially negative, through the analysis of large volumes of PB (for example, 50 ml), confirms this hypothesis; the exact frequency remains to be determined in this as well as in the other younger age-groups. Interestingly, among these cases, a high frequency of subjects in whom both sIgκ+ and sIgλ+ CLL-like B-cells were present, was found. In-line with these findings, recent results in relatives of CLL patients also found multiclonal CLL-like B-cells in 4/6 MBL cases with low MBL counts.[8] Altogether, these results support the hypothesis that the presence of CLL-like B-cells may be a physiological phenomenon in adults with normal lymphocyte counts. If this holds true, it could be expected that (i) CLL-like B-cell numbers would be lower among multiclonal vs monoclonal cases and (ii) the frequency of CLL-associated genetic abnormalities would significantly decrease at lower MBL counts. In-line with this hypothesis, in 14/35 fluorescence-activated cell sorter-purified CLL-like B-cell populations investigated for cytogenetic abnormalities (purity >97%) by interphase fluorescence in situ hybridization, cytogenetic changes were detected—trisomy 12 in 2 (6%) cases and del(13q) in 12 (36%) subjects— and these MBL cases showed an absolute number of CLL-like B-cells significantly higher than that found for the other 21 MBL cases in whom no cytogenetic abnormalities were detected by interphase fluorescence in situ hybridization: median of 32 (minimum–maximum

values of 8 and 56) and 11.5 (minimum–maximum values of 0.55 and 1172) CLL-like B-cells per μl in those cases carrying trisomy 12 and del(13q), respectively, vs a median of 0.56 (minimum–maximum values of 0.1 and 8) CLL-like B-cells per μl for the other cases with no cytogenetic abnormalities by interphase fluorescence in situ hybridization ($P=0.001$); notably, none of the cases carrying trisomy 12 and only 2/12 cases with del(13q) showed less than 1 PB CLL-like B-cell per μl, whereas 11/21 cases without detectable genetic abnormalities were 'low-count' MBL (<1 circulating CLL-like B-cell per μl). Whether the higher tendency to be monoclonal vs polyclonal, and to have detectable genetic abnormalities, as the number of CLL-like MBL cells increases would be relevant in the outcome of MBL, requires further investigation. Further studies in which the functional role of these CLL-like cells is investigated are necessary to confirm this hypothesis and determine the potential nature of the chronic antigen stimuli leading to the emergence of CLL-like B-cells in healthy adults and transformation to CLL; however, these are not easy to perform, because of the limited number of cells that can be obtained in cases with very low MBL counts.

Conflict of interest

The authors declare no conflict of interest.

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Non-malignant B cells and chronic lymphocytic leukemia cells induce a pro-survival phenotype in CD14+ cells from peripheral blood

Chronic lymphocytic leukemia (CLL) is a malignant disease characterized by the accumulation of mature CD5+ B cells in the blood, bone marrow and secondary lymphoid organs.[1] Unlike most tumor entities, there is only a small proportion of CLL cells that proliferate.[2] This has led to the hypothesis that the accumulation of CLL cells in vivo is not due to increased proliferation rates, but rather due to resistance to apoptosis.[1] In CLL, resistance to apoptosis is supported by microenvironmental signals, as CLL cells cultured without support in vitro rapidly undergo apoptosis. In vitro, apoptosis of CLL cells can be prevented by co-culture with supporting cells, either indirectly through soluble factors and/or by direct interaction through cell-surface receptors.[3]

From peripheral blood monocytes (PBMCs) of CLL patients, a subset of cells (CD14+ cells) differentiate in vitro into large, round, adherent cells ('Nurse-like cells', NLCs) that assist in survival of CLL cells.[4] Cells that are morphologically and immunophenotypically similar to NLCs can also be differentiated from CD14+ PBMCs of healthy donors by cultivating them in vitro with CLL cells.[5] In vivo, NLCs have been described to be present in the spleen and lymph nodes of CLL patients, suggesting that NLCs might promote survival of CLL cells also in these tissues.[5,6] Several proteins that are involved in the pro-survival capabilities of NLCs have been identified: CXCL12,[4] CXCL13,[6] BAFF (B-cell activating factor of the tumor necrosis factor family),[7] APRIL (a proliferation-inducing ligand)[7] and CD31 (PECAM1).[8] NLCs have an expression profile of surface and cytoplasmic antigens that is distinct from that of other cells of the myelo-monocytic lineage.[5] NLCs have a significantly higher CD68 expression compared with monocytes, macrophages and dendritic cells from healthy donors.[5] What remains unclear is whether the differentiation of NLCs is specific to CLL or whether non-malignant B cells can also convert CD14+ PBMCs to cells with pro-survival capacities.

We hypothesized that if non-malignant B cells can convert CD14+ PBMCs to cells similar to NLCs, then lymphatic tissues of both CLL patients as well as healthy donors should be positive for cells expressing markers present on NLCs like CD14 and CD68. In order to validate that cells presenting these markers are also present in vivo, we stained lymph node sections of CLL patients for cells positive for CD68 (Figures 1a–c) and CD14 (Figures 1e–g). We found that NLCs might indeed help in the survival of CLL cells in the lymph node as reported previously.[6] Interestingly, non-malignant tonsil and lymph node sections from healthy individuals were positive for these markers as well (Figures 1d and h). Although it is known that NLCs have higher CD68 intensities than macrophages and dendritic cells, immunohistochemistry is not well suited for a quantitative analysis. Ideally, markers specific to NLCs will be required to make a definitive statement about the exclusive presence of NLCs in the lymphatic tissues of CLL. Nevertheless, our results give rise to the possibility that NLC-like cells are present in the lymphatic tissues of healthy individuals as well as CLL patients.

For functional analysis of a CLL-specific phenotype of NLCs, we generated these cells as previously described[5] (please refer to Figure 2a for a schematic of the experimental setup). Similar to NLCs, after culture, in vitro CD14+ cells differentiated by CLL cells (CD14$_{CLL}$ cells) as well as CD14+ cells differentiated by non-malignant B cells (CD14$_B$ cells) were positive for CD68, CD14 and negative for both CD1a (blood-derived dendritic cell marker) and CD15 (granulocyte marker) (data not shown).

One of the central phenotypic capabilities of NLCs is their potential to support survival of CLL cells. However, so far it is unclear whether this competence is specific for CLL or can also be induced in monocytes by non-malignant B cells. In order to test whether CD14$_B$ cells are capable of supporting survival, long-term allogeneic co-cultures of either CLL cells or non-malignant B cells with CD14+ PBMCs from healthy donors were initiated (Figure 2a). After 18 days of co-culture, survival of suspension cells was compared between continued co-culture on differentiated adherent cells (NLCs, CD14$_{CLL}$ cells and CD14$_B$ cells) and culture of suspension cells with medium alone.

All cultures with medium alone led to significant loss of viability of suspension cells (34.6 ± 16.1% (without