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Methylation status regulates lipoprotein lipase expression in chronic lymphocytic leukemia

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Abstract

Among different prognostic factors in chronic lymphocytic leukemia (CLL), we previously demonstrated that lipoprotein lipase (LPL) is associated with an unmutated immunoglobulin profile and clinical poor outcome. Despite the usefulness of LPL for CLL prognosis, its functional role and the molecular mechanism regulating its expression are still open questions. Interaction of CLL B-cells with the tissue microenvironment favors disease progression by promoting malignant B-cell growth. Since tissue methylation can be altered by environmental factors, we investigated the methylation status of the LPL gene and the possibility that overexpression could be associated with microenvironment signals. Our results show that a demethylated state of the LPL gene is responsible for its anomalous expression in unmutated CLL cases and that this expression is dependent on microenvironment signals. Overall, this work proposes that an epigenetic mechanism, triggered by the microenvironment, regulates LPL expression in CLL disease.

Keywords: CLL, methylation, prognostic factors

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease, with survival ranging from months to decades. The development of the Rai and Binet staging systems has allowed the division of patients with CLL into three prognostic groups: good, intermediate and poor prognosis. The two staging systems have improved the identification of patients who need immediate treatment. However, neither the Rai nor the Binet staging system can predict which patients among the good prognosis group will have progressive disease [1]. Major progress has been made to identify molecular and cellular markers that predict disease progression in patients with CLL. Among these markers, cytogenetic abnormalities including 11q or 17p deletions in the leukemic B cells, or the presence of an unmutated (Um) status of the immunoglobulin heavy chain genes (*IGHV*), are the better predictors of rapid progression and shorter survival [1].

Gene-expression profiling (GEP) has introduced a new dimension into our understanding of CLL biology and clinical behavior. Results from GEP in CLL led us to propose that aberrant overexpression of the lipoprotein lipase (LPL) gene is a surrogate marker of the mutational status of *IGHV* [2]. This observation was extensively confirmed by other groups [3–7]. Thus, LPL mRNA overexpression is currently demonstrated to be associated with Um status and clinical poor outcome, and also it appears to be the most powerful prognostic tool among RNA-based markers in CLL [8].

Previous work suggests a role for LPL expression in CLL disease, not only in gene expression changes but also in functional pathways related to fatty acid degradation and signaling, which may influence CLL cell behavior [9]. Despite these studies and though the prognostic value of the LPL gene is well established, the functional consequences of LPL overexpression in CLL pathogenesis as well as the molecular mechanism regulating its expression remain elusive.

Aberrant DNA methylation has been shown to play a strong role in tumorigenesis, where genome-wide hypomethylation and regional hypermethylation of tumor suppressor gene promoters are characteristic hallmarks of many cancers. Tissue specific patterns of methylated cytosine residues can be altered by environmental factors, and are often abnormal in tumor disorders [10].

The main goal of this investigation was to obtain deep insight into the molecular mechanisms responsible for the high expression of LPL in Um CLL B cells. Along this line, we investigated the methylation status of the LPL gene promoter region, as well as the possibility that its expression could be related to specific signals delivered by the microenvironment.

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Our results suggest that the demethylated status of the LPL gene is responsible for the anomalous expression of this prognostic marker in Um CLL, and that this epigenetic mechanism can be induced in the leukemic clone by microenvironment signals.

Materials and methods

Patient samples and clinical data

Peripheral blood mononuclear cells (PBMCs) were obtained from 26 patients with CLL. Samples were characterized at a molecular level through CD38, LPL expression, and *IGHV* status as well as fluorescence *in situ* hybridization (FISH) analysis (Supplementary Table I to be found online at http://infor-mahealthcare.com/lal/doi/10.3109/10428194.2013.796057). All patients were followed at the Hospital Maciel, Montevideo, and provided informed consent in accordance with the ethical regulations of Uruguay and the Declaration of Helsinki.

RNA extraction and analysis of LPL transcripts by PCR

Genomic DNA and RNA extraction, cDNA synthesis, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (Q-PCR) for LPL amplification were performed as described [2].

Methylation analysis and 5-aza-2′-deoxycytidine treatment

The CpG island in the 5′-region of the LPL gene was identified by EMBOSS/CpGPlot/CpGReport/Isochore software. Five hundred ng of genomic DNA was treated with sodium bisulfite and amplified using bisulfite-sequencing primers. PCR products were cloned and five clones from each patient with CLL were sequenced to evaluate the methylation status of the CpG island. In order to confirm the specificity of changes in LPL methylation status, the Daudi cell line was cultured over 3 and 5 days in supplemented RPMI medium containing 10 μM 5-aza-2′-deoxycytidine (5-Aza-dC) (Sigma Aldrich, St Louis, MO). Medium was changed every 24 h and bisulfite analysis was carried out as describe above.

CLL B cell stimulation with different microenvironment signals

Stimulation of PBMCs from patients with negative LPL expression with autologous T cells or with recombinant CD40L and interleukin 4 (IL-4) was performed as described previously [11,12]. In turn, B cell receptor (BCR) stimulation was achieved by anti-immunoglobulin M (IgM) (Jackson ImmunoResearch, West Grove, PA) immobilized on culture plates at 15 μg/mL, and the cells collected after 4 days.

Flow cytometry analysis

A total of 1×10^6 PBMCs were incubated for 45 min at 4°C with anti-CD19–peridinin chlorophyll protein complex (PerCP), anti-CD25–phycoerythrin (PE), anti-CD80–PE and anti-CD86–fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, CA). The cells were then washed and analyzed. Negative isotype controls were performed with irrelevant antibody in the same conditions. Results were analyzed using Summit v4.3 software (Dako Inc., Carpinteria, CA).

Epifluorescence microscopy

For intracellular detection of LPL, an indirect immunofluorescence technique was performed using anti-LPL monoclonal antibody 5D2 (kindly provided by Dr. J. Brunzell, University of Washington). PBMCs from patients with CLL were isolated using a B-Cell Isolation Kit for B-CLL (http://www.miltenyibiotec.com), washed with phosphate buffered saline (PBS) and 5 U/mL heparin (Sigma Aldrich), fixed with 3.7% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Non-specific binding was blocked with 5% fetal bovine serum (FBS) in PBS, incubated with anti-LPL antibody overnight at 4°C, and finally with Alexa Fluor® 488-labeled goat anti-mouse antibody (Invitrogen, Carlsbad, CA) for 1 h. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). After washes, preparations were visualized (×100) and photographed using an Olympus CKX31 inverted microscope.

Statistical analyses

Expression of LPL mRNA, CD25, CD80 and CD86 as activation control proteins and methylation percentage of the R1-LPL region were compared between control samples and different activation subsets using either a paired Wilcoxon signed-rank test or two-tailed unpaired Student's *t*-test. Variables with *p*-values less than 0.05 were considered to be significant. The Spearman rank correlation coefficient was calculated to determine the strength of association between LPL expression and methylation percentage. p-Values ≤ 0.01 were considered significant. All analyses were done using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA).

Results

First exon/intron within core promoter of LPL gene is differentially methylated in patients with mutated and unmutated CLL

In order to address whether the LPL gene can be regulated epigenetically through CpG methylation, we analyzed the CpG sites in the LPL gene. This analysis revealed CpG-rich sequences encompassing a classical CpG island of 1163 bp with 112 CpG dinucleotides. This region is located within the first exon and first intron of the LPL gene. To better characterize this CpG island we focused on the methylation status of CpG dinucleotides in two different regions (R1 = 248 bp, from +87 bp to +335 bp and R2 = 261 bp, from +446 bp to +707 bp) [Figures 1(A)–1(C)]. Methylation of the first exon is critical for transcriptional silencing [10]. In accordance with this, our initial results comparing methylation changes between these two regions in six CLL samples showed that the main differences appeared to be restricted to R1 (exon 1, CpG dinucleotides numbers 1–18) [Figure 1(C)]. Following this, 26 patients with CLL (14 Um expressing LPL and 12 mutated [Mut] negative for LPL mRNA, Supplementary Table I to be found online at http://infor-mahealthcare.com/lal/doi/

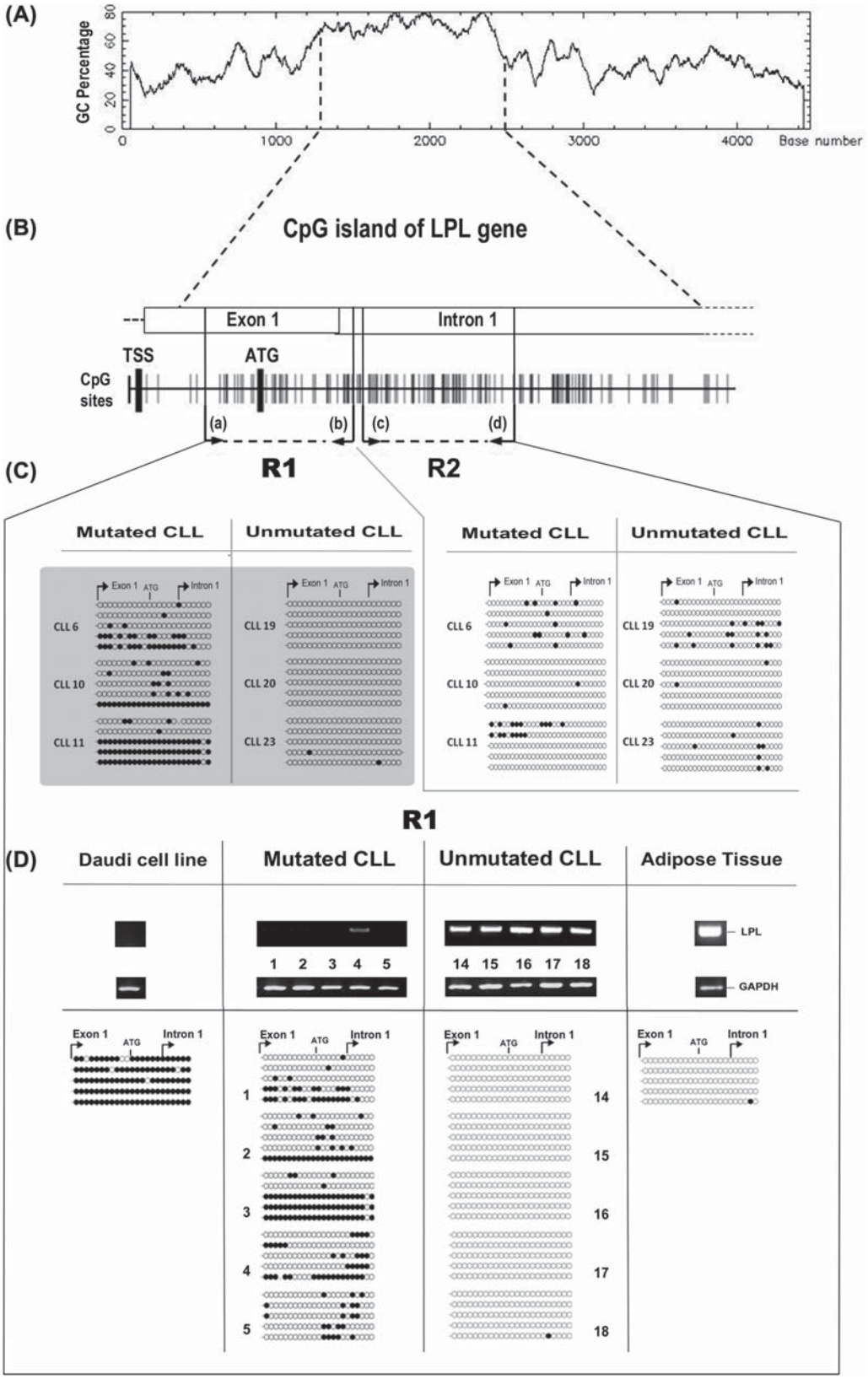

Figure 1. CpG island analysis of LPL gene in patients with CLL. (A) Localization of CpG island of LPL gene. The CpG island was defined as the region containing over 50% of CpG dinucleotides by EMBOSS/CpGPlot/CpGReport/Isochore software. (B) Schematic representation of exon 1 and intron 1 within CpG island of LPL gene. The position of exon 1, intron 1, transcription start site (TSS) and PCR primers for R1 and R2 are depicted as (a), (b), (c) and (d). (C) R1 and R2 profile methylation differences. Methylation analysis of R1- and R2-LPL regions was performed in six CLL cases (three Mut and three Um) in order to visualize the main changes in methylation status of both subsets. Shaded squares highlight the fact that major differences concerning the methylation profile are located in R1 and not R2. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. (D) Differential expression of LPL gene and methylation status in patients with Mut and Um CLL. Results from five representative patients with Mut (1–5) and five with Um (14–18) CLL, Daudi cell line and adipose tissue samples as negative and positive controls, respectively, are depicted. LPL mRNA expression evaluated by RT-PCR is shown in agarose gel stained with ethidium bromide. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in all cases as internal control. Methylation status evaluated by bisulfate sequencing of R1-LPL region is shown as lollipop diagrams. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively.

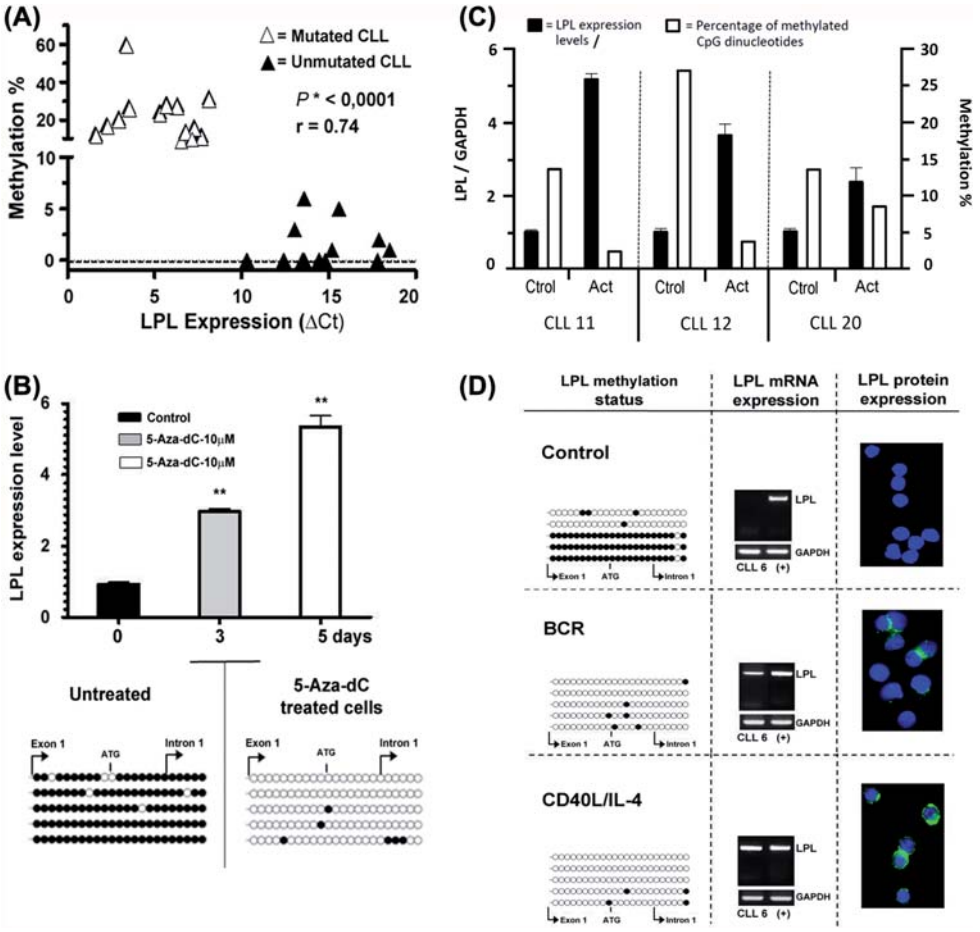

Figure 2. LPL expression and methylation change analysis after incubation with demethylated agents or microenvironment signals. (A) Correlation between LPL mRNA expression of 14 Mut and 12 Um CLL evaluated by quantitative reverse transcription PCR and methylation percentage in R1-LPL region. Statistical analysis indicating a significant correlation by Spearman's rank (p-values ≤ 0.001) is shown. In this case LPL expression was correlated to methylation status ($p < 0.00015$; Spearman's rank coefficient $p = 0.72$) in 26 CLL samples. (B) Activation of LPL expression and DNA demethylation after 5-Aza-dC treatment. LPL expression level ($2^{-\Delta\Delta Ct}$) by QRT-PCR after 5-Aza-dC treatment on Daudi cell line is depicted (error bars indicate range factor difference). The histogram represents mean of three replicates (**$p < 0.01$ by unpaired two-tailed Student's t-test). DNA methylation status of R1-LPL region in untreated and treated cells by 5 days is shown. Black and white circles represent methylated and unmethylated cytosines, respectively, and each row represents one bacterial clone. (C) Autologous T cell activation on CLL B cells. Black bars depict LPL expression at mRNA levels in control cultures (Ctrol) and in stimulated T cell cultures (Act). Quantification was performed by QRT-PCR ($2^{-\Delta\Delta Ct}$). Expression levels of control samples were normalized to 1 as expression relative unit. White bars represent the percentage of methylated CpG dinucleotides in control and activated T cell cultures. (D) Representative CLL sample after stimulation with different microenvironment signals. LPL methylation status: DNA methylation profile of R1-LPL region before and after different activation signals. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. LPL mRNA expression: LPL expression by RT-PCR is depicted in agarose gel stained with ethidium bromide. Um/LPL(pos) CLL sample was used as positive control and GAPDH was used as endogenous control. LPL protein expression: protein expression was visualized by epifluorescence microscopy. Green: antibody anti-LPL, blue dye: DAPI. Successful activations with autologous T cells, CD40L/IL-4 and anti-BCR were corroborated by cytometry flow analysis with anti-CD19–PerCP, anti-CD25–PE anti-CD80–PE and anti-CD86–FITC.

10.3109/10428194.2013.796057) were analyzed by bisulfite DNA conversion and sequencing methodology focusing on R1 of the LPL CpG island [Figure 1(C)]. We found that Mut CLL cases that did not express, or expressed minimal levels of LPL mRNA had a methylated R1 profile. In contrast, Um CLL cases expressing higher levels of LPL mRNA displayed a demethylated profile of this region [Figure 1(D)]. Overall, these results suggest that differential methylation status is responsible for LPL gene expression in patients with Mut and Um CLL.

LPL expression correlates with methylated status of CpG island and depends on microenvironment signals

To confirm previous results linking LPL expression with demethylation status, we studied the LPL mRNA expression of these 26 patients with CLL by QRT-PCR. A significant correlation ($p < 0.0001$) between LPL expression and demethylated status in patients with Um CLL and absence of LPL expression and methylated status in patients with Mut CLL [Figure 2(A)] was found. To further analyze the epigenetic mechanism of LPL expression we exposed LPL-negative Daudi cells to 5-Aza-dC, a DNA methyltransferase inhibitor. Results showed that exposure to this drug triggered mRNA LPL expression at significant levels compared with untreated cells (3 and 5 days, $p < 0.01$), and was also able to induce demethylation of the R1-LPL region [Figure 2(B)]. These data confirm that demethylation in exon 1/intron 1 of the LPL gene is correlated with the expression of this prognostic marker in leukemic CLL B-cells. Additionally, we queried whether LPL anomalous expression in leukemic B-cells could be related to the microenvironment signals. To obtain deep

insight into this hypothesis, PBMCs from three LPL-negative samples were incubated with or without autologous T-cells, activated through T cell receptor (TCR) cross-linking. After 4 days, CD19/CD5 positive cells were purified, for LPL mRNA expression and methylation status of the R1-LPL region were evaluated. As depicted in Figure 2(C), we found that following autologous T cell activation, the leukemic clone expressed LPL and altered the methylation status of the R1-LPL region to a mostly unmethylated pattern. To confirm this result we also stimulated another six LPL-negative samples through CD40 and IL-4 receptors or through the BCR. Both activation protocols were able to induce LPL mRNA and protein expression, and this was associated with DNA demethylation of the R1-LPL region. Results for a representative patient with LPL-negative CLL with or without stimulation are shown in Figure 2(D). Overall, these results suggest that LPL expression in CLL is related to proliferative microenvironment signals that appear to induce a demethylation process in the leukemic clone.

Discussion

Although it has traditionally been assumed that CLL is the consequence of long-lived tumor cell accumulation, evidence indicates that disease evolution results from the balance between proliferating cells in a specialized tissue microenvironment and circulating cells resisting apoptosis [13]. This equilibrium is finely tuned by a set of surface molecules expressed by CLL B cells and modulated in response to environment signals [14].

Since there is no expression of LPL in normal B cells, the presence of high levels of LPL in Um CLL B cells is a very interesting issue. This anomalous expression not only constitutes a suitable prognostic marker for the disease but could also be helpful to understand the heterogeneous proliferative behavior of CLL B cells.

At a physiological level, the largest expression of the LPL gene has been reported in adipose tissue, skeletal muscle, heart tissue, dendritic cells and CD33 myeloid cells [9]. LPL is a protein located on the luminal side of the wall vessels, where it is anchored to heparan sulfate proteoglycans and contains binding sites for heparan sulfate chains and apoproteins [15]. Furthermore, LPL has a bridging function in the formation of a trimolecular complex including a lipoprotein particle, LPL and heparan sulfate proteoglycans from different cells [15]. This is an interesting characteristic of LPL because, independent of its catalytic function, LPL expression in patients with Um CLL may be associated with cell spreading and the migratory capacity of the proliferative cell subset [5,15]. If this is the case, LPL might also act as a cross-talk factor, facilitating specific interactions with accessory cells in the tissue microenvironment.

The results presented here link tumor cell proliferation with epigenetic changes on the CpG island of LPL DNA, suggesting that LPL expression in CLL is related to microenvironment signals that appear to induce a demethylation process in the leukemic clone. These data are supported by the fact that anomalous LPL expression is a hallmark of patients with Um CLL.

In conclusion, this work shows that an epigenetic mechanism, triggered by the microenvironment, is responsible for the anomalous LPL expression in patients with Um CLL.

Supplementary material available online

Table showing clinical and molecular characteristics of patients

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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