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High expression of Cathepsin D in Non-Hodgkin's Lymphomas negatively impacts on clinical outcome

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Abstract. The lysosomal protease Cathepsin D (CD) has been implicated in the homeostasis of lymphatic tissues. We investigated whether the level of CD expression influences the progression and the clinical outcome in Non-Hodgkin's Lymphomas (NHLs). The expression of CD was assessed by immunohistochemistry and immunofluorescence in biopsies of Diffuse Large B Cell Lymphomas (DLBCL, 35 cases), Follicular Lymphomas (FL, 9 cases of grade I-II plus 14 cases of grade IIIB), Chronic Lymphocytic Leukaemias (CLL, 17 cases) and Peripheral T-cell Lymphomas (PTCL, 5 cases). CD staining showed a cytoplasmic punctate pattern compatible with its lysosomal localization. Based on the level of CD expression and the proportion of positive cells, lymphomas were classified as 'low expressing' ($<$ 20% of tumor cells) or 'highly expressing' ($\geqslant$ 20% of tumor cells). Lymphomas highly expressing CD were associated with a worse stage (III-IV) at diagnosis (31/34 cases; $p = 0.002$) and with a poor clinical outcome (i.e., partial remission and death; 28/34 cases; $p = 0.03$). In the subgroup of aggressive/high grade of malignancy lymphomas (i.e., DLBCL, FL IIIB and PTCL), the Kaplan-Meier curve revealed a very low cumulative overall survival probability ($\sim$20% at 5 year) for patients bearing a NHL with $>$ 40% CD-positive cells compared to that of patients bearing a NHL with $<$ 20% CD-positive cells ($\sim$70% at 5 year). This correlation was statistically significant (log-rank test, $p = 0.01$). In Cox multivariate analysis CD failed to be a prognosticator independent of pathologic stage, though the hazard ratio confirmed the association of low expression with a better survival probability. These data indicate that the presence of a high percentage of CD-positive tumor cells negatively reflects on the progression of NHLs.

Keywords: Cathepsin D, tumor marker, prognosis, lymphomas

Abbreviations

CD:	Cathepsin D
CLL:	(B-cells) Chronic lymphocytic leukaemia
DLBCL:	Diffuse large B-cell lymphoma
FL:	Follicular (center) lymphoma
PTCL:	Peripheral T-cell Lymphoma
IF:	immunofluorescence
IHC:	immunohistochemistry
NHL:	Non Hodgkin's Lymphoma
OS:	overall survival
WHO:	World Health Organisation.

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1. Introduction

Non-Hodgkin's Lymphomas (NHLs) encompass malignant lymphoproliferative disorders that originate

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from B or T-lymphocytes (and rarely form Natural Killer cells), and that are heterogeneous for morphology, immunophenotipic and cytogenetic profiles, and biological characteristics. On the basis of the clinical presentation and the patient's life expectancy, oncologists categorize NHLs in 'aggressive' or 'indolent' lymphomas, which correspond to the intermediate/high grade of malignancy and low grade of malignancy subgroups, respectively (reviewed in 1,2). Beside the five factors of the International Prognostic Index (age, stage, serum LDH, performance status and number of extranodal sites) commonly used, the gene expression signature of the NHL greatly concurs to better assess the prognostic profile of the patient. Accordingly, a wide range of proteins that monitor or directly control cell proliferation, cell differentiation or cell death (e.g., bcl-6, bcl-2, p53, Ki-67, nm-23, c-FLIP and survivin) have been proposed as prognostic markers in NHLs [3–9].

Cathepsin D (CD) is a lysosomal aspartic protease ubiquitously expressed in human tissues, in which it can be present under three molecular forms: the precursor (enzymatically inactive), the intermediate single-chain and the mature double-chain polypeptides (both the latter are enzymatically active). CD was shown of vital importance for cell survival and tissue homeostasis, as deduced from the phenotype of mice homozygously knocked-out for CD [10]. These mice are not vital beyond the third-fourth week after birth and show atrophy of primary lymphoid organs, intestine and heart [10]. CD has been shown to play an active role in the apoptotic response of cancer cells to chemotherapy drugs [11–14] or death-inducing cytokines [15,16], following its translocation into the cytosol. The precursor of CD, which is highly secreted by cancer cells, has been shown to stimulate the growth and the activation of peripheral human lymphocytes [17,18]. The regulatory function of CD in cell proliferation and apoptosis in lymphoid tissues potentially implicates this molecule in the pathogenesis and progression of NHLs. To test this hypothesis, we assessed the expression of CD in NHL tumor cells and searched for possible correlations between the proportion of CD-positive lymphoma cells and the clinico-pathological parameters at diagnosis and the response to therapy. CD expression was analyzed by immunohistochemistry (IHC) and immunofluorescence (IF) in a wide series of NHLs, which included 54 cases classified as 'aggressive' or high grade of malignancy and 26 cases classified as 'indolent' or low grade of malignancy. Our findings indicate that the presence of a high percentage of CD-positive tumor cells negatively reflects on the clinical response to chemotherapy of NHLs.

2. Materials and methods

2.1. Patient cases and histological diagnosis

A series of paraffin-embedded biopsies was selected in the years 2000–2006 from the files of the Department of Pathology of Università del Piemonte Orientale (Novara, Italy). This included the following histologic types classified according to the current WHO Classification of Lymphoid Neoplasms [19]: 35 Diffuse Large B-Cell Lymphomas (DLBCL), 23 Follicular (center) Lymphomas (FL), 17 B-cells Chronic Lymphocytic Leukaemia (CLL) and 5 Peripheral T-cell Lymphomas (PTCL). Routine immunophenotyping with a panel of antibodies corroborated the histological diagnosis.

All patients were subjected to chemotherapy and/or radiotherapy, following standard criteria based on clinical stage, histologic type and patient performance status. The study was conducted in conformity of the rules currently approved by the local ethic committee.

2.2. Immunohistochemistry and Immunofluorescence

Immunohistological analysis was performed independently by four investigators (GV and SK for IHC; CI and GN for IF) on biopsies obtained prior to any therapy. Formalin-fixed paraffin-embedded tissue sections were cut from all NHL cases. IHC was performed using a standard procedure as previously described in details [7]. CD was revealed by incubating the sections for 30 min with a mouse monoclonal antibody (diluted 1: 500; clone BC011) purchased from Calbiochem (San Francisco, CA). After washing out the unbound antibody, the sections were further incubated for 30 min with a peroxidase-conjugated polymer carrying an antibody against mouse immunoglobulins (ChemMate DAKO EnVision kit; DAKO Cytomation Glostrup, Denmark). After washing out the excess of antibody, a reaction product was developed by using the ChemMate Diaminobenzidine as chromogen (DAKO Cytomation). Negative control was made either by omitting the primary antibody and by using in the first step an isotype matched polyclonal antibody specific for HBcAg (Dako; diluted 1:200) (not shown). Finally, the sections were counterstained with hemalume (Merck, Germany). Non neoplastic cells were excluded from the evaluation. Macrophages were identified by staining with an anti-CD68 monoclonal antibody (Dako).

Representative areas were imaged using a microscope (Leitz Diaplan, Germany) equipped with a digital camera. The number of CD-positive tumor cells was evaluated by manual counting, in agreement with criteria well established in the pathologists community. At least 10 fields for each case were considered. The cells were judged as positive when the reaction product was well detectable at low magnification.

For IF staining, the sections (prepared as above) were incubated first with the primary antibody (1: 100 in phosphate buffer saline supplemented with 0,1% Triton X-100 and 4% Fetal Calf Serum, for 16 h in a humid chamber at 4°C) and then with Texas Red- or FITC-conjugated secondary antibodies (diluted 1:200 as above, for 1 h at room temperature in a humid chamber). Excess of unbound antibody was removed at each step by two washes with phosphate buffer saline. As negative control, the primary antibody was omitted and the sections were incubated only with the secondary antibody. The nucleus was evidenced by staining the chromatin with the fluorescent dye Propidium Iodide (PI), as previously reported [7]. Stained sections were mounted with Slow-FAD (Light AntiFADE kit, Molecular Probes) and observed under a laser confocal immunofluorescence microscope (Leica Fluorescent Microscopy DMI600, Heidelberg, Germany). Each biopsy was tested at least three times. At least four fields per section were imaged and evaluated by manual counting. Macrophages, which expressed CD at high level, were identified by double-staining with both anti-CD and anti-CD68 monoclonal antibody (Dako) and excluded from evaluation. Representative images are shown.

2.3. Western blotting

Pieces from frozen biopsy were cut and finely homogenized by several cycles of freeze-thawing and sonication in a phosphate buffer containing detergents (Triton X-100 and Na-deoxycholate) and a cocktail of protease inhibitors. A 50 μg of protein homogenate was resolved by SDS-polyacrylamide (13.5%) gel electrophoresis under reducing conditions. Electrotransfer and immunodetection were carried out as previously described [14]. CD polypeptides were detected with the same monoclonal antibody used for IHC and IF (Calbiochem, San Francisco, CA). Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-anti-mouse antibody (1:20.000) and subsequent peroxidase-induced chemiluminescence reaction (Biorad, Hercules, CA, USA).

2.4. Statistical analysis

Data were statistically analysed with the Microsoft Excel XLStat 2008 software to calculate the Odds Ratio (O.R.) and to perform the Chi-square test. The Fisher's exact test was also employed for pair wise comparison of distributions of categorized groups. The probability of overall survival (OS) for patients was estimated by the Kaplan-Meier method [20]. OS was calculated from the date of initial diagnosis until the date of last follow-up or patient's death. Surviving patients were censored at the date of last contact and mathematically removed from the curve. Patients who died were censored at the date of death. The significance of differences between survival curves was calculated by using the log-rank test. To determine the relative influence of CD and of pathologic stage on OS, univariate and multivariate analyses were also performed using the Cox proportional hazard model [21]. These analyses yielded the chi-square, hazard ratios, the confidential intervals and p values. To indicate statistical significance the threshold for p values was taken at 5% level. Statistics was performed using the XL-STAT 2010 software.

3. Results

3.1. Patients' clinical parameters

The relevant clinical data of the patients diagnosed with NHL included in the present study are reported in Table 1. The following information were available: clinical stage at first diagnosis, histological type, objective response to chemo- and radiotherapy, and clinical outcome. On pathologic bases, the histologic types belonged to a subgroup classified as NHLs of intermediate/high grade of malignancy and to a subgroup classified as NHLs of low grade of malignancy. The first subgroup encompassed DLBCL, FL of grade IIIB (showing a higher proportion of centroblasts and a partially diffuse pattern) and PTCL. These neoplasms are characterized by an aggressive clinical behaviour (reviewed in 2). The second subgroup encompassed CLL and FL of grade I and II. These neoplasms show an indolent clinical behavior (reviewed in 1). Response to therapy regimen was evaluated according to the international guidelines. Clinical outcomes were classified as complete remission (CR, disappearance of any evidence of disease for at least 24 months), partial remission (PR, =/> 50% decrease of tumor lesions for at least 24 months) and exitus (DdD, Death due to the

Table 1
Clinical characteristics of NHL cases. Data set of NHL patients included in the present study. Abbreviations used are: CLL, Chronic Lymphocytic Leukaemia; DLBCL, diffuse large B-cell lymphoma; FL, Follicular Lymphomas; PTCL, Peripheral T-cell Lymphomas. Pathological staging (*one lacking data) and clinical outcome are indicated. CR, Complete Remission.

Histologic type	Sex	n. cases	age (range)	Median age	Stage		CR rate	Survival rate (5y)
					I-II	III-IV		
DLBCL (35)	m	19	44–78	67	14*	20	43%	60%
	f	16	27–81	73				
FL I-II (9)	m	1	69	63	2	7	22%	77%
	f	8	48–73					
FL IIIB (14)	m	6	35–75	62	5	9	50%	86%
	f	8	49–79	73				
CLL (17)	m	9	34–81	69	1	16	12%	76%
	f	8	66–85					
PTCL (5)	m	3	49–73	68	0	5	0%	0%
	f	2	73–74	73.5				

Disease). Poor clinical outcome included no response at all (progression and exitus) or only partial response to therapy (PR). The survival rate was calculated at 5 years post-diagnosis.

3.2. Cathepsin D expression in tumor cells of NHL tissue sections

The expression of CD was analyzed by IHC and by IF in biopsy sections obtained from patients diagnosed for NHL and not yet subjected to therapy. Results from both techniques overlapped, though some cases judged negative on IHC appeared faintly positive on IF owing to the highest sensitivity of the latter technique. Representative images of CD expression and cellular distribution, as assessed by IHC and IF in selected cases, are shown in Figs 1 and 2, respectively. CD was detected in the cytoplasm of neoplastic cells. At high magnification, CD positivity appeared as distinct spots in the cytoplasm, a pattern consistent with its intra-lysosomal localization. The nuclei of tumor cells were constantly negative for CD. In most of the cases, CD-positive spots appeared thoroughly distributed in the cytoplasm, whereas in few other cases the reaction product appeared localized in a small cytoplasmic area proximal to the nucleus. Macrophages infiltrating the tumor tissue were strongly positive for CD. These cells, easily recognizable on the basis of their morphology and dimension, were identified as CD68 positive. Apoptotic cells within the tumor context were recognized on the basis of chromatin condensation and fragmentation, as evidenced by PI staining (see inset in Fig. 2).

In order to validate the specificity of the anti-CD antibody, we analyzed by western blotting the molecular forms of CD expressed by tumor cells. The analysis was performed in 20 cases, for which a frozen biopsy was available. A selection of these cases, representative of different levels of CD expression, is shown in Fig. 3. Specific bands revealed by the antibody correspond to the following molecular forms of CD: i) the precursor (P), enzymatically inactive, of $\sim$53 kDa; ii) the intermediate single-chain (I) of $\sim$48 kDa; and iii) the large-chain of the mature (M, $\sim$31 kDa) double-chain. The latter is by far the most represented molecular form in all samples. In some cases, the large-chain of the double-chain form appears as a doublet (of 33 and 31 kDa), likely representing micro-heterogeneities generated by aminoacid trimming within the lysosomes.

CD expression was evaluated semiquantitatively by IHC in all samples. Only tumor cells were considered. CD-positive macrophages, recognized by morphology and by their CD68 positivity, were excluded from the counting. Based on the proportion of CD-positive cells within the tumor tissue, the extent of CD expression was initially stratified into four ranges of positivity: 0 to 10%; 10 to 20%; 20 to 40% and more than 40%. Negative expression, that is a basal expression faintly detectable or under the detection limit of the method, was classified as $< 10\%$. More than 50% of the tumors fell into the 0-20% range of expression (Table 2A). The final threshold was set at 20% CD-positive cells. The percentage of CD-positive tumor cells in the various NHL histologic types examined is reported in Table 2B. Altogether, 46 tumors showed a low percentage ($<$ 20%) of cells over-expressing CD and 34 tumors were characterized by a high ($\geqslant$ 20%) percentage of positive cells. No statistically significant correlation was found between the extent of CD expressing cells and the histologic type. We note, however, that the large majority of DLBCL (27/35) were characterized by a low proportion of cells positive for CD. When tumors were categorized for pathological malignancy, it was

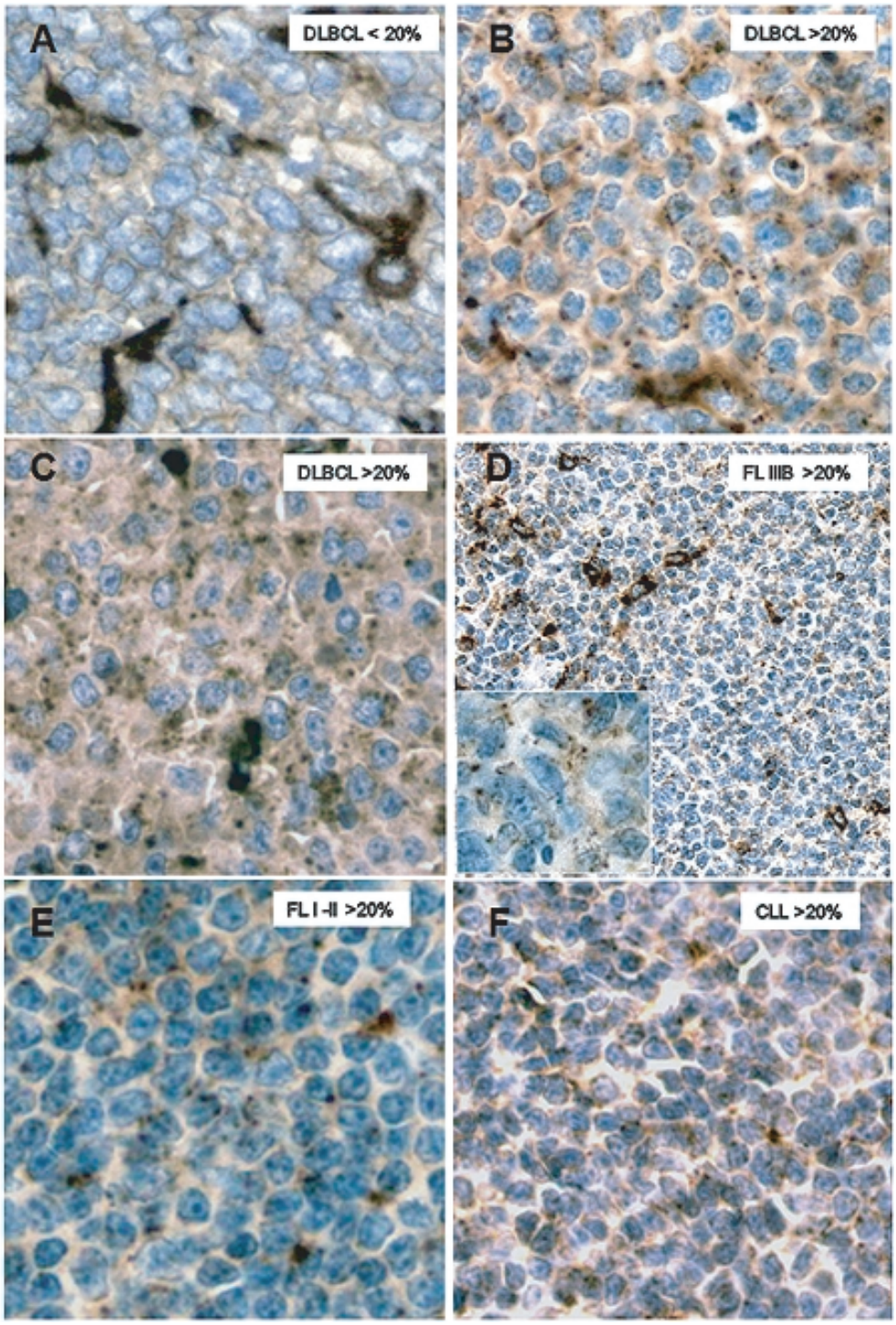

Fig. 1. Cathepsin D immunohistochemical expression in tissue sections of NHLs. IHC images refer to representative fields of selected cases of different histologic types. Acronymus for NHL histologic types as follow: DLBCL, Diffuse Large B Cell Lymphoma; FL, Follicular Lymphoma; CLL, Chronic Lymphocytic Leukaemia. The reaction product, indicative of positivity, show a punctate pattern scattered in the cytoplasm of tumor cells. The Cathepsin D (CD) expression score assigned to the case is reported. The inset in panel D at higher magnification shows the punctate staining of CD, indicative of lysosomal localization. Original magnification: 440x in panels A, B, C, E, F and 220x in panel D).

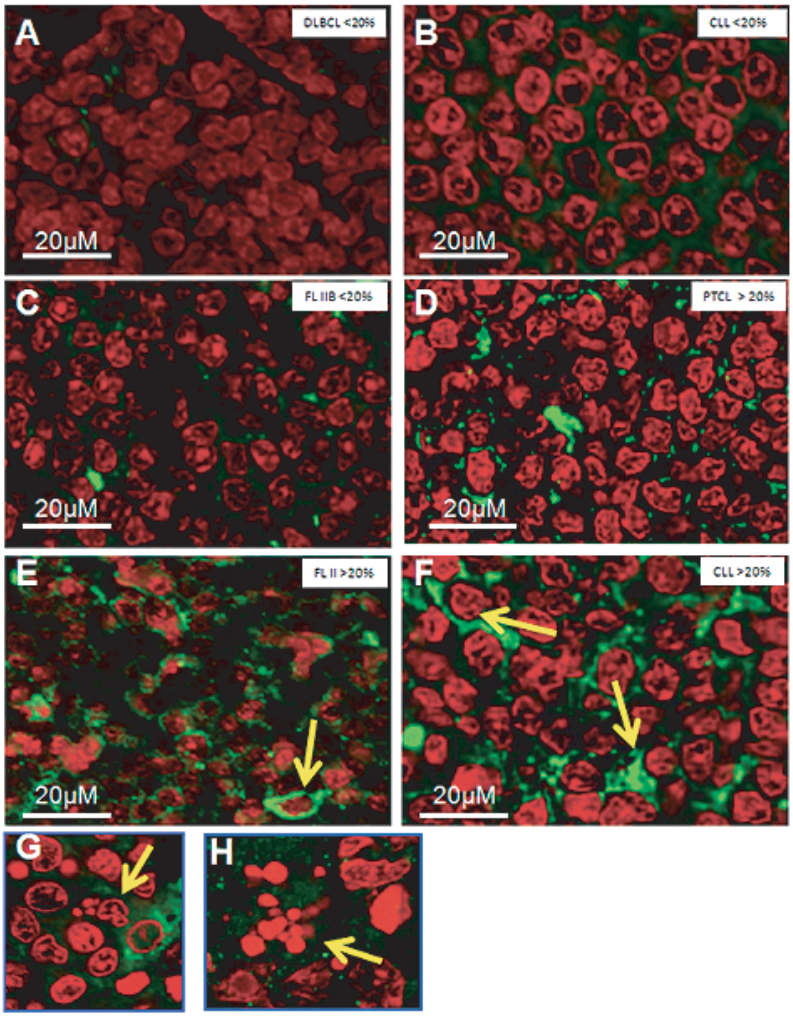

Fig. 2. Immunofluorescence staining of Cathepsin D in tissue sections of NHLs. Immunofluorescence staining of CD and propidium iodide (PI) counter-staining of nuclei of NHL sections. Acronymus for NHL histologic type as follow: DLBCL, Diffuse Large B Cell Lymphoma; FL, Follicular Lymphoma; CLL, Chronic Lymphocytic Leukaemia, PTCL, Peripheral T Cell Lymphoma. The proportion of CD-positive cells is indicated. The arrows point to macrophages highly expressing CD. The punctate pattern of CD staining can be appreciated in panel F. Panels G and H show examples of apoptotic cells with condensed and fragmented chromatin, as stained by PI. Scale bar = 20 μm.

found that approximately one-third of the high-grade (Hg) of malignancy subgroup and two-third of the low-grade (Lg) of malignancy subgroup presented with a high proportion of CD-positive cells (Table 2C).

3.3. Correlation of CD expression with clinico-pathological parameters at diagnosis and clinical outcome

We asked about the biological significance of our finding and investigated whether the level of CD expression reflected on clinico-pathological features of NHLs. First, we checked for any correlation with the pathological stage at diagnosis (Table 3A). Strikingly, 31 out of 34 (91%) NHLs expressing a high percentage of CD-positive cells were diagnosed at stages III and IV (O.R. = 7; $p = 0.002$). When aggressive (high grade of malignancy, Hg-NHL) and indolent (low grade of malignancy, Lg-NHL) subgroups of NHLs were considered separately, only the former subgroup showed a statistically significant correlation (O.R. = 16; $p = 0.005$) between CD expression and pathological staging (Tables 3B–C).

Next, we performed a statistical analysis to correlate the IHC data with the clinical outcome. From the data reported in Table 4, we note that NHLs highly express-

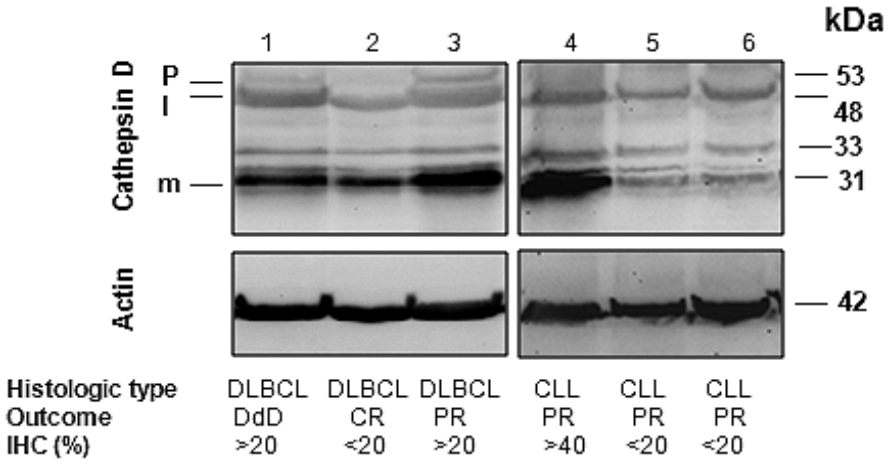

Fig. 3. Western blotting analysis of cathepsin D expression. Representative western blotting of CD in DLBCL and CLL expressing different proportion of CD-positive cells within the tumor context. The level of CD expression as assessed by IHC is reported. Based on IHC, samples 3 and 4 presented macrophage infiltration amounting to 5% and 8%, respectively. Molecular forms of CD: P, precursor (∼53 kDa); I, intermediate single-chain (∼48 kDa); M, large-chain (∼31 and 33 kDa) of the mature double-chain. The blot was stripped and re-probed for actin as marker for control loading of tissue proteins in the lanes. Data on CD expression as assessed by IHC and clinical outcome of the tumor analyzed are also reported (abbreviations as in Tables 1 and 3).

ing CD associated with a poor clinical outcome of patients ($p = 0.03$). In this group, only 6 patients (17.6%) underwent complete remission (CR), whereas in the group of patients bearing a NHL with a low positivity for CD as many as 20 out of 46 (43.5%) underwent CR. These correlations were statistically significant ($p = 0.05$) also when clinical outcome was considered separately as CR, PR and Death-due-to-Disease (DdD). The aggressive and indolent subgroups of NHLs were then considered separately (Tables 5A–B). Both in the two subgroups, only 3 patients out 17 bearing a NHL highly expressing CD experienced a CR. However, it was found that only in the subgroup of aggressive histologic types the correlation between the percentage of CD-positive tumor cells and the clinical outcome was statistically significant ($p = 0.04$).

We further investigated whether in the Hg-malignancy subgroup of NHLs the expression of CD could be of prognostic significance. To address this point, we estimated the overall survival (OS) probability of NHL patients by the Kaplan-Meier method. Figure 4 shows the OS Kaplan-Meier curves relative to the group of patients bearing an aggressive, Hg-NHL. Log-rank test was applied to test the statistical significance of such correlation. Patients bearing a Hg-NHL that expresses a low percentage ($< 20\%$) of CD-positive cells showed a ∼72% OS at 5-year, regardless of the NHL histologic type (Fig. 4). By contrast, Hg-NHLs highly expressing CD ($\geqslant 20\%$ of CD-positive cells) associated with a low probability of OS of patients. In particular, in these patients the 5-year OS probability was ∼34% (Fig. 4A). Statistical significance determined by the log-rank test was $p = 0.06$. To see whether OS probability was related to the proportion of CD-positive cells within the tumor, we reconsidered the stratification of CD expression in the Hg-NHLs highly expressing CD setting the cut-off at $> 40\%$. No one of the patients bearing a NHL with such a high proportion of CD-positive cells underwent CR. We calculated the Kaplan-Meyer curve for these patients (i.e., bearing a NHL with $> 40\%$ CD-positive tumor cells) in comparison with patients bearing a NHL expressing a low percentage ($< 20\%$) of CD-positive cells (Fig. 4B). Patients bearing a very-high CD-expressing Hg-NHL ($n = 11$) revealed at 5 year an OS probability of ∼20%. In this case, log-rank test indicated that the different OS probability among the two groups of patients was statistically significant ($p = 0.01$; Fig. 4B). To see whether the correlation between CD expression and OS was associated with individual histologic subtypes the univariate Kaplan-Meier analysis was applied to DLBCL, the most represented lymphoma in our series. At 5 years, patients ($n = 27$) whose tumor contained less than 20% of CD-positive cells showed an OS probability of 65%, whereas the patients ($n = 8$) whose tumor contained $\geqslant 20\%$ CD-positive cells showed an OS probability of 20% (data not shown). However, owing to the small numbers, the difference in OS between groups did not reach the statistical significance (log-rank test, $p = 0.23$).

Table 2
Cathepsin D (CD) expression in NHLs. The number of CD-positive tumor cells were evaluated by immunohistochemistry in serial sections from NHL tissues (see Fig. 1) and the score was assigned as explained in Materials and Methods. A) Stratification of CD positivity in the high-grade (Hg) and Low-grade (Lg) of malignancy sub-groups of NHLs. B) CD positivity in the various histologic types of NHLs examined. Abbreviations used as described in the legend to Table 1. C) Distribution of CD positivity in Hg- and Lg-NHLs.

A

CD expression	Hg-NHLs	Lg-NHLs	n. cases
0-20%	37	9	46
20-40%	6	3	9
40-60%	5	1	6
>60%	6	13	19
Total	54	26	80

B

HISTOLOGIC TYPE / CD expression	DLBCL	FL I-II	FL IIIB	CLL	PTCL	n. cases
< 20%	27	3	10	6	0	46
≥ 20%	8	6	4	11	5	34
Total	35	9	14	17	5	80

C

% CD positive	Hg-NHLs	Lg-NHLs	n. cases
< 20%	37	9	46
≥ 20%	17	17	34
Total	54	26	80

Chi square= 6.926
DF=1
p=0.0085
Fisher exact test p=0.007

3.4. *Overall survival as influenced by Cathepsin D expression and pathologic stage in multivariate analysis*

As predictable, inferior pathologic stage was associated with better clinical outcome (Table 6). Of the 22 patients with NHL staged I-II, only 3 died (∼14%) and as many as 14 (∼64%) underwent CR; whereas, of the 57 patients with NHLs staged III-IV, 23 died (∼40%) and only 12 (∼21%) underwent CR. Similarly, of 19 patients with Hg-NHL staged I-II, only 3 died (∼16%) and 13 (∼68%) underwent CR; whereas, of the 34 patients with Hg-NHL staged III-IV, 17 died (50%) and only 9 (∼26%) underwent CR. We then calculated the OS probability with the Kaplan-Meier method in the groups of patients staged I-II and III-IV. In the whole series, the OS probability of patients staged I-II ($n = 22$) and of those staged III-IV ($n = 57$) were ∼88% and ∼42%, respectively (curves not shown) (log-rank test, $p = 0.021$). In the Hg-NHL series, the OS probability of patients staged I-II ($n = 19$) and of those staged III-IV ($n = 34$) were ∼85%

Table 3
Correlation between the level of Cathepsin D expression and pathological staging. Statistical correlation between the proportion of Cathepsin D-positive cells in NHL and the pathologic stage at presentation. In the whole series (panel A), Fisher exact test indicated that NHL with $>$ 20% of CD-positive cells statistically associated with stages III-IV at diagnosis ($p = 0.001$). Panels B and C refer to the subgroups of high grade of malignancy (Hg) and of low grade of malignancy (Lg) NHLs, respectively. (*one lacking data)

A

Total-NHLs

STAGE / % CD positive	STAGE I-II	STAGE III-IV	n. cases
< 20%	19	26	45*
≥20%	3	31	34
Total	22	57	79*

Chi square=9
DF=1
p=0.002
Fisher exact test p=0.001
O.R.=7

B

Hg-NHLs

STAGE / % CD positive	STAGE I-II	STAGE III-IV	n. cases
< 20%	18	18	36*
≥20%	1	16	17
Total	19	34	53*

Chi square=7.9
DF=1
p=0.005
Fisher exact test p=0.002
O.R.=16

C

Lg-NHLs

STAGE / % CD positive	STAGE I-II	STAGE III-IV	n. cases
< 20%	1	8	9
≥20%	2	15	17
Total	3	23	26

Chi square=0.3
DF=1
p=0.5

*one lacking data

and ∼45%, respectively (Fig. 5A) (log-rank test, $p = 0.014$). Of the 34 patients with a Hg-NHL staged III-IV, 18 were bearing a tumor with $<$ 20% CD-positive cells (10 survivors plus 8 DdD) and 16 were bearing a tumor with $\geqslant$ 20% CD-positive cells (7 survivors plus 9 DdD). The Kaplan-Meier OS curves of these patients (Fig. 5B) revealed a lower OS probability for the high CD-expressing group ($p = 0.78$).

Above data revealed that Hg-NHLs with a high proportion of CD-positive cells were linked to a high pathologic stage at diagnosis (Table 3) and to poor OS (Fig. 4). To determine whether the prognostic value of CD persisted even when also the pathologic stage was considered, we performed a multivariate analysis of OS using the Cox proportional hazards model. The univariate analysis for CD expression and stage shown in Table 7A confirmed the prognostic value of CD expression in Hg-NHLs ($p = 0.07$ with cut-off = 20%; $p = 0.03$ with cut-off = 40%) and of the pathologic stage in the whole series ($p = 0.03$) and in the Hg-NHLs subgroup ($p = 0.02$). We then carried out the Cox multiple regression to test the prognostic value of CD expression adjusting for pathologic stage. Statistical data reported in Table 7B indicate that the pathologic stage is an independent parameter significantly correlated with OS in the whole series of NHLs ($p = 0.02$), whereas in the Hg-NHLs sub-group the p value does not reach the statistical significance ($p = 0.07$). In the whole series of NHLs, CD expression was not significantly correlated with OS ($p = 0.32$), as expected. In the Hg-NHLs subgroup, because of the small numbers, CD failed to be significantly associated to OS as an independent prognosticator, still the hazard ratio yielded confirmed that tumors low expressing CD (adjusted for the stage) were associated to a higher probability of survival compared to those highly expressing CD.

Table 4
Correlation between the level of Cathepsin D expression and clinical outcome. Statistical correlation between the proportion of Cathepsin D-positive cells in NHL and the clinical outcome (CR, complete remission; PR, partial remission; DdD, death-due-to-disease; other = PR plus DdD). Statistics was calculated for CR vs. poor outcome assumed as PR + DdD (upper panel) or PR and DdD separately (lower panel). Chi-square test indicated a statistically significance correlation between tumors expressing $\geqslant$ 20% CD-positive cells and a poor prognosis.

Total-NHL

Clinical outcome / % CD positive	CR	OTHER	n. cases	
< 20%	20	26	46	
≥ 20%	6	28	34	
Total	26	54	80	Chi square=4.8 DF=1 p=0.03

Clinical outcome / % CD positive	CR	PR	DdD	n. cases	
< 20%	20	10	16	46	
≥ 20%	6	12	16	34	
Total	26	22	32	80	Chi square=6 DF=1 p=0.05

4. Discussion

As in other tissues, homeostasis in the lymphoid tissue is strictly dependent on the balance between cell proliferation and apoptotic cell death. The lysosomal protease CD has been implicated in the control of both these events. In these respect, it is worth noting that mice homozigous knocked-out for CD present with atrophy of lymphoid organs [10]. Moreover, the secreted precursor of CD has been shown to activate and stimulate the growth of peripheral human lymphocytes [17, 18]. Thus, the potential regulatory function of CD in cell proliferation and apoptosis in lymphoid tissues may implicate this molecule in the pathogenesis and progression NHLs. Previous reports outlined the potential of CD as a biomarker of tumor progression in various epithelial cancers [22–30]. We interrogated on the potential prognostic value of CD in lymphomas. To this end, we have studied the expression of CD in a series of different NHL histologic types and searched for any correlation between the extent of CD-positive tumor cells and the clinico-pathological parameters and patient's clinical outcome after therapy. CD expression was assessed by IHC and IF on biopsy specimes obtained from patients prior to any treatment. CD staining in NHL tumor cells showed a punctate pattern, which is compatible with its confinement within lysosomes. Western blotting analysis validated the specificity of the antibody and also the staining assessment of CD expression. Western blotting analysis revealed that the mature double-chain form, which is resident within lysosomes, is the main CD molecular form expressed in NHLs, regardless of the histotype and of clinical features. The literature gives no uniform recommendation for a cut off point of CD expressed as percentage of tumor cells positive in IHC. In esophageal squamous cell carcinomas, it was found that $>$ 10% of CD-positive cells significantly associated with cell proliferation, invasive tumor growth and patient's poor prognosis [28]. As no data are available for lymphomas, we initially chose three different cut off values for CD expression, namely $>$ 10%, $\geqslant$ 20% (high expression) and $\geqslant$ 40% (very high expression). The 20% threshold of CD expression discriminated in our series two different populations with differences in clinico-pathological characteristics, prognosis and survival. In this view, we have chosen 20% as the cut off value for the main statistical analyses. It is to be stressed that in our study only tumor cells were considered for CD expression. In fact, stromal reactive cells (mainly macrophages) infiltrate the tumor and clearly contribute to the actual level of

Table 5
Correlation between the level of Cathepsin D expression and clinical outcome in aggressive and indolent NHLs groups. Statistical correlation between the proportion of Cathepsin D-positive cells in NHLs and the clinical outcome (CR, complete remission; PR, partial remission; DdD, death-due-to-disease; other = PR plus DdD) in the subgroups of patients bearing high-grade of malignancy (Hg) or low-grade of malignancy (Lg) NHLs (panels A and B, respectively). Statistics was calculated for CR vs. poor outcome assumed as PR + DdD (upper panel) or PR and DdD separately (lower panel). Chi-square test indicated a statistically significance correlation between tumours expressing $\geqslant$ 20% CD-positive cells and a poor prognosis only in the subgroup of patients bearing a Hg-NHL.

A

Hg-NHL

Clinical outcome / % CD positive	CR	OTHER	n. cases
< 20%	19	18	37
≥ 20%	3	14	17
Total	22	32	54

Chi square=3.4
DF=1
p=0.04

Clinical outcome / % CD positive	CR	PR	DdD	n. cases
< 20%	19	7	11	37
≥ 20%	3	4	10	17
Total	22	11	21	54

Chi square=5.9
DF=1
p=0.05

B

Lg-NHL

Clinical outcome / % CD positive	CR	OTHER	n. cases
< 20%	1	8	9
≥20%	3	14	17
Total	4	22	26

Chi square=1
DF=1
p=0.9

Clinical outcome / % CD positive	CR	PR	DdD	n. cases
< 20%	1	3	5	9
≥20%	3	8	6	17
Total	4	11	11	26

Chi square=1
DF=2
p=0.6

CD. This is of particular relevance, since it has recently been reported that the expression of CD of macrophage origin in DLBCL, assessed both by genome array and IHC, is higher in tumors from patients considered cured after primary chemotherapy compared to those from patients showing chemoresistance [31].

We found that patients bearing a NHL with a high percentage of CD-positive cells were more frequently diagnosed at stage III or IV and experienced a poor clinical outcome (PR or DdD). In this group, only 6 out

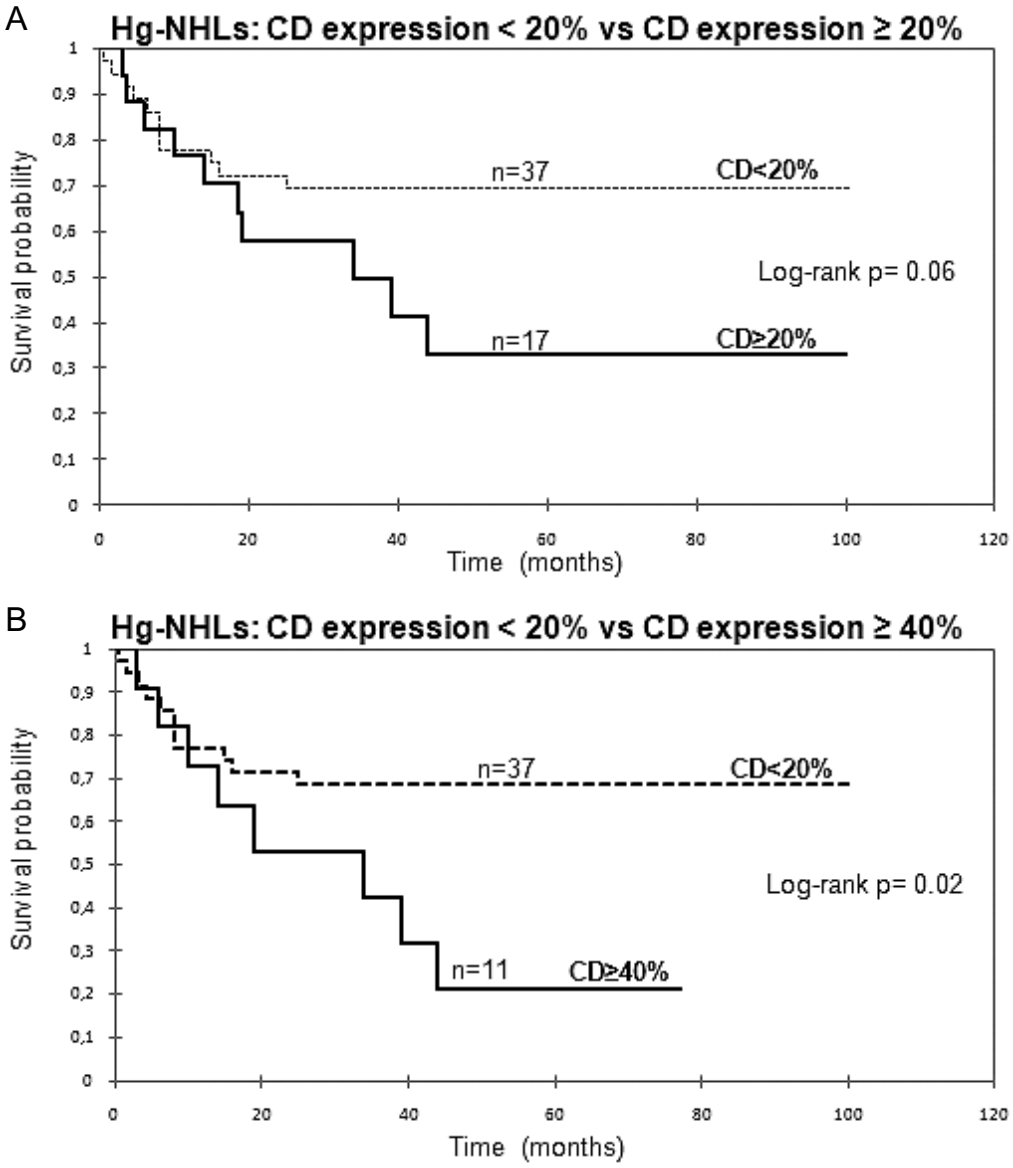

Fig. 4. Kaplan-Meier curves of overall survival in Hg-NHL patients as a function of Cathepsin D expression. In A) comparison of OS in patients bearing Hg-NHLs expressing a low ($<$ 20%) and a high ($\geqslant$ 20%) proportion of CD-positive tumor cells. Difference in OS probability is not statistically significant ($p = 0.06$). In B) comparison of OS in patients bearing NHLs expressing a low ($<$ 20%) and a very high ($\geqslant$ 40%) proportion of CD-positive tumor cells. Difference in OS probability is statistically significant ($p = 0.01$). p, statistical value for significance as calculated by log-rank test. The number of patients per group is indicated.

of 34 patients underwent CR. Patient's poor prognosis in the group bearing a NHL with a high proportion ($\geqslant$ 20%) of CD-positive cells was not associated with a particular histologic type (7 DLBCL, 8 FCL, 8 CLL and 5 PTCL), nor with a specific therapy regimen (regardless of whether this included or not rituximab).

On the whole, these data indicate that the probability to experience a CR is much higher in patients bearing a NHL with a low percentage of CD-positive cells than in patients bearing a NHL with a high percentage of CD-positive cells. When the aggressive (high grade of malignancy) and indolent (low grade of malignancy) NHLs were considered separately, only the former subgroup of NHLs showed a statistically significant correlation between CD expression and pathological staging. In addition, as estimated by the Kaplan-Meyer

Table 6
Correlation between the pathologic stage and clinical outcome in NHLs. Statistical correlation between the pathologic stage and the clinical outcome in the whole series of NHLs (panel A) and in the Hg-NHLs sub-group (panel B). Superior pathologic stage is significantly associated with a low rate of CR and a high rate of death.

A

Total-NHLs

Stage	survivors	dead	n. cases	
I-II	19	3	22	
III-IV	34	23	57	
Total	53	26	79*	Chi square=3.9 DF=1 p=0.046
Clinical outcome / Stage	**CR**	**other**	**n. cases**	
I-II	14	8	22	
III-IV	12	45	57	
Total	26	53	79*	Chi square=11.2 DF=1 p=0.0008

B

Hg-NHLs

Stage	survivors	dead	n. cases	
I-II	16	3	19	
III-IV	17	17	34	
Total	33	20	53*	Chi square=6 DF=1 p=0.013
Clinical outcome / Stage	**CR**	**other**	**n. cases**	
I-II	13	6	19	
III-IV	9	25	34	
Total	22	31	53*	Chi square=7 DF=1 p=0.007

*One lacking data

curves, the survival probability of Hg-NHL patients was inversely related to the proportion of CD-positive cells. In particular, patients bearing an Hg-NHL with $>$ 40% of CD-positive cells showed a low OS probability ($\sim$20%) in comparison to that of patients bearing an Hg-NHL expressing detectable CD in a low proportion of tumor cells ($\sim$70%). This difference was statistically significant (log-rank test, $p = 0.01$). Pathologic stage was also significantly correlated with OS, as expected. Since a high proportion of CD-positive cells was also associated with a worse staging at diagnosis, we applied the Cox regression model to assess the relative prognostic significance of CD expression and of pathologic stage. Univariate analyses confirmed the prognostic value of CD expression. Because the number of patients very highly expressing CD was small in the present study, CD could not be shown to be an independent factor for OS in the multivariate analysis. How-

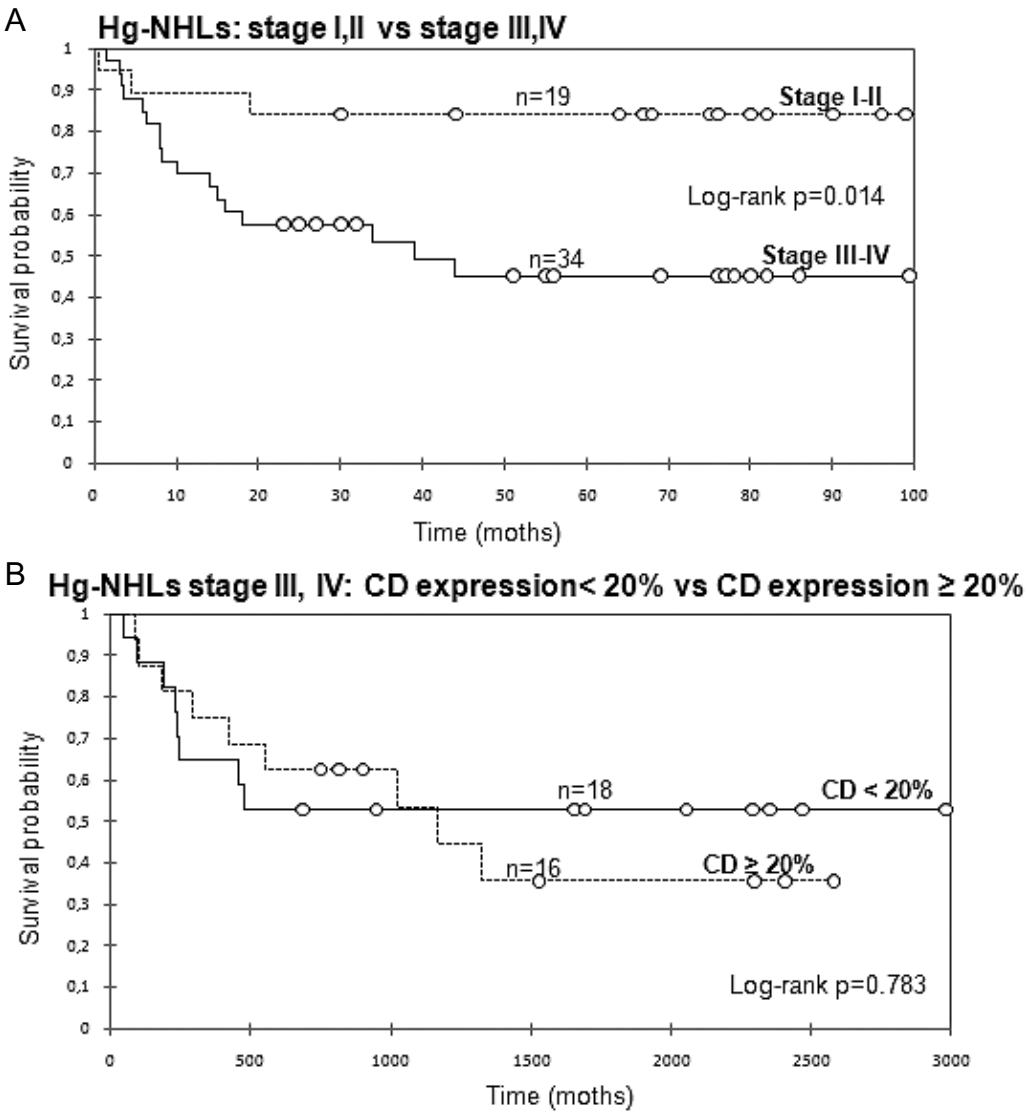

Fig. 5. Kaplan-Meier curves of overall survival in Hg-NHL patients as a function of pathologic stage and Cathepsin D expression. In A) comparison of OS in patients bearing Hg-NHLs staged as I-II ($n = 19$) or III-IV ($n = 34$) (one data lacking). Difference in OS probability is statistically significant ($p = 0.014$). In B) comparison of OS in patients bearing NHLs staged III-IV and expressing a low ($< 20\%$) or high ($\geqslant 20\%$) proportion of CD-positive tumor cells. Difference in OS probability is not statistically significant ($p = 0.783$). p, statistical value for significance as calculated by log-rank test. The number of patients per group is indicated.

ever, the hazard ratio indicated a better probability of survival for patients bearing a tumor with $< 20\%$ CD-positive cells. It is to note that of the 34 patients with Hg-NHL staged III-IV, 18 were low-expressing and showed an OS of 52% and 16 were highly-expressing and showed an OS of 35% ($p = 0.78$). Though the numbers were not sufficient for statistical significance, the trend indicates that patients with Hg-NHLs staged III-IV and highly expressing CD have a very low OS probability. It can be hypothesized that, during progression of NHLs, the emergence of increased numbers of CD-positive cells leads to a more aggressive clinical course of the disease. This aggressiveness manifests clinically with a worse staging at diagnosis and, later on, with a failure of the chemotherapy treatments.

In the Lg-NHLs sub-group, two-third (17/26) of tumors expressed CD in more than 20% of the cells and the majority (15/17) of these tumors staged III-IV. Still, CD expression showed no significant correlation with clinico-pathological characteristics in this sub-group of

Table 7
Cox regression analysis of survival as influenced by Cathepsin D expression and pathologic stage. The Cox proportional hazard model was applied to determine the prognostic significance of Cathepsin D expression and of pathologic stage in the whole series of NHLs and in the Hg-NHL sub-group. A) Univariate analysis of CD expression ($<$ 20% vs. $\geqslant$ 20% or $\geqslant$ 40%) and of pathologic stage (I–II vs. III–IV). B) Multivariate analysis of CD expression adjusted for pathologic stage.

A

PROGNOSTICATOR	UNIVARIATE *p*
CD expression <20% vs ≥ 20%	
Total NHLs (n= 80)	0.88
Hg-NHLs (n= 54)	0.07
CD expression <20% vs ≥ 40%	
Total NHLs (n= 74)	0.84
Hg-NHLs (n= 48)	0.03
Pathologic Stage	
Total NHLs: I-II (n=22) vs III-IV (n= 57)	0.03
Hg-NHLs: I-II (n=19) vs III-IV (n=34)	0.02

B

PROGNOSTICATOR	MULTIVARIATE			
	Wald Chi-square	p*	Hazard ratio	95% confidential interval
Hg-NHLs				
CD <20% (n= 36) vs CD ≥ 20% (n= 17)	-0.39-0.66	0.41	0.68	0.26-1.73
Stage I-II (n=19) vs stage III-IV (n=34)	-1.21-3.21	0.07	0.30	0.08-1.12

NHLs. We have not a clear explanation for this, though one principal reason likely resides in the small number of Lg-NHLs examined.

In conclusion, we have shown, for the first time, that CD can be over-expressed by NHL cells and that the presence of a high percentage of CD-positive tumor cells negatively reflects on the clinical response to chemotherapy and on the progression of NHLs. We found that over-expression of CD in a large proportion of tumor cells in Hg-NHL identifies a subgroup of lymphomas with poor prognosis and short OS. Further studies will be necessary to definitively assess whether CD is indeed a reliable prognostic marker in NHLs. We plan to extend our analysis to a larger cohort of NHL-bearing patients with a number of indolent and aggressive NHL histologic types sufficient to reach conclusions statistically significant on the correlation between CD expression and OS.

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