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Peripheral neuropathies in chronic lymphocytic leukemia: a single center experience on 816 patients

Chiara Briani[1*], Andrea Visentin[2*], Alessandro Salvalaggio[1], Silvia Imbergamo[2], Francesco Piazza[2], Mario Cacciavillani[3], Marta Campagnolo[1], Federica Frezzato[2], Gianpietro Semenzato[2], Livio Trentin[2].

[1]Neurology Unit, Department of Neuroscience, University of Padova. [2]Hematology and Clinical Immunology Unit, Department of Medicine, University of Padova; [3]CEMES, Data Medica Group, Padova, Italy. *****BC and VA contributed equally to the manuscript

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‡**Corresponding authors**

Chiara Briani, MD
Department of Neurosciences, University of Padova
Via Giustiniani 5, 35128 Padova – Italy
Tel.: +39-049-8213600 Fax: +39-049-8751770
Email: chiara.briani@unipd.it

Livio Trentin, M.D.
Hematology and Clinical Immunology Unit
Department of Medicine, University of Padua
Via Giustiniani, 2 - 35128 Padova
e-mail: livio.trentin@unipd.it
phone: 0039 049 821 2298 fax: 0039 049 821 1970

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Chronic lymphocytic leukemia (CLL) is the most common leukemia in the adulthood with an estimated incidence of 5 cases/100.000/year and a median overall survival of almost 21 years (1). CLL is a very heterogeneous disease characterized by patients with slowly increasing lymphocytes to patients with rapidly progressive and sometimes life threating disease (2).

CLL is also associated with several immunological abnormalities (3) that may predispose to autoimmune (4) as well as to infectious diseases (5). Neurological complications are uncommon, but may involve both central and peripheral nervous system (6, 7). The pathogenesis of these complications varies from CLL spreading, opportunistic infections, immune-mediated acute or chronic polyradiculoneuropathies, iatrogenic neuropathies, or may simply be coincidental.

While central nervous system complications of CLL have recently been investigated (8), peripheral nervous system illnesses are generally overlooked or considered secondary to chemotherapies or infections.

The aim of our longitudinal cohort study was to assess the prevalence and characteristics of peripheral neuropathy (PN) in a wide population of CLL patients, and their correlation with CLL specific clinical and biological prognostic markers [cytogenetic analyses, TP53 aberrations (17p deletion and/or TP53 mutation), immunoglobulin heavy chain variable region (IGHV) mutational status, CD38 and ZAP70 expressions] (4-5).

Time to peripheral neuropathies (TTPN) was calculated from the date of CLL diagnosis to PN occurrence (event) or last available follow-up (censored). Detailed information on prognostic markers evaluation and statistical methods are reported in supplementary data. All the patients who reported symptoms suggestive of PN underwent neurological and neurophysiological evaluations. Sera from patients with PN were tested for the presence of antibodies to peripheral nerve antigens (gangliosides and sulfatides) as previously reported (9). The occurrence of VZV reactivation, which causes radiculopathies, as well as peripheral facial nerve palsy, a mononeuropathy, has been also

recorded. The role of chemo-immunotherapy therapies, infections reactivation or genetic associations will also be discussed.

The study was approved by the local ethic committee and was made according to the declaration of Helsinki. Informed consent was obtained by all the patients.

Eight hundred sixteen patients affected with CLL and regularly followed at the Hematology and Clinical Immunology Unit of Padova University have been recruited and their features are summarized in Table I.

Nineteen (2.2%, Figure 1A) out of 816 patients, mainly men (63%), suffered from PN during a median follow-up of 99 months, as confirmed by extensive neurophysiological studies (supplementary data; 4 other subjects reported symptoms suggestive of PN, that was however not confirmed by neurological and neurophysiological evaluation). The median age at PN occurrence was 69±12 years and 4 (22%) patients had a Rai stage ≥2. Ninety percent of the PN cases were identified after CLL diagnosis and in 3 patients PN occurred within the first 6 months from CLL diagnosis (Figure 1B). Of the 19 patients with PN, the majority (10/19) had sensory axonal PN, 5 sensory-motor axonal PN, one had a multiple mononeuropathy, and 3 fulfilled the clinical and neurophysiological criteria of chronic inflammatory polyradiculoneuropathy (CIDP). For 3 patients (one affected by CIDP, one with sensory axonal PN and the patient with multiple mononeuropathy), the neuropathy represented the symptom at onset of CLL. Serum antibodies to peripheral nerve antigens were absent in all 19 subjects.

When investigating comorbidities that might have caused or contributed to the PN (i.e. diabetes mellitus, HCV infection), we observed that predisposing conditions were present in 5 patients (3 diabetes mellitus and 2 HCV). In 2 patients the sensory axonal neuropathy was present before CLL diagnosis and likely secondary to type 2 diabetes mellitus. In 3 patients an iatrogenic cause of neuropathy has been identified, namely lenalidomide for 2 patients and ibrutinib (10) for

the third patient. In all the 3 cases neuropathic symptoms occurred after the beginning of chemotherapy. After excluding diabetic and iatrogenic PN, 12/816 (1.5% of all the cohort) patients with PN remain in whom other identifiable causes of PN (e.g. infections, vitamin deficiency) had been ruled out. Population-based data for PN are lacking, but the overall prevalence of chronic PN in the general population is supposed to be around 1%. Interestingly, the presence of CIDP in our sample is high (0.37%) compared to general population (11), as it had already been described in a large European study on lymphoma-associated paraneoplastic neuropathies (12).

Since the risk of developing PN is a time-dependent variable, we performed Kaplan-Meier analysis (Figure 1C). The risk of developing PN increases over time, with an estimated TTPN of 2.1% after 10 years from the diagnosis and 6.9% after 20 years of follow-up.

We also investigated the association with clinical and biological prognostic markers in patients with and without PN. Interestingly, CLL subjects who developed PN harbored high-cytogenetic risk by FISH (i.e. 11q and 17p deletion, p=0.0210) and at diagnosis CD38 (p=0.0055) and ZAP70 (p=0.0360) were more often expressed, which are known to be negative prognostic markers. A higher percentage of monoclonal proteins ($p=3.9236x10^{-11}$) in patients with PN (Table I) was also found. The median level of M proteins among patients with PN was 2.44g/L; 6 were IgM/k, 4 IgG/k, 2 IgG/λ, 1 IgM/λ and 1 exclusively λ. The occurrence of monoclonal protein may be coincidental. However 10% of patients with idiopathic PN have a monoclonal serum protein (13), prevalence which is much higher than that in-general population. Conversely, the prevalence of PN in patients with monoclonal protein is close to 5% for IgG paraproteins, 15% for IgA, and 30–50% for IgM, the latter being often associated with antibody reactivity to peripheral nerve antigens (14).

It is interesting to note that PN was more common in previously treated patients (Table 1, $p=4.9820x10^{-5}$), although CLL chemo-immunotherapeutic agents (fludarabine, bendamustine,

cyclophosphamide, clorambucil, rituximab, etc.) are not known to cause iatrogenic PN. It is likely that duration and severity of the disease may play a role.

Furthermore, we want to point out that 2 out of 12 subjects (17%) treated with lenalidomide developed PN after a median treatment of 28 months (range 6-32 months). This percentage is high when compared with the incidence of PN in lenalidomide-treated multiple myeloma patients (15, 16).

The Kaplan-Meier analysis showed that the estimated 10 years overall survival (OS) for patient with and without PN was 73% and 78% (Log-rank test, p=0.9236), respectively (Figure 1D). As a consequence, patients with PN do not harbour and increased risk of death than patients without PN.

Among our cohort of patients, 36 (4.4%) experienced VZV re-activation causing zoster radiculopathy. Interestingly, in one of these 36 cases both sensory and motor fibers were involved as documented by severe muscle weakness and electromyography. Involvement of motor fibers in VZV radiculopathy is a rare event, which might have been favored by the derangements in the immune regulation that characterizes CLL patients. Furthermore, we also identified 4 (0.5%) cases of idiopathic facial nerve palsy.

Neurologic manifestations in patients with CLL may be numerous and various (gait disorder, paresthesia, headache etc.). Recently, a group from Mayo clinic has extensively described the Central Nervous System (CNS) complications in CLL patients, showing that the most common etiologies were infections (1%), autoimmune/inflammatory diseases (0.7%), direct CNS involvement by CLL (0.4%), CNS Richter syndrome (0.3%) and other cancers (0.2%). The authors concluded that in almost 80% of patients neurologic symptoms are due to causes different from CLL (3).

However, the exact relationship between neurological manifestations and CLL remains to be elucidated, and may be difficult to establish whether they are exclusively neoplastic, paraneoplastic, inflammatory, iatrogenic or simply incidental (17). It is likely that different pathogenic mechanisms take part in the genesis of neurological diseases.

We herein provide evidence that PN is not a so rare complication in patients with CLL. After ruling out PN due to concomitant diseases or iatrogenic, a percentage of CLL-associated PN still remains. Its incidence increases during follow-up and occurs more commonly in subjects with unfavorable biological prognostic makers, especially in those who present a monoclonal protein. The high percentage of CIDP in our cohort (25% of the patients with neuropathy) deserves consideration and is consistent with the frequent occurrence of demyelinating polyradiculoneuritis in patients with non-Hodgkin lymphoma (12).

AUTHORS' CONTRIBUTIONS

CB and AV designed the study, evaluated patients and wrote the article; AV performed statistical analysis; AS, SI, MartaC and FP provided intellectual inputs and evaluated the patients; MarioC performed neurophysiological studies; FF performed immunophenotypic and molecular analysis. GS and LT evaluated patients, provided intellectual input and reviewed the article. All the authors declare that they have no competing interests.

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Table I. Clinical and biological characteristics of the whole population, patients with and without peripheral neuropathies (PN and no-PN, respectively).

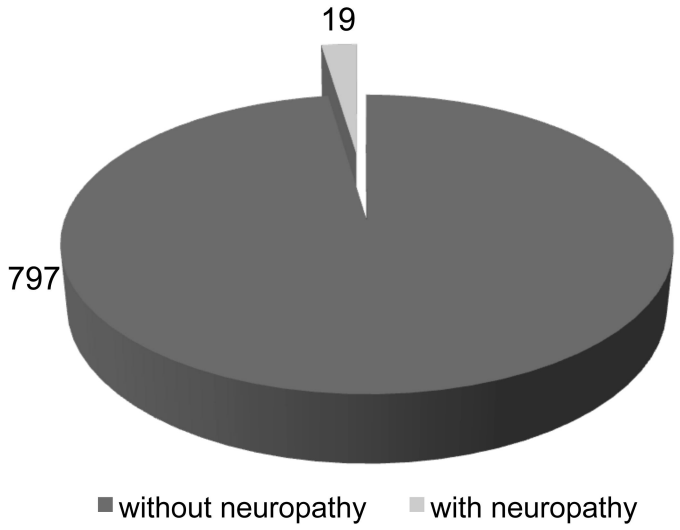
	Population (n=816)	PN (n=19)	No-PN (n=797)	p value
GENDER				
female	327 (40%)	4 (21%)	323 (40%)	0.1006
male	489 (60%)	15 (79%)	474 (60%)	
AGE AT DIAGNOSIS				
<65	439 (54%)	9 (47%)	430 (54%)	0.6447
≥65	377 (46%)	10 (53%)	367 (46%)	
TREATMENT				
treated	317 (39%)	16 (84%)	301 (38%)	4.9820×10^{-5}
not treated	499 (61%)	3 (16%)	496 (62%)	
MONOCLONAL PROTEINS				
Yes	84 (10%)	14 (74%)	70 (9%)	3.9236×10^{-11}
No	732 (90%)	5 (26%)	727 (91%)	
STAGE AT DIAGNOSIS				
0-I	638 (78%)	14 (74%)	624 (78%)	0.1482
II	131 (16%)	2 (10%)	129 (16%)	
III-IV	47 (6%)	3 (16%)	44 (6%)	
IGHV				
M-IGHV	310 (60%)	6 (55%)	304 (60%)	0.7623
U-IGHV	209 (40%)	5 (45%)	204 (40%)	
FISH				
13q- & N	389 (72%)	8 (47%)	380 (73%)	0.0210
+12	62 (12%)	2 (12%)	60 (11%)	
11q- & 17p-	87 (16%)	7 (41%)	84 (16%)	
CD38				
<30%	493 (74%)	6 (37%)	487 (75%)	0.0022
≥30%	176 (26%)	10 (63%)	166 (25%)	
ZAP70				
<20%	308 (57%)	3 (23%)	305 (58%)	0.0201
≥20%	234 (43%)	10 (77%)	224 (42%)	
TP53 ABN				
yes	53 (10%)	3 (18%)	50 (10%)	0.2294
no	485 (90%)	14 (82%)	471 (90%)	

PN= peripheral neuropathy. IGHV = immunoglobulin heavy chain mutational status; unmutated IGHV (U-IGHV) was defined by a homology sequence >98%, otherwise the gene was considered mutated (M-IGHV). ABN = abnormalities. TP53 ABN included 17p13 deletion and/or TP53 mutation.

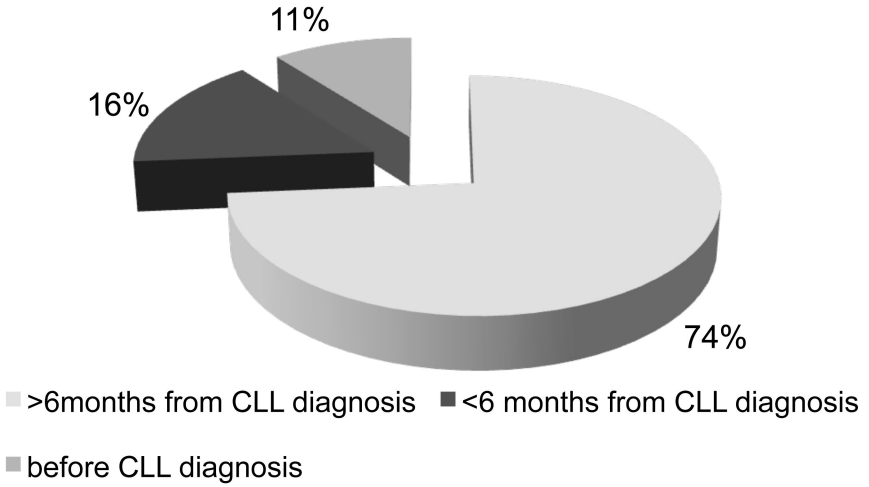
Legend to figure

The upper left panel (A) shows the prevalence of neuropathy among the whole population; 19 subjects suffered from peripheral neuropathy. The upper right panel (B) shows the distribution of onset of peripheral neuropathy according to the diagnosis of CLL: 2 of 19 patients (11%) developed PN before CLL, 3 (16%) and 13 (74%) subjects before and after 6 months from CLL diagnosis, respectively. The bottom left panel (C) shows the Kaplan-Meier curve for time to peripheral neuropathy. In this analysis we did not include the 3 patients who developed PN before CLL diagnosis. The bottom right panel shows the Kaplan-Meier curves for overall survival between patients with and without neuropathy (p=0.9236).

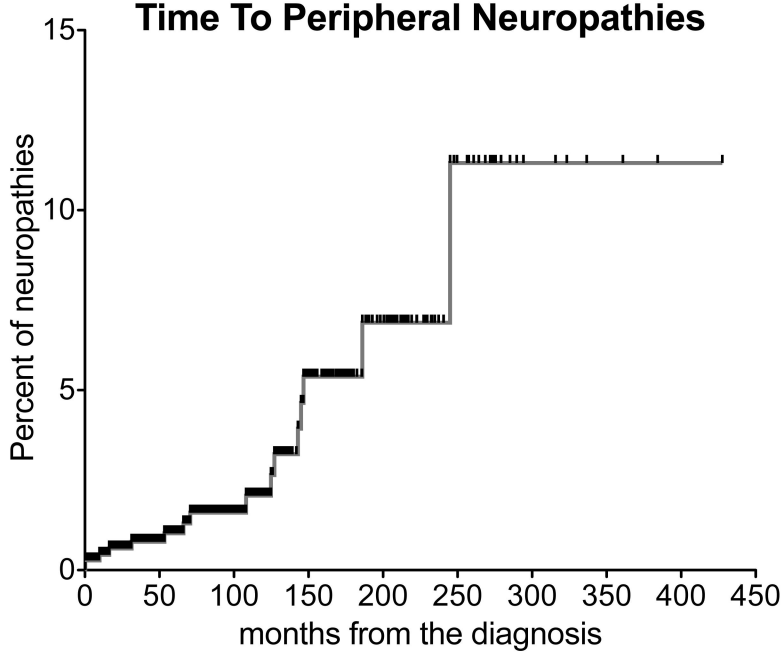
A


19
797
without neuropathy
with neuropathy

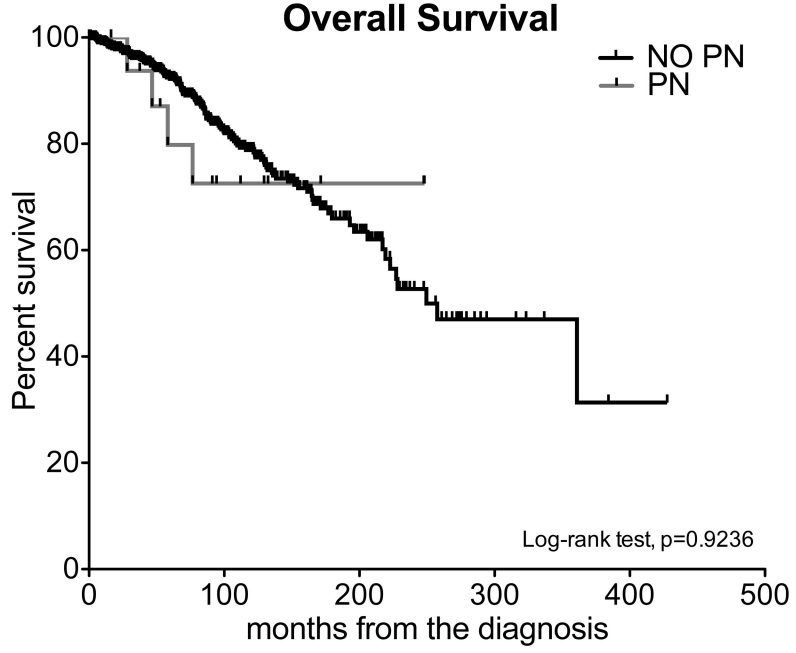
B


11%
16%
74%
>6months from CLL diagnosis
<6 months from CLL diagnosis
before CLL diagnosis

C


Time To Peripheral Neuropathies
Percent of neuropathies
0
5
10
15
0
50
100
150
200
250
300
350
400
450
months from the diagnosis

D


Overall Survival
NO PN
PN
Percent survival
0
20
40
60
80
100
0
100
200
300
400
500
months from the diagnosis
Log-rank test, p=0.9236

SUPPLEMENTARY METHODS

Prognostic factors were evaluated on fresh sample or on purified frozen chronic lymphocytic leukemia (CLL) cells harvested in dimethyl sulfoxide (DMSO), collected before chemo-immunotherapy according to recent guidelines[1].

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization (FISH) was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridized with the multicolor probe sets LSI p53/LSI ATM, LSI D13S319/LSI 13q34/ CEP12 and RP11-177O8 (Vysis-Abbott, Des Plaines, IL, USA) according to the manufacturer's protocol. Three hundred interphase nuclei were analyzed for each probe. According to the literature, cut-off for positive values (mean of normal control ±3 standard deviation) was 4% for centromere 12 trisomy, and 10% for deletion of 11q22.3, 13q14.3 and 17p13.1 [2,3].

Immune globulin heavy chain variable region mutation (IGHV)

To perform IGHV studies, RNA was extracted from $2x10^6$ B cells using the RNeasy™ Total RNA kit (Qiagen) and reverse transcribed using the SuperScript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc.). The CLL cell VH gene family was assigned as previously described [3] using a sense VH family-specific framework region (FR) primer in conjunction with the appropriate antisense CH primer. VH gene sequences were determined by amplifying 5µl of the original cDNA using the appropriate VH leader and CH primers. PCR products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using IMGT/VQUEST and BLAST software [4] to detect VDJ junction. Sequences homology ≤98%, from the corresponding germline gene, were considered mutated, as opposite to unmutated cases [3,5].

CD38 expression

Analyses of CD38 expression on CLL cells was carried out by incubating whole blood with 5µl of the following antibodies: anti-CD5 FITC (BD Biosciences, New-Jersey, USA), anti-CD38 PE (BD Biosciences, New-Jersey, USA), and anti-CD19 RPECy5 (BD Biosciences, New-Jersey, USA), for 20 minutes and at least 100,000 events were counted. Each sample was run with the appropriate isotype control antibody to define the negatively stained cells. The percentage of $CD38^+$ cells was defined as the percentage of $CD19^+$ $CD5^+$ that were $CD38^+$. The threshold for CD38 expression was set at 30%; values ≥30% were defined as $CD38^+$ and <30% as $CD38^-$ [3,6].

ZAP70 expression

Cytoplasmic ZAP70 expression was determined by flow cytometry. Permeabilized cells were analyzed with the anti-ZAP70 antibody Alexa Fuor488 (Caltag, Buckingham, UK), anti-CD3-phycoerythrin (PE), anti-CD56-PE (BD Biosciences, New-Jersey, USA), anti-CD19-peridinin chlorophyll protein-cytochrome 5,5 (Caltag, New-Jersey, USA) and anti-CD5 APC (BD Biosciences, New-Jersey, USA). After appropriate lymphocyte gating, cytoplasmic ZAP70 expression was determined in $CD19^+$ $CD5^+$ CLL cells. The threshold level for ZAP70 was set at 20% [3,7].

Statistical analysis

Categorical variables were compared by Chi-square (Stage and FISH) and Fisher exact test (all the other variables), when indicated, while continuous variables were compared by Mann-Whiney test. Time to peripheral neuropathy (PN) (TTPN) and overall survival (OS)

were calculated from the date of CLL diagnosis to PN or death (event), respectively, or last available follow-up (censored). Survival analyses were performed by Kaplan-Meier method and Log-rank test was used to compare OS curves between groups. Statistical analysis was performed with R (an open source statistical package downloadable from http://www.r-project.org).

Neurological and Neurophysiological evaluation

Neurologic assessment included strength, sensory, gate and cranial nerve evaluation; neurophysiological evaluation included electromyography, motor and sensory nerve conduction velocity, and short-latency somatosensory-evoked potentials.

Electrophysiological investigation included motor and sensory nerve conduction studies (NCS) and needle electromyography (EMG).

Compound motor action potential (CMAP) amplitude, motor conduction velocities (MCV) and distal motor latency of median, ulnar, deep fibular and tibial nerves were performed. Presence of conduction blocks were ascertained at and outside the compression sites.

Sensory nerve action potential (SNAP) amplitude and sensory conduction velocities (SCV) of median, ulnar, radial, sural and dorsal sural nerves (the most distal branch of sural nerve) were performed bilaterally.

EMG was performed using a concentric electrode: spontaneous activity, motor unit potential configuration, and recruitment were scored semi-quantitatively. The skin temperature was maintained at ≥32°C throughout the study.

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