High frequency of chronic lymphocytic leukemia-like low-count monoclonal B-cell lymphocytosis in Japanese descendants living in Brazil

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries. However, it is much less frequent in Asians[1,2] including in Asians living in Western countries.[3] At present, it is well-established that virtually every CLL case is preceded by an indolent stage known as monoclonal B-cell lymphocytosis (MBL) of CLL phenotype (CLL-like MBL).[4] MBL is categorized as high-count (MBL[hi]) or low-count MBL (MBL[lo]), based on the presence of more *versus* less than 500 CLL-like clonal B cells/µL in peripheral blood (PB), respectively. MBL[hi] cases have a probability of progression to CLL requiring therapy of ~1–2% patients/year.[5] In contrast, the clinical significance of MBL[lo] remains to be determined, with rare progression to MBL[hi] and CLL in the medium-term (5–7 years).[6] CLL-like MBL[lo] is present in 7-14% individuals from Western adult (>40 years) populations with an overall median (range) MBL clone size of ~1 cell/µL (0.1-10 cells/µL), based on high-sensitive multiparameter flowcytometry (MFC).[7] In turn, the only study performed in Asian populations reported a lower prevalence of MBL of 1.3% in Taiwan, after analyzing 500,000 PB cells/case.[8] Even though the precise mechanisms (*e.g.* genetic, environmental or both) that lead to such ethnical differences on the prevalence of CLL still remain unknown, they might be related either to a significantly lower frequency of MBL and/or to a lower rate of malignant transformation of CLL-like MBL to CLL. Therefore, the investigation of the prevalence of CLL-like MBL in Asian populations might contribute to better understand such ethnical differences and to shed light on the pathogenesis of CLL.

Here, we investigated the frequency and characteristics of CLL-like MBL clones among Japanese descendants living in a Western environment through high-sensitive MFC. Overall, 258 healthy adult (≥40 years) Japanese descendants living in Sao Paulo (Brazil), without race miscegenation –108 males (42%) and 150 females (58%), with a median (range) age of 66 years (40-96 years) and no differences in the sex-distribution per age ranges, were randomly invited to participate in the study at the outpatient laboratory sample collection facility of two general hospitals (Hospital Santa Cruz and Hospital S. Paulo/UNIFESP) in Sao Paulo (Brazil) (*Online*

Supplementary Materials and Methods and *Online Supplementary Table S1*), and sequentially followed for between 1-5 years after the study had been approved by the corresponding local Ethics Committees. Detection of MBL cells and enumeration of the main normal residual PB lymphocyte populations were based on high-sensitivity 8-color MFC analysis of ≥5x10^{6} leukocytes/sample (*Online Supplementary Materials and Methods* and *Online Supplementary Table S2*). Whenever enough sample was available, clonal B cells were sorted to investigate CLL-associated cytogenetic abnormalities by interphase fluorescence *in situ* hybridization (iFISH) and the mutational status of their immunoglobulin heavy chain (IGVH) sequences.

In total, 27 of 258 (10.5%) individuals showed MBL clones, including a greater frequency of males (16 of 108, 15%) *versus* females (11 of 150, 7%) (*P*=0.05) (Table 1). The frequency of MBL cases progressively increased with age from 4% in subjects aged 40–59 years to 22% among older (≥80 years) adults (*P*=0.02). Of note, such an increase was significantly higher in males than females (10x *vs.* 3x, *P*=0.002) (Figure 1; Table 1). Interestingly, the MBL clone size was systematically <500 cells/µL (<0.5 cells/µL in 92% cases) with median (range) counts of 0.12 cells/µL (0.03-20 cells/µL) (Table 1; *Online Supplementary Table S3*). Of note, MBL clones were significantly larger in men *versus* women, both in absolute (*P*=0.04) and in relative numbers (*P*=0.02), and they progressively increased with age (*P*=0.04 and *P*=0.05, respectively) in parallel to the greater frequency of MBL (Figure 1). None of the eight CLL-like MBL clones studied carried any CLL-associated cytogenetic abnormality, while 4 of 6 cases in which enough purified MBL cells were available displayed mutated *IGHV* gene sequences (*Online Supplementary Table S4*).

Altogether, these results show that, despite the similarly high prevalence of MBL[lo] here reported for the Japanese population living in Sao Paulo and other series of healthy MBL[lo] Caucasians, Japanese presenting with MBL[lo] displayed unique features. These included: i) greater predominance of MBL[lo] in men *versus* women (men/women ratio of 2 *vs.* 0.71-1.7);[7,9] ii) almost four-fold smaller MBL[lo] clone sizes (median of 0.12 cells/µL *vs.* 1 cell/µL; range: 0.1-34 cells/µL),[7,10] even when high-sensitive MFC methods had been used;[7] and iii) greater predominance of CLL-like *versus* non-CLL-like MBL[lo] pheno-

Table 1. Frequency and number of chronic lymphocytic leukemia-like monoclonal B-cell lymphocytosis cells in adult (≥40 years) Japanese descendants living in Brazil according to sex and age.

| Japanese descendants living in Brazil	Sex				Age range (years)			
	Total	Male	Female	*P*	(40 – 59)	(60 – 79)	(≥80)	*P*
Total N.	258	108	150		75 (26 M/ 49 F)	156 (72 M/ 84 F)	27 (10 M/ 17 F)	
MBL[lo] N. (%)	27 (10.5%)	16 (15%)	11 (7.3%)	0.05 (4.1%)	3 (12%)	18 (22%)	6	0.02
Clonal B cells in MBL[lo] cases								
% MBL cells/total B cells	0.08 (0.01 – 20)	0.14 (0.02 – 20)	0.05 (0.01 – 0.20)	0.02	0.02 (0.01 – 0.14)	0.06 (0.01 – 0.42)	0.26 (0.02 – 20)	0.05
N. clonal B cells/µL	0.12 (0.03 – 20)	0.21 (0.04 – 20)	0.09 (0.03 – 0.4)	0.04	0.07 (0.03 – 0.51)	0.15 (0.04 – 0.5)	0.32 (0.03 – 19)	0.04

Results expressed as median (range) for continuous variables and as number of cases (percentage) for 495 categorical variables. CLL: chronic lymphocytic leukemia; F: female; M: male; MBL: monoclonal B-cell lymphocytosis; MBL[lo]: low-count 496 monoclonal B-cell lymphocytosis; N.: number.

types (98% *vs.* 66-85%);[7,9,10] associated with, iv) a strikingly high frequency of biclonal MBL[lo] cases (*Online Supplementry Table S3*) (13 of 27; 48% *vs.* 2.6-19%).[7,9,11,12] The (significantly) smaller MBL clone sizes here detected might contribute to explain the higher frequency of MBL cases found to have ≥2 MBL clones, since oligoclonal expansions of ≥2 unrelated B-cell clones is frequently observed in ~20% MBL[lo] cases,[7] particularly among those with lower PB MBL counts (<150 cells/μL), whereas its frequency decreases from MBL[lo7,9-11] to MBL[hi10,11] and CLL, as clonal B-cell counts in PB increase. In addition, these findings would also support the progressively greater frequency (and size) of MBL[lo] clones during adulthood reported here for the Japanese population living in Brazil. In line with this hypothesis, no cytogenetic alterations were detected in a (small) subset (n=8/27) of MBL clones investigated, which also showed a high frequency of mutated IGHV BCR sequences (*Online Supplementary Table S4*). Similarly to what has been previously reported in follow-up studies of MBL[lo] subjects from European and North-American populations,[6,7] all CLL-like MBL[lo] clones except two, persisted after a 1-5 year follow-up, without significant size variations (*data not shown*).

Altogether, the above findings suggest a potentially longer time to progression for MBL[lo] in the Japanese *versus* other (*e.g.* Caucasian) populations and might contribute to explain the lower prevalence of CLL among Asian populations, including Asian migrants to Western countries. Although direct comparison of the prevalence of MBL in the Japanese population versus the general (*e.g.* non-Japanese) population in Brazil would be of utmost interest, the actual prevalence of CLL-like MBL[lo] and CLL in Brazil was not investigated here and still needs to be determined. Despite this, preliminary data

suggest that prevalence of CLL in Japanese living in Brazil is also lower than in other ethnic groups living in the same region. Thus, based on the Brazilian CLL registry data, only 0.42% Japanese patients have been included among all CLL cases registered in the State of Sao Paulo,[13] a region where 690,600 Japanese descendants live among an overall (age-matched) population of 45,230,000 individuals (1.5% of the population living in Sao Paulo), further supporting an ethnical/genetic background, more than an environment-related trigger in determining the lower prevalence of CLL in Japan[2] *versus* Europe and North-America. However, in a recent study in Taiwan, a lower prevalence of MBL of 1.3% (4 of 302) has been reported.[8] Although the lower prevalence of MBL in this *versus* our series might be related to the lower sensitivity of the MFC screening technique used (with 5×10^5 cells analyzed per PB sample *vs.* $>5 \times 10^6$ cells in our series) and the slightly lower age of the Taiwanese population, the potential impact of different environmental factors on the occurrence of MBL in individuals living in Taiwan *versus* those from a Western country like Brazil, deserves further investigations.

In this regard, in our study clear evidence for early emergence of alterations in the PB compartments of T and natural killer (NK) cells was observed, similar to those reported in Caucasian populations.[6] Thus, the median number of PB, T and NK cells was already significantly increased in MBL[lo] *versus* non-MBL subjects (Table 2). Despite the relatively small sample size (n=27 MBL cases) and the overall tendency of NK cells to increase with age in non-MBL subjects, such an increase was more pronounced in MBL[lo] *versus* non-MBL subjects (*Online Supplementary Figure S1*). These findings are fully in line with similar results found in Caucasian popula-
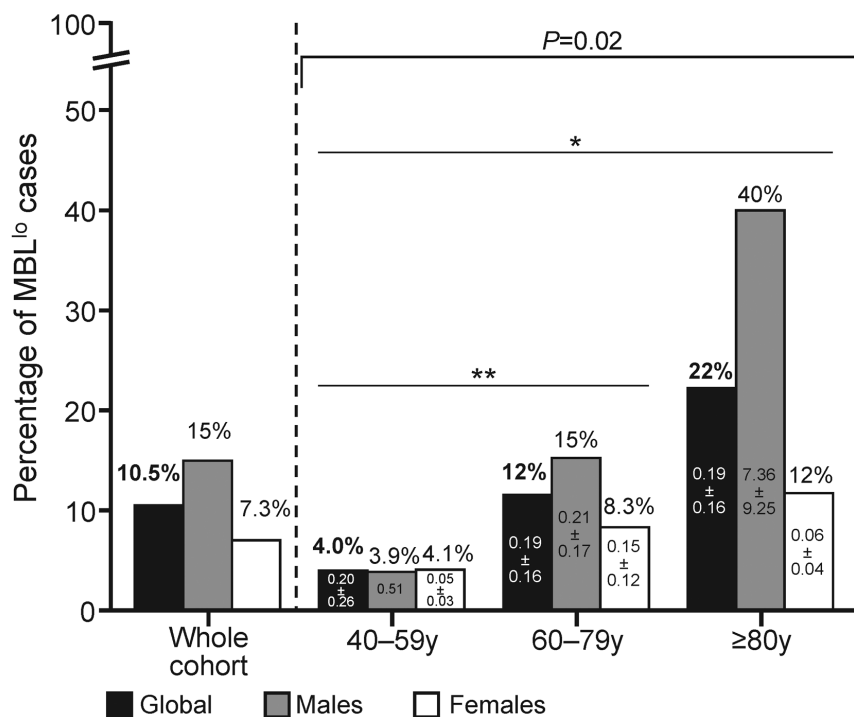
Figure 1. Frequency of low-count monoclonal B-cell lymphocytosis cases (n=27) among 258 healthy adult (≥40 years) Japanese descendants analyzed, distributed per age group and sex. *P*-value=0.004 for the 40–59 years *versus* ≥80 years groups; ***P*-value=0.06 for the 40–59 years *versus* 60–79 years groups. Mean number of peripheral blood circulating clonal B cells/μL (± 1 standard deviation) is shown inside each bar for 40-59 years, 60-79 years and ≥80 years categories, both for males and females. MBL[lo]: low-count monoclonal B-cell lymphocytosis; y: years.

Table 2. Distribution of peripheral blood leukocytes, lymphocytes and their T-cell, B-cell and natural killer-cell populations according to the presence *versus* absence of monoclonal B-cell lymphocytosis in healthy adult (≥40 years) Japanese descendants.

	Total n=258	MBL[lo] n=27	Non-MBL n=231	P
Age (years)	66 (40 – 96)	70 (42 – 96)	65 (40 – 93)	0.04
N. of leukocytes (x10⁹/L)	5.7 (2.8 – 14)	5.8 (4.4 – 7.4)	5.7 (2.8 – 14)	NS
N. of lymphocytes (x10⁹/L)	1.8 (0.66 – 4.6)	1.9 (0.79 – 2.8)	1.8 (0.66 – 4.6)	NS
N. of T cells (x10⁹L)	1.1 (0.35 – 3.2)	1.2 (0.56 – 2.5)	1.1 (0.35 – 3.2)	0.05
N. of CD4⁺ T cells (x10⁹/L)	0.7 (0.19 – 2.8)	0.78 (0.39 – 1.5)	0.69 (0.19 – 2.8)	NS
N. of CD8⁺ T cells (x10⁹/L)	0.29 (0.05 – 1.2)	0.38 (0.10 – 0.89)	0.29 (0.06 – 1.2)	NS
N. of NK cells (x10⁹/L)	0.27 (0.05 – 1.1)	0.32 (0.12 – 0.74)	0.26 (0.05 – 1.1)	0.002
N. of B cells (x10⁹/L)	0.19 (0.003 – 0.84)	0.18 (0.05 – 0.51)	0.19 (0.003 – 0.84)	NS
N. of plasma cells (x10⁹/L)	3.4 (0.12 – 79)	2.0 (0.23 – 18)	3.4 (0.12 – 79)	NS
B-cell subsets	**Total n=143**	**MBL[lo] n=21**	**Non-MBL[lo] n=122**	**P**
N. of immature B cells (x10⁶/L)	11 (0.003 – 147)	12 (0.54 – 76)	11 (0.003 – 147)	NS
N. of naïve B cells (x10⁶/L)	144 (1.7 – 714)	111 (23 – 373)	150 (1.7 – 714)	NS
N. of memory B cells (x10⁶/L)	38 (1.9 – 130)	42 (14 – 129)	38 (1.9 – 130)	NS
N. of unswitched memory B cells (x10⁶/L)	12 (0.48 – 80)	15 (4.0 – 37)	12 (0.48 – 80)	NS
N. of switched memory B cells (x10⁶/L)	26 (1.0 – 94)	27 (7.0 – 93)	26 (1 – 94)	NS

Results expressed as median values (range); B-cell subsets expressed in cells x10⁶/L. MBL: monoclonal B-cell lymphocytosis; NK: natural kiler; N.: number; NS: not statistically significantly different (*P*>0.05).

tions showing greater NK- and T-cell numbers in PB of MBL.[6] Likewise, similar anti-cytomegalovirus (anti-CMV) and anti-Epstein-Barr virus (anti-EBV) antibody (Ab) titers were found in MBL[lo] *versus* non-MBL Japanese (*Online Supplementary Table S5 and Supplementary Figure S2*), as previously described for MBL[lo] Caucasians.[14]

Altogether, these findings suggest that similar (*i.e.* common) genetic (*e.g.* sex) and/or environmental factors (*e.g.* multiple diverse antigenic determinants) might be involved in the earliest stages of development of (biclonal/oligoclonal) MBL expansions in both the Japanese and Western European/North-American populations.[9,15] However, expansion and transformation of such CLL-like MBL[lo] clones into MBL[hi] and CLL would require additional genetic and/or environmental triggers, including those involved in acquisition of CLL-associated genetic alterations,[15] that might differ among Asian and Caucasian populations.

In summary, here we show that Japanese descendants (≥40 years) living in Sao Paulo (Brazil) display a similar frequency of MBL to that found among Western Caucasian populations, at the expense of smaller MBL[lo] clones, and higher frequency of oligoclonality, suggesting earlier stages of development with potentially lower rates of progression to MBL[hi] and CLL. Further studies in larger cohorts of Japanese (or Asian) *versus* non-Japanese control populations living in the same -as well as distinct geographical regions- are necessary to gain insight into the potential genetic and microenvironmental factors that determine these differences.

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