LPL protein in Chronic Lymphocytic Leukaemia have different origins in Mutated and Unmutated patients. Advances for a new prognostic marker in CLL

Daniel Prieto,[1,2] Noé Seija,[1,3] Angimar Uriepero,[1] Thais Souto-Padron,[4,†] Carolina Oliver,[5] Victoria Irigoin,[5] Cecilia Guillermo,[5] Marcelo A. Navarrete,[6] Ana Inés Landoni,[7] Guillermo Dighiero,[7] Raúl Gabus,[7] Mirta Giordano[8] and Pablo Oppezzo[1]

[1]Chronic Lymphocytic Leukaemia Laboratory, Institut Pasteur de Montevideo, Montevideo, Uruguay, [2]Department of Developmental Neurobiology, Instituto de Investigaciones Biologicas Clemente Estable, Montevideo, Uruguay, [3]Departamento de Inmunobiología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay, [4]Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, CCS, Rio de Janeiro, Brazil, [5]Cátedra de Hematología, Hospital de Clínicas, Universidad de la República, Montevideo, Uruguay, [6]School of Medicine, University of Magallanes, Punta Arenas,Chile, [7]Hospital Maciel, Administración Servicios de Salud del Estado, Ministerio de Salud, Montevideo, Uruguay and [8]Laboratorio de Inmunología Oncológica, Instituto de Medicina Experimental, Academia Nacional de Medicina, Buenos Aires, Argentina

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*Correspondence: Oppezzo Pablo, Institut Pasteur de Montevideo, Research Laboratory on Chronic Lymphocytic Leukaemia, Mataojo 2020, Montevideo 11400, Uruguay.
e-mail: poppezzo@pasteur.edu.uy
[†]Deceased 10 July 2017

Summary

Lipoprotein lipase (*LPL*) mRNA expression in chronic lymphocytic leukaemia (CLL) is associated with an unmutated immunoglobulin profile and poor clinical outcome. We evaluated the subcellular localization of LPL protein in CLL cells that did or did not express *LPL* mRNA. Our results show that LPL protein is differently located in CLL cells depending on whether it is incorporated from the extracellular medium in mutated CLL or generated *de novo* by leukaemic cells of unmutated patients. The specific quantification of endogenous LPL protein correlates with mRNA expression levels and mutational *IGHV* status, suggesting LPL protein as a possible reliable prognostic marker in CLL.

Keywords: Chronic Lymphocytic Leukaemia, LPL, prognostic marker, *IGHV* profile, flow cytometry.

Chronic lymphocytic leukaemia (CLL) is an indolent B cell neoplasm with monoclonal CD5 positive cells that accumulate in lymphoid organs (LO) and bone marrow and move into peripheral blood (PB) (Oppezzo & Dighiero, 2013). A proliferating pool of cells resides in LO, feeding the accumulation pool in the PB (Palacios *et al*, 2010; Calissano *et al*, 2011). Signalling from the microenvironment regulates this proliferative/quiescent dynamics and is probably one of the major causes of the biological and clinical CLL heterogeneity (Oppezzo & Dighiero, 2013). While the mutational status of immunoglobulin heavy chain variable (*IGHV*) genes is considered a good prognostic marker in CLL (Parikh *et al*,

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2016), its heterogeneity justifies further exploration of new biomarkers that can predict therapy response (Rosenquist *et al*, 2013). We described that Unmutated (Um) CLL patients, usually associated with poor clinical outcome, could be differentiated from mutated (Mut) ones by expression of the lipoprotein lipase gene (*LPL*) (Oppezzo *et al*, 2005; Vasconcelos *et al*, 2005). Since then, multiple evidence has confirmed *LPL* mRNA expression as one of the most robust molecular markers in CLL (Rombout *et al*, 2016).

Besides the potential relevance of *LPL* as a prognostic marker, there are still unsolved questions regarding the role of the enzyme it encodes in CLL pathogenesis. Under physiological conditions the main role of LPL is related to lipid metabolism and transport. It has been shown that *LPL* expression confers a survival advantage to CLL cells (Rozovski *et al*, 2015), although other work suggests that LPL is catalytically inactive in CLL (Mansouri *et al*, 2010; Porpaczy *et al*, 2013). A comprehensive characterization of this protein inside the tumour lymphocyte is essential to confirm the different roles of LPL expression in the leukaemic cloneumour. We hereby analysed the sub-cellular localization of LPL protein in CLL, comparing leukaemic cells expressing *LPL* mRNA ($LPL^{mRNA/pos}$) or not ($LPL^{mRNA/neg}$). Additionally, our results led us to study whether measuring LPL protein content by flow cytometry could become a reliable *IGHV* surrogate.

We studied the PB of 42 patients with a confirmed CLL diagnosis. PB mononuclear cells (PBMC) were isolated and the mutational status and *LPL* mRNA expression performed as described (Oppezzo *et al*, 2005). Clinical and molecular characterization of the patients is depicted in Supplementary Table S1. Full details of the methods used are provided in supplementary data.

CLL cells that expressed *LPL* mRNA-transcripts ($LPL^{mRNA/pos}$) or not ($LPL^{mRNA/neg}$) showed marked differences in LPL subcellular localization (Fig 1). Whereas the $LPL^{mRNA/pos}$ group showed a perinuclear LPL sub-localization (Fig 1A, white arrows), $LPL^{mRNA/neg}$ patients and B cells from healthy donors, showed lower intensity of LPL staining with peripheral localization, (middle and bottom panels, Fig 1A).

Co-immunostaining of LPL with the endoplasmic reticulum (ER) cisternae marker calnexin (CLX), and LAMP1, as a marker of late endosomes/lysosomes, showed that LPL was associated with ER in $LPL^{mRNA/pos}$ cells, evidenced by overlapping of LPL and CLX signals (Fig 1B, arrows in top panels), whereas small quantities of LPL protein are associated with LAMP1. In contrast, in $LPL^{mRNA/neg}$ co-localization of LPL protein with CLX is barely detected, and the majority of LPL overlaps with LAMP1 (Fig 1B, bottom panel, white arrows). These results were validated in six CLL cases of each group ($CLL^{mRNA/pos}$ and $CLL^{mRN/-neg}$) and four samples from healthy donors. Subsequently, these findings were confirmed by immuno-electron microscopy, with immunogold labelling of LPL being mainly observed in cisternae of ER in three $LPL^{mRNA/pos}$ samples, whereas in $LPL^{mRNA/neg}$ samples ($n = 3$) labelling was mainly found in vesicles, (Supplementary Fig 1A).

The presence of LPL protein inside endosomes of CLL cells that lack *LPL* mRNA expression suggests the incorporation of external protein into tumour cells. To explore this hypothesis, we incubated CLL cells from both groups of patients for 24 h in serum-free medium (SFM) or in autologous plasma and evaluated the presence and localization of LPL. Autologous plasma incubation seemed to increase the proportion of LPL positive cells as compared to incubation with SFM (Fig 1C). After validation of this finding by flow cytometry and fluorescent microscopy (Supplementary Fig 1B), we tested the subcellular colocalization of LPL after incubation with autologous plasma. In the $LPL^{mRNA/pos}$ cells, LPL colocalized with CLX and LAMP1 (Fig 1D, top panels, white and red arrows, respectively). In $LPL^{mRNA/neg}$ patients LPL appears to be internalized and remains excluded from ER being mainly associated with LAMP1 vesicles (white arrows in Fig 1D, bottom panels).

The incorporation by CLL cells of either human LPL from plasma or bovine LPL from fetal bovine serum (FBS) represents a challenge for the development of a reliable prognostic method because frozen PBMCs, a usual form of preserving CLL cells, could lead to equivocal results. To overcome this issue, we optimized concentrations, temperatures and times of heparin incubation of CLL PBMCs for removal of cell-surface attached LPL (data not shown) and compared this protocol between fresh and frozen PBMCs from the same patients. Our results demonstrated that heparin treatment detaches LPL protein from the CLL cell surface, but endogenous LPL, either in intracellular vesicles of $LPL^{mRNA/neg}$ cases or in the ER of LPL $^{mRNA/pos}$ group, remains (Fig 2A, white arrows in heparin-treated panels, and representative histograms). These results further confirm that external LPL could be incorporated by CLL cells and show that heparin treatment is not enough to achieve an accurate LPL measure.

As frozen cells could be a source of unspecific LPL immunostaining (Fig 1C) we established the optimal conditions in which LPL protein detection displayed the best specificity/sensitivity properties. To this aim we analysed 20 patients, 10 $LPL^{mRNA/neg}$ and 10 $LPL^{mRNA/pos}$ and evaluated different conditions: (i) frozen/thawed PBMCs; (ii) frozen/thawed PBMCs plus heparin; (iii) fresh PBMCs; and (iv) fresh PBMCs plus heparin. Supporting our previous observations, heparin treatment in fresh PBMCs was the best option to improve cytometric detection of LPL (Fig 2B, bottom right panel). To confirm these results, LPL protein and mRNA expression was analysed in 42 CLL cases using fresh samples. Our results showed that LPL protein measurement by flow cytometry had an optimal cut-off value of 25% for *IGHV* mutational status prediction, achieving a sensitivity of 95.24 (95% confidence interval [CI] 76.18–99.88) and a specificity of 100 (95% CI: 83.89–100). Using this strategy, the only discordant case was CLL_37 in which LPL expression was 21%. (Fig 2C).

Finally, we explored the potential predictive prognostic power of LPL protein measurement using time to first treatment (TTFT) as primary endpoint and compared it with

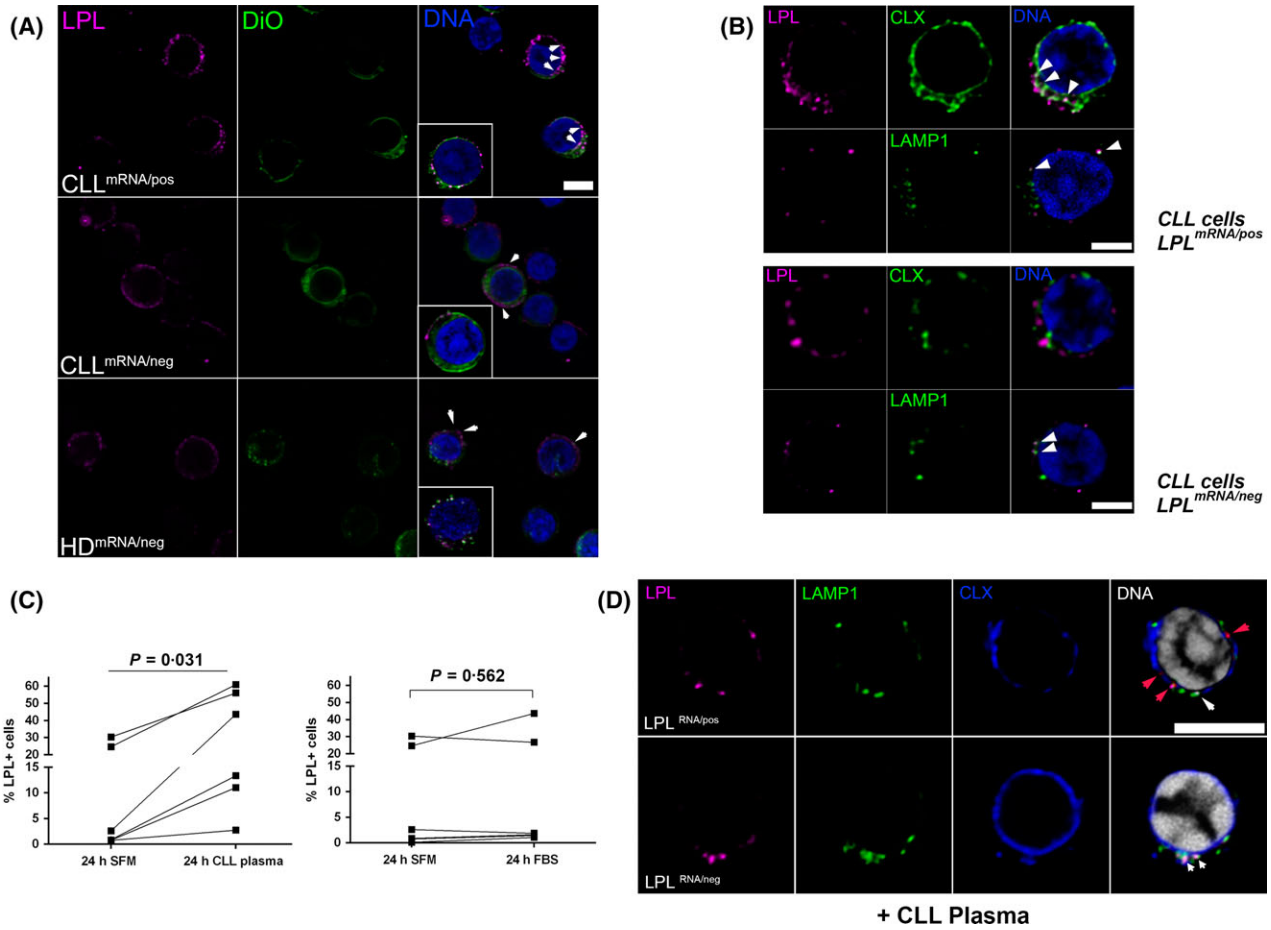

Fig 1. Subcellular localization of LPL and internalization from plasma or FBS of this protein in CLL B-cells. (**A**) Subcellular distribution of LPL immunoreactivity in relation to cellular membranes. LPL signal shows a different pattern in cells from LPL$^{mRNA/pos}$ patients than in those from LPL$^{mRNA/neg}$ patients. A perinuclear membrane localization was found in cells from LPL$^{mRNA/pos}$ patients. Insets show a representative cell with typical LPL distribution in LPL$^{mRNA/pos}$, LPL$^{mRNA/neg}$ patients cells and B cells from a representative healthy donor (HD) (white arrows). (**B**) Cells from LPL$^{mRNA/pos}$ patients display two different populations of LPL, one localized in the cisternae of the endoplasmic reticulum (ER) -as shown by calnexin (CLX) staining (arrowheads, upper panels), and the other punctually excluded from the ER of which some coincided with LAMP1 positive vesicles (arrowheads, lower panels). LPL immunomarcation was only located in LAMP1 positive vesicles in cells from LPL$^{mRNA/neg}$ patients (white arrows in second panel B). (**C**) Percentage of LPL positive cells measured by flow cytometry after 24-h culture in serum-free medium (SFM) or in chronic lymphocytic leukaemia (CLL) plasma (left panel). The number of LPL-positive cells increased when cultured in CLL plasma (P = 0.031, n = 6, Wilcoxon test, two tailed). Cells cultured in fetal bovine serum (FBS) showed a trend to internalization, but this did not reach statistical significance. (**D**) LPL internalization after culturing LPL$^{mRNA/pos}$ (upper panels) or LPL$^{mRNA/neg}$ (lower panels) cells in CLL plasma. In LPL$^{mRNA/pos}$ LPL (top panels), labelling (magenta) is mainly visualized into calnexin-positive zones (Blue), depicted with red arrows, whereas a small amount of LPL protein was located in LAMP1 (green) positive vesicles (white arrows). In bottom panel, LPL$^{mRNA/neg}$ cells display LPL colocalization with LAMP1 positive vesicles (white arrows) but is excluded from calnexin-positive zones. Scale bar B, C: 5 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

mutational *IGHV* status and *LPL* mRNA expression. Median TTFT for the evaluated cohort (n = 36) was 25 months in CLLs displaying Um *IGHV* genes (P = 0.001), 20 months in CLLs expressing *LPL* mRNA (P = 0.005) and 25 months for CLLs expressing LPL protein, (P = 0.02). Median TTFT was not reached in Mut CLLs, *LPL* mRNA/negative and LPL protein/negative subgroups (Supplementary Fig 2).

This study provides deep insight into the subcellular localization of LPL within different prognostic subgroups of CLL to gain new perspectives about the functional role of this protein. We describe for the first time that two different origins could account for LPL expression in the leukaemic clone: (i) an internal source from *LPL* mRNA transcription that exists mainly in Um CLL, and (ii) an external source from plasma or FBS from where LPL appears to be taken up in Um and Mut cases, being specifically recognized by the anti-LPL 5D2 monoclonal antibody (mAb), an antibody originally produced against bovine LPL (Peterson *et al*, 1992). These findings support and complement previous results (Heintel *et al*, 2005) explaining why the expression of *LPL* mRNA in leukaemic cells of Um patients does not correlate with a higher expression of LPL protein.

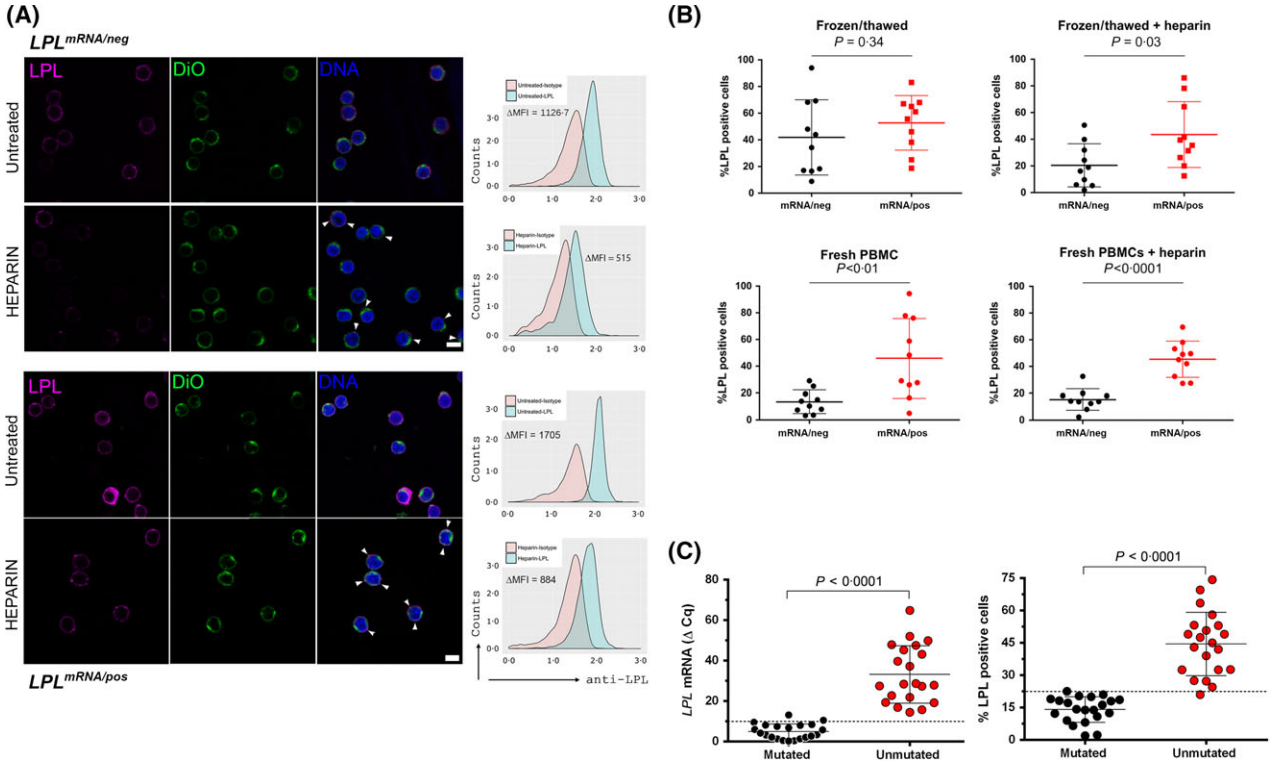

Fig 2. Different protocol conditions to develop a new methodology for measure endogenous LPL protein in CLL. (**A**) In situ immunolocalization of LPL (magenta) shows staining of both untreated LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ cells (upper panels). A decrease in LPL staining in both subgroups is observed after heparin treatment, uncovering a punctate pattern with noticeable differences between LPL in both subgroups (arrowheads, lower panels). LPL signal was excluded from membrane structures stained by DiOC16 (green). Flow cytometry analysis of median fluorescent staining intensities (MFI) reveals an improvement in LPL protein discrimination. Representative density plots depict MFI differences between anti-LPL labeled cells and its isotype control (delta MFI, right column, log scale). (**B**) Heparin treatment as previously optimized using frozen PBMC appears not to be enough to obtain a clear cut-off that correctly discriminate LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ (P values = 0.34 and 0.03 for frozen/thawed and frozen/thawed plus heparin respectively, n = 20). Freshly collected cells without heparin treatment improve discrimination of LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ cells (right panel, P = 0.01, n = 20). However best discrimination is obtained with the combination of both variables (heparin treatment and fresh PBMC), as is depicted in the right panel, ($P < 0.0001$, n = 20). Each dot represents the percentage of IgM and LPL positive cells from an individual patient. All these conditions were equally tested using the anti-LPL 5D2 mAb in flow cytometry. (**C**) *LPL* at the mRNA level by qRT-PCR with a threshold of 10 as previously described in (Heintel *et al*, 2005) discriminated 21/21 Um cases and 19/21 Mut cases. This threshold was confirmed using the Youden index method. *LPL* at the mRNA level showed a 100% sensitivity with 90% specificity for the identification of Um cases; and 90% sensitivity with 100% specificity for Mut cases. Endogenous LPL protein levels assessed by flow cytometry in freshly collected heparin-treated CLL cells in an expanded cohort show significant differences between *IgVH* Mut and Um groups (n = 42, P = 0.0001, unpaired t test with Welch's correction). LPL protein measurement by flow cytometry has an optimal cutoff value for *IgVH* mutational status prediction of 25%, as assessed by the Youden index. Mutated cases showed a mean of 14.16% ± 1.2% LPL positive cells whereas Um cases a mean of 44.51% ± 3.1%. The mutational status was accurately predicted in 21/21 (100%) Mut and 20/21 (95%) Um cases. The overall concordance was 95%, the two discordant cases, CLL 16 and CLL 11, had values of 13.2 and 10.5 respectively, (Supplementary Table 1). [Colour figure can be viewed at wileyonlinelibrary.com]

Secondly, we evaluated whether LPL protein assessment could serve as a possible prognostic tool in CLL, if the necessary conditions are set up. To this aim, we first established the use of fresh CLL cells and heparin surface peeling as optimal conditions for a flow cytometry LPL measurement. Although we show here the feasibility of this technology, there are still some technical challenges to be solved for its widespread application. Note: (i) staining was performed with the 5D2 mAb (Peterson *et al*, 1992) and the performance of commercially available 5D2 mAb remains to be assessedl; (ii) While there was a high concordance with the immunoglobulin mutational status and univariate TTFT analysis indicates some potential clinical value, the prognostic and clinical value of the here proposed methodology should be assessed in a larger prospective cohort.

Finally, our data appear to reinforce the hypothesis that LPL protein in progressive cases could have a catalytic function, providing metabolic survival advantages for the tumour clone. In this case, LPL protein is generated *de novo* and appears to be correctly expressed in a classical ER/Golgi-dependent secretory pathway. Conversely, the hypothesis suggesting that membrane-bound LPL could support proliferation, migration and spread for progressive patients seems unlikely, considering that both Mut and Um patients are able to bind or internalize LPL from plasma indiscriminately.

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Authorship contributions

P.D. performed biological studies, collected CLL samples, prepared figures and wrote the paper. U.A.m, S.N. and G.M. performed experiments and collected CLL samples. S.-P.T. supervised electron microscopy analysis. O.C., I.V., C.G., L.A.I. and G.R. performed clinical activities and data collection of CLL patients. N.M. performed data collection of CLL patients and data analysis. D.G. performed clinical activities and wrote the paper. O.P. designed research, coordinated the study and data analysis and wrote the paper.

Conflicts of interest

The authors declare no competing interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Subcellular localization of LPL protein in CLL.

Figure S2. Like mRNA levels and *IGHV* mutational status, endogenous LPL protein levels could predict time to first treatment in CLL.

Table S1. Clinical and molecular characterization of CLL patients.

Data S1. Supplementary methods.

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