



EUROPEAN
HEMATOLOGY
ASSOCIATION



Ferrata Storti
Foundation

miR-125b and miR-532-3p predict the efficiency of rituximab-mediated lymphodepletion in chronic lymphocytic leukemia patients. A French Innovative Leukemia Organization study

Anne-Laure Gagez,^{1,2*} Isabelle Duroux-Richard,^{3*} Stéphane Leprêtre,⁴ Frédérique Orsini-Piocelle,⁵ Rémi Letestu,⁶ Sophie De Guibert,⁷ Edouard Tuailon,⁸ Véronique Leblond,⁹ Olfa Khalifa,³ Valérie Gouilleux-Gruart,¹⁰ Anne Banos,¹¹ Olivier Tournilhac,¹² Jehan Dupuis,¹³ Christian Jorgensen,^{3,14} Guillaume Cartron^{1,2} and Florence Apparailly^{3,14}

Haematologica 2017
Volume 102(4):746-754

¹CNRS UMR 5235, University of Montpellier; ²Department of Clinical Hematology, University Hospital Montpellier; ³INSERM, U1183, Institute of Regenerative Medicine and Biotherapy, University Hospital Montpellier; ⁴Henri Becquerel Center, Rouen; ⁵Department of Clinical Hematology, Hospital Center of Annecy, Pringy; ⁶Department of Biological Hematology, APHP, GHUPSSD, Avicenne Hospital, Bobigny; ⁷Department of Clinical Hematology, Pontchaillou Hospital, Rennes; ⁸Department of Bacteriology-Virology, University Hospital Montpellier; ⁹Department of Hematology, La Pitié Salpêtrière Hospital, Paris; ¹⁰CNRS UMR 7292, François Rabelais University, University Hospital Tours; ¹¹Department of Hematology, Cote Basque Hospital, Bayonne; ¹²Department of Clinical Hematology, University Hospital Estaing, Clermont-Ferrand; ¹³Unit of Lymphoid Hematologic Malignancies, Henri Mondor Hospital, Créteil and ¹⁴Clinical department for Osteoarticular Diseases, University Hospital Lapeyronie, Montpellier, France

*A-LG and ID-R contributed equally to this work

ABSTRACT

The underlying *in vivo* mechanisms of rituximab action remain incompletely understood in chronic lymphocytic leukemia. Recent data suggest that circulating micro-ribonucleic acids correlate with chronic lymphocytic leukemia progression and response to rituximab. Our study aimed at identifying circulating micro-ribonucleic acids that predict response to rituximab monotherapy in chronic lymphocytic leukemia patients. Using a hierarchical clustering of micro-ribonucleic acid expression profiles discriminating 10 untreated patients with low or high lymphocyte counts, we found 26 micro-ribonucleic acids significantly deregulated. Using individual real-time reverse transcription polymerase chain reaction, the expression levels of micro-ribonucleic acids representative of these two clusters were further validated in a larger cohort (n=61). MiR-125b and miR-532-3p were inversely correlated with rituximab-induced lymphodepletion ($P=0.020$ and $P=0.001$, respectively) and with the CD20 expression on CD19⁺ cells ($P=0.0007$ and $P<0.0001$, respectively). *In silico* analyses of genes putatively targeted by both micro-ribonucleic acids revealed a central role of the interleukin-10 pathway and CD20 (MS4A1) family members. Interestingly, both micro-ribonucleic acids were negatively correlated with MS4A1 expression, while they were positively correlated with MS4A3 and MSA47. Our results identify novel circulating predictive biomarkers for rituximab-mediated lymphodepletion efficacy in chronic lymphocytic leukemia, and suggest a novel molecular mechanism responsible for the rituximab mode of action that bridges miR-125b and miR-532-3p and CD20 family members. (*clinicaltrials.gov Identifier: 01370772*).

Correspondence:

g-cartron@chu-montpellier.fr

Received: July 20, 2016.

Accepted: January 23, 2017.

Pre-published: January 25, 2017.

doi:10.3324/haematol.2016.153189

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/4/746

©2017 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>,

sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



Introduction

Micro-ribonucleic acids (miRNAs) are a class of small noncoding RNAs that regulate gene expression at the post-transcriptional level and play an important regulatory role in many cellular processes.¹ Deregulated expression of miRNAs could play a critical oncogenic or tumor-suppressor role and has therefore been associated with cancer, including hematological malignancies and, especially, B-cell lymphomas.²⁻⁴ MiRNAs correlate with the clinical characteristics or outcome of chronic lymphocytic leukemia (CLL) patients, allowing the identification of CLL subgroups with worse outcomes.⁵⁻⁷ Some of these miRNAs contribute to the deregulation of pathways involved in CLL oncogenic processes, such as PI3K/Akt (miR-22), NF- κ B (miR-9 family), or toll-like receptor 9 (miR-17~92 family).⁸⁻¹⁰ The B-cell receptor (BCR) signaling pathway was recently shown to be directly regulated by miR-34, miR-150, and miR-155 in CLL,^{11,12} in addition to BCL2 (miR-15a/16), TCL1 (miR-29 and miR-181), P53 (miR-15a/miR-16-1 cluster, miR-17-5p, miR-29c and miR-34a), or PTEN (miR-26a and miR-214).¹³⁻¹⁷ Response to a CLL treatment could also be regulated by miRNAs. Thus, patients refractory to fludarabine exhibit significantly higher expression levels of miR-21, miR-148a and miR-222 than fludarabine sensitive patients.¹⁸ The activation of P53-responsive genes was found only in fludarabine responsive patients, suggesting a possible link between abnormal miRNA expression and P53 pathway dysfunction in non-responder patients. Links between miRNAs, fludarabine-refractory CLL and genomic abnormalities were further demonstrated, underlying the crucial role of *MYC* and *P53* regulatory networks in determining cell response to fludarabine in CLL.¹⁹ Finally, in a prospective clinical trial aiming at evaluating the contribution of 17p deletion or *TP53* mutation in fludarabine-refractory CLL, miR-34a expression at baseline was lower than in a control cohort of CLL non-refractory patients.²⁰ The detection of miRNAs in serum and other body fluids under physiological and pathological conditions raises the possibility of using them as diagnostic or prognostic biomarkers.²¹⁻²³ Visone *et al.* found that blood expression levels of miR-181b decreased in progressive CLL patients but not in patients with stable disease.²⁴ Recently, we have shown that high miR-125b blood concentration can predict clinical benefit of rituximab (MabThera®, Rituxan®) treatment in patients with rheumatoid arthritis, and preliminary results suggested a similar prognostic value of miR-125b in B-cell lymphoma patients.²⁵ Interestingly, the expression of miR-125b is high in hematopoietic stem cells (HSCs) and decreases in committed progenitors.²⁶ In addition, overexpression of miR-125b in mice HSCs is associated with the development of lymphoproliferative disease.²⁷ Finally, miR-125b expression is reduced in CLL patients as compared with healthy donors.²⁸ Altogether, these studies suggest that low expression of circulating miR-125b might be associated with lymphoproliferation and poor treatment response to rituximab.

The *in vivo* mechanisms of rituximab action remain incompletely understood, and could differ depending on the subtype of B-lymphoproliferative disorders. *In vitro* data demonstrate that rituximab is able to induce apoptosis, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Although the influence of Fc γ RIIIa-V158V on rituximab response

in follicular lymphoma patients strongly suggests that ADCC occurs *in vivo*, there is a lack of evidence for the other immune mechanisms.²⁹ One of the main barriers to improve our knowledge is the scarcity of clinical situations in which rituximab is used alone. Indeed, chemotherapy associated with rituximab pollutes any definitive conclusions in studies attempting to analyze *in vivo* rituximab mechanisms of action. Recently, we conducted a clinical phase II study testing a new approach of dose-dense rituximab pre-phase before immunochemotherapy.³⁰ This study was based on increased rituximab elimination observed in CLL patients compared to lymphoma patients in different clinical trials.³¹ Thus, this study provided a unique opportunity to dissect the mechanisms of action of rituximab in CLL patients. The study herein aimed at identifying a blood-based miRNA signature at diagnosis, before rituximab monotherapy, that predicts rituximab efficacy in CLL patients. It also aimed at shedding light on the miRNA-mediated molecular mechanisms involved in rituximab's mode of action *in vivo*.

Methods

CLL2010FMP protocol

A prospective, randomized, open-label, phase II study (CLL2010FMP, *clinicaltrials.gov* Identifier: 01370772) included 140 treatment-naïve patients (aged 18-65 years) diagnosed with confirmed chronic lymphocytic leukemia, according to the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) 2008 criteria, and Binet stage C, or with active Binet stage A or B.³² An additional inclusion criteria was the absence of 17p deletion, assessed by fluorescence *in situ* hybridization (FISH) (<10% positive nuclei). Each patient provided written informed consent before enrolment. Participating centers are listed in the *Online Supplementary Information*. Patients were stratified according to *IGHV* mutational status, FISH analysis (11q deletion or not) and were randomly assigned to receive either 6 cycles of chemoimmunotherapy combining fludarabine, cyclophosphamide, and rituximab (FCR) (rituximab 375mg/m² for the first course, Day (D)1 and 500mg/m² for the others, fludarabine 40mg/m²/d D2-4, cyclophosphamide 250mg/m²/d D2-4) every 28 days, or Dense-FCR with an intensified rituximab pre-phase (500mg on D0, and 2000mg on D1, D8 and D15) before initiating the standard FCR treatment. The main objective was to increase the complete response rate with undetectable minimal residual disease three months after treatment, as published previously.³⁰ In the study herein we have explored miRNA signature in a cohort of 123 patients, 61 receiving rituximab pre-phase before immunochemotherapy (Dense-FCR arm) and 62 receiving the FCR chemotherapy (standard arm). The study was approved by the institutional ethics committee of each participating center according to the principles of the Declaration of Helsinki.

Gene expression analysis

Details are described in the *Online Supplementary Information*.

FCGR3A genotyping

Single-step multiplex allele-specific PCR assays were performed as initially described by Dall'Ozzo *et al.* introducing minor modifications (for details see the *Online Supplementary Information*).

IL-10 competent CLL cells identification

Interleukin (IL)-10-competent CLL cell counts were determined by flow cytometry analysis of IL-10 production. Peripheral blood

Table 1. Patients' characteristics for the protocol CLL2010FMP.

	Cohort (n=123)		FCR (n=62)		Dense FCR (n=61)	
	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)
Age (years)	–	58.45 (52.82-61.83)	–	59.95 (52.32-62.00)	–	58.34 (53.22-61.36)
Women	33 (26.83)	–	16 (25.81)	–	17 (27.87)	–
Binet stage AB	91 (73.98)	–	44 (70.97)	–	47 (77.05)	–
ECOG 0	86 (69.92)	–	41 (66.13)	–	45 (73.77)	–
<i>IGHV</i> unmutated	75/119 (63.03)	–	39/59 (66.10)	–	36/60 (60.00)	–
Cytogenetic abnormalities						
Del(13q)	54/96 (56.25)	–	29/48 (60.42)	–	25/48 (52.08)	–
Del(11q)	24/120 (20.00)	–	12/61 (19.67)	–	12/59 (20.34)	–
Trisomy 12	9/82 (10.98)	–	5/40 (12.50)	–	4/42 (9.52)	–
$\beta 2$ microglobulin (mg/L)	114 (92.68)	3.05 (2.35-4.00)	56 (90.32)	3.22 (2.42-4.20)	59 (96.72)	2.80 (2.32-3.68)
IL-10-competent B cells	44 (35.77)	2.97 (0.98-9.58)	–	–	44 (72.13)	2.97 (0.98-9.58)
<i>FCGR3A</i>						
VV	14 (12.17)	–	8 (14.04)	–	6 (10.34)	–
V/F	55 (47.83)	–	26 (45.61)	–	29 (50.00)	–
F/F	46 (40.00)	–	23 (40.35)	–	23 (39.66)	–

IQR: interquartile range; FCR: fludarabine, cyclophosphamide, and rituximab; ECOG: Eastern Cooperative Oncology Group; IL-10: interleukin-10.

mononuclear cells (PBMCs) were purified from peripheral blood samples of the Dense-FCR arm of the protocol using Ficoll-Hypaque density gradients (Eurobio, Courtaboeuf, France).³³ Clonal CLL cells were identified as CD19⁺ CD5⁺ CD20^{int} lymphocytes with a previously described protocol.³⁴ Analyses were performed on a CyAnTM ADP flow cytometer (Beckman Coulter, Brea, CA, USA).

Cell surface CD20 expression analysis

CD20 expression was quantified using CD20-PE QuantiBRITE™ reagents (Ratio 1:1) according to manufacturer's recommendations (BD Biosciences, Le Pont-de-Claix, France). Calibration and quantification were performed using a FAC-SCANTO II cytometer (BD, Biosciences, Le Pont-de-Claix, France), and details are in the *Online Supplementary Information*. Circulating CD20 antigen was evaluated by considering the lymphocyte count and blood volume for each patient.

Statistical analysis

The distribution of data was tested with the Shapiro-Wilk test. A χ^2 or Fisher's test were used to compare categorical data. For numerical data, medians were compared using a Student's *t*-test or Mann-Whitney test. All variables with a *P* value <0.10 in univariate analysis were included in an intermediate model. The final model variables were determined by backward selection using a Student's *t*-test (*P*<0.05 as significant model). The Spearman's correlation test was used to assess the association between two numerical data. All statistical analyses were performed at the conventional two-tailed α level of 0.05 using R software version 3.0.2.10.

Results

Patients' characteristics

Sixty-one CLL patients were allocated to receive rituximab pre-phase. Their clinical and biological characteristics are presented in Table 1. They did not differ from the entire cohort. Median age was 58 years (interquartile range (IQR): 53-61), 28% were females and 77% were

Table 2. List of the 26 miRNAs differentially expressed in CLL patients with low or high lymphocyte counts at DO.

miRNA ID	Ct value in high lymphocyte count (mean)	Fold Change low/high lymphocyte count (mean)	<i>P</i>
Cluster 1			
hsa-miR-323-3p	31.8	9.6	0.022
hsa-miR-99b	26.5	8.7	0.003
hsa-miR-326	31.3	7.5	0.001
hsa-miR-365	31.6	7.5	0.004
hsa-miR-125b	28.6	5.2	0.033
hsa-miR-494	28.4	4.3	0.022
hsa-miR-193b	25.6	3.3	0.009
hsa-miR-211	28.0	2.8	0.012
hsa-miR-193a-5p	26.5	2.1	0.020
hsa-miR-212	26.4	1.8	0.012
hsa-miR-184	29.1	0.2	0.041
Cluster 2			
hsa-miR-328	22.9	5.1	0.015
hsa-miR-486	18.9	4.7	0.013
hsa-miR-532-3p	22.1	4.4	0.010
hsa-miR-484	17.4	3.7	0.028
hsa-miR-324-3p	22.2	2.9	0.004
hsa-miR-92a	17.2	2.9	0.001
hsa-miR-339-5p	23.3	2.8	0.010
hsa-miR-223	14.5	2.5	0.034
hsa-miR-423-5p	22.8	2.3	0.013
hsa-miR-652	21.2	2.2	0.038
hsa-miR-30c	16.6	2.0	0.029
hsa-miR-16	16.6	0.4	0.044
hsa-miR-26a	17.6	0.3	0.031
hsa-miR-29c	19.6	0.1	0.002
hsa-miR-29a	18.9	0.1	0.002

The cycle threshold (Ct) value is defined as the number of cycles required for the fluorescent signal to cross the background level. Fold Change is the fold ratio of the geometric means of miRNA expression from low and high lymphocyte count patients. The *P* value is determined by *t*-test and considered significant for *P*<0.05.

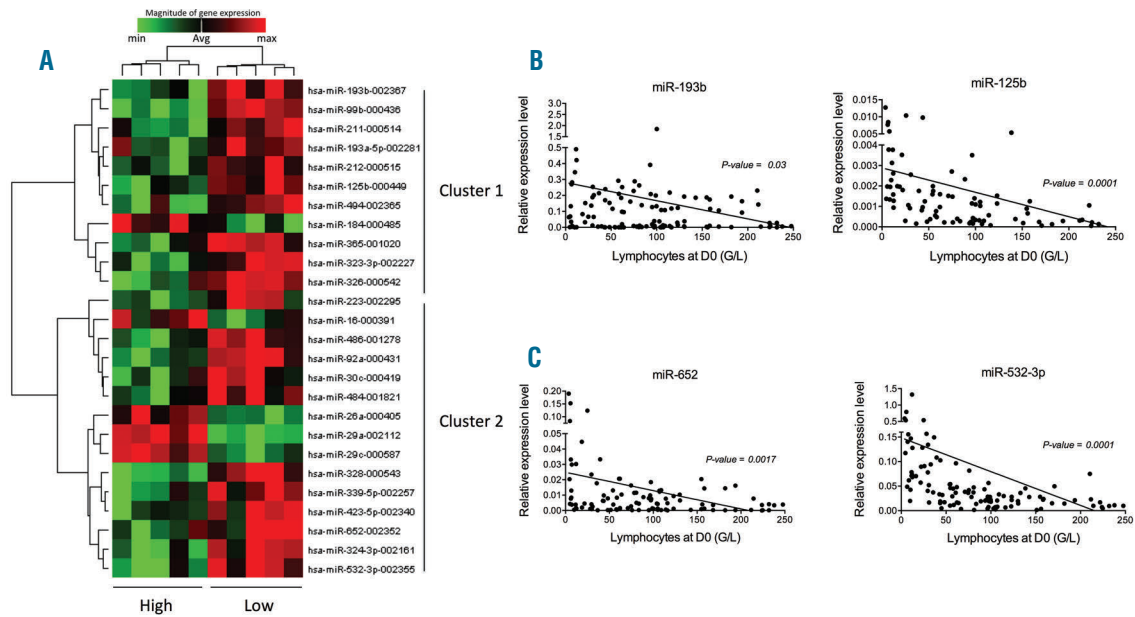


Figure 1. MicroRNAs expression profile discriminates CLL patients with low or high lymphocyte counts before treatment. (A) The profiles of 26 microRNAs significantly differently expressed ($P < 0.05$) between high and low lymphocyte concentration samples isolated from CLL patients ($n = 5/\text{group}$) were visualized using a supervised heatmap (average linkage and Pearson's correlation). Relative miRNA expression was calculated using the comparative threshold cycle (Ct) method. For normalization, the mean Ct value of all miRNA targets was used. Dendrograms indicated the correlation between groups of samples and miRNAs. Samples are in columns and miRNAs in rows. Each column represents an individual sample and each row represents a single miRNA. The heat map shows relative levels of miRNA expression in a green (low relative expression) to red (high relative expression) scale. (B-C) Expression levels of 4 miRNAs representative of each cluster, miR-193b and miR-125b for cluster 1 (B), and miR-532-3p and miR-652 for cluster 2 (C), were measured for 123 CLL patients included in the CLL2010FMP protocol, using RT-qPCR. A significant inverse correlation was observed depending on the lymphocyte counts for miR-193b ($r = -0.19$), miR-125b ($r = -0.39$), miR-652 ($r = -0.30$) and miR-532-3p ($r = -0.34$) (Spearman's correlation test).

Binet stage A or B. Cytogenetic analysis demonstrated del11q in 20% of patients and median lymphocyte count was 89 G/L (IQR: 43-123).

Blood miRNA expression profile discriminates CLL patients with high and low lymphocytosis

Because miR-125b expression is reduced in CLL patients compared with healthy donors and is a key regulator of lymphoproliferation,²⁸ we performed real-time PCR-based high-throughput miRNA arrays comparing CLL patients with high lymphocyte counts ($>93.93\text{G/L}$, Q3) versus low lymphocyte counts ($<11.67\text{G/L}$, Q1) at baseline. Q1 and Q3 were interquartile values of lymphocyte counts at D0 for patients of the Dense-FCR arm analyzed by TaqMan low-density array (TLDA). After filtering (fold change ≥ 1.5 and cycle threshold (Ct) values < 32) on the differentially expressed miRNAs, we found 5 miRNAs downregulated and 21 miRNAs upregulated in CLL patients with low lymphocyte counts compared with those with high lymphocyte counts ($P < 0.05$) (Table 2). The heat map showed results of the unsupervised hierarchical clustering based on the significantly differentially expressed miRNAs (Figure 1A). Two patterns of miRNA expression profile named cluster 1 and cluster 2 were clearly identified according to lymphocyte counts. To confirm this finding, we selected four miRNAs (two from each cluster) based on technical criteria ($21 < \text{Ct} < 29$ and 2-fold difference between high and low lymphocyte count at D0 between two miRNAs in each cluster), and on the literature.^{28,35,36} We therefore assessed their expression in a cohort of 123 CLL patients (Figure 1B). Expression patterns of miR-193b, miR-125b, miR-652 and miR-532-3p were consistent with

the array data. Scatter plots confirmed that increased lymphocyte counts were inversely correlated with the expression levels of miR-193b and miR-125b ($P = 0.03$ and $P = 0.0001$, respectively) for cluster 1, and miR-652 and miR-532-3p ($P = 0.0017$ and $P = 0.0001$, respectively) for cluster 2 (Figure 1B). No significant correlation was found between individual miRNAs (miR-125b, miR-193b, miR-532-3p, miR-652) and clinical (age, Binet stage, Eastern Cooperative Oncology Group (ECOG)) or biological (*IGHV* mutation, cytogenetic abnormalities (del11q, del13q, trisomy 12), B10 frequency, or Fc γ RIIIa-158V/F polymorphism) parameters (*data not shown*). Finally, our results demonstrated that all 4 miRNAs were markedly downregulated in the blood of CLL patients displaying high lymphocyte counts.

miR-125b and miR-532-3p expression levels correlate with lymphodepletion observed after rituximab treatment

We hypothesized that lymphocyte depletion observed after rituximab infusions was related to *in vivo* rituximab activity, and then monitored the lymphocyte depletion in our CLL cohort following four rituximab infusions at D22. We thus determined whether a correlation existed between the miRNA expression profile that discriminates CLL patients with high lymphocytosis before treatment and the efficacy of lymphocyte depletion with rituximab. The median lymphocyte count was 89G/L (range: 4-351) before rituximab treatment and 3G/L (range: 0.1-189) after four rituximab infusions, bringing the median lymphocyte depletion after rituximab treatment up to 95.9% (range: -5.0-99.6).

Only 2 out of the 4 validated miRNAs, namely miR-125b and miR-532-3p, were significantly correlated with lymphodepletion, whereas miR-652 and miR-193b did not correlate with lymphodepletion. Thus, the lymphodepletion rate was inversely correlated with the expression levels of miR-125b and miR-532-3p ($P=0.020$ and $P=0.001$, respectively), as shown in the scatter plots in Figure 2. However, no correlation was found between miRNAs and the clinical response assessed 3 months after immunochemotherapy by FCR.

Our group recently showed that the frequency of IL-10-competent B cells adversely impacts lymphodepletion following rituximab treatment of CLL patients and also correlates with clinical response assessed 3 months after immuno-chemotherapy by FCR.³⁴ Because we reported that the IL-10-competent regulatory B cells frequency predicts efficacy of rituximab-mediated lymphodepletion and clinical CLL outcome, we integrated this third variable in our analysis. Logistic regression analyses showed that only IL-10-competent B cells frequency and miR-532-3p were associated with 90% lymphodepletion after rituximab monotherapy (odds ratio (OR)=0.87; 95% confidence interval (CI)=0.76-0.97; $P=0.014$, and OR=0.0002; 95% CI=<10-4-0.34; $P=0.029$, respectively). Receiver operating characteristic curve (ROC) using IL-10-competent B cells frequency and miR-532-3p expression levels showed a highly discriminative power (area under the curve (AUC)=0.795; 95% CI=0.652-0.939), allowing one to predict patients who will have more than 90% of lymphodepletion after rituximab pre-phase.

Putative and validated target genes of miR-125b and miR-532-3p

Using the miRWalk database, a tool that compares miRNAs binding sites resulting from 5 main existing miRNA-target prediction programs (DIANA, RNA22, PicTar, miRanda and TargetScan), we investigated putative target genes for the two miRNAs associated with rituximab-induced lymphodepletion.³⁷ Two lists of putative target genes were obtained: 5053 genes for miR-125b and 6652 for miR-532-3p. The Venny program, an interactive tool for comparing lists, identified 3151 common genes targeted by both miR-125b and miR-532-3p.³⁸ We then

compared them with transcriptomic datasets available for IL-10-competent B cells.³⁹ Among the 104 genes differentially expressed in the study that compared IL-10⁺ and IL-10⁻ human regulatory B cells, 33 and 46 genes overlapped with miR-125b and miR-532-3p putative targeted genes, respectively.³⁹ Importantly, 26 genes were common targets of both miRNAs (Figure 3A). Consequently, in the context of rituximab, which is known to target the pan-B-cell marker CD20/MS4A1, we wondered whether this gene could also be targeted by miR-125b and miR-532-3p. We found that both miRNAs were predicted to target MS4A1.

Pathway enrichment analysis was performed using the web-based bioinformatics application Ingenuity Pathway Analysis (IPA Ingenuity Systems) based on the *in silico* 26 predicted target genes common to miR-125b, miR-532-3p and differentially regulated in human IL-10⁻/IL-10⁺ regulatory B cells, as well as on MS4A1. A hierarchical layout was built with only miRNA/mRNA interactions displaying high predicted scores and for which the correlation was experimentally observed in humans (Figure 3B). All the 9 genes presented in this figure were associated with the IL-10 pathway (*EGR3*, *IL1A*, *IL10*, *IL10RA*, *IRF4*, *IRF5*, *MS4A1*, *TLR7* and *TSC22D3*).

Expression of CD20 family members, miR-125b and miR-532-3p on CLL cells

We analyzed the association between miR-125b and miR-532-3p expression levels and the CD20 surface expression on CD19⁺/CD5⁺ CLL cells. In both cases, a significant inverse correlation was observed between CD20 protein and miRNA expression levels (Figure 4). A high expression level of CD20 at the surface of CLL cells correlated with a low expression level of miR-125b and miR-532-3p ($P=0.0007$ and $P<0.0001$, respectively). Since miRNAs are negative regulators of gene expression, we monitored the mRNA level of CD20 in the blood of CLL patients. CD20 (MS4A1) mRNA tended to be inversely correlated with both miR-125b and miR-532-3p (Figure 5A). Interestingly, the expression of two other members of the CD20 family, namely MS4A3 and MS4A7, might also be controlled by miR-125b and miR-532-3p. Indeed, both MS4A3 and MS4A7 mRNAs present putative binding sites for miR-125b and miR-532-3p, not only at the 3'UTR

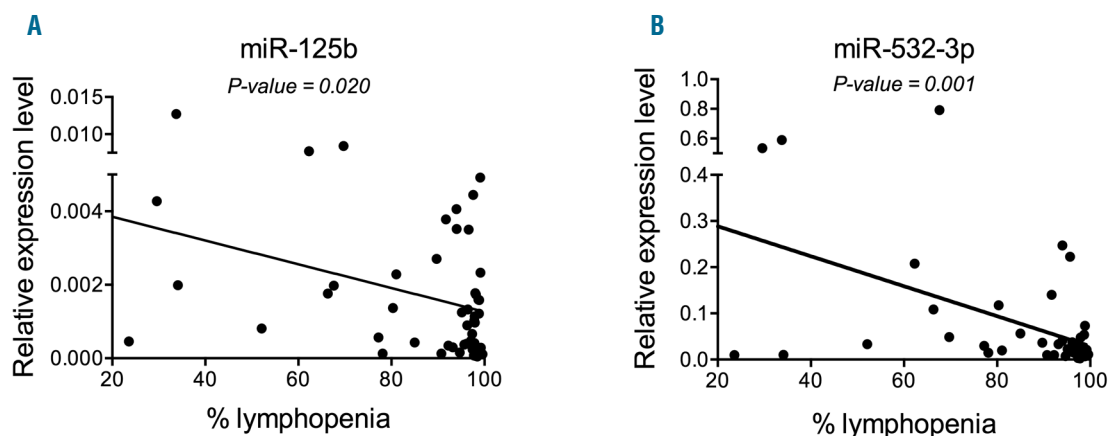


Figure 2. Efficacy of lymphodepletion after rituximab treatment inversely correlates with miR-125b and miR-532-3p expression levels. miR-125b ($r=-0.42$) (A) and miR-532-3p ($r=-0.49$) (B) expression levels were quantified by RT-qPCR. The lymphodepletion was measured 22 days after 4 doses of rituximab infusion and correlated with miRNAs (Spearman's correlation test) ($n=61$).

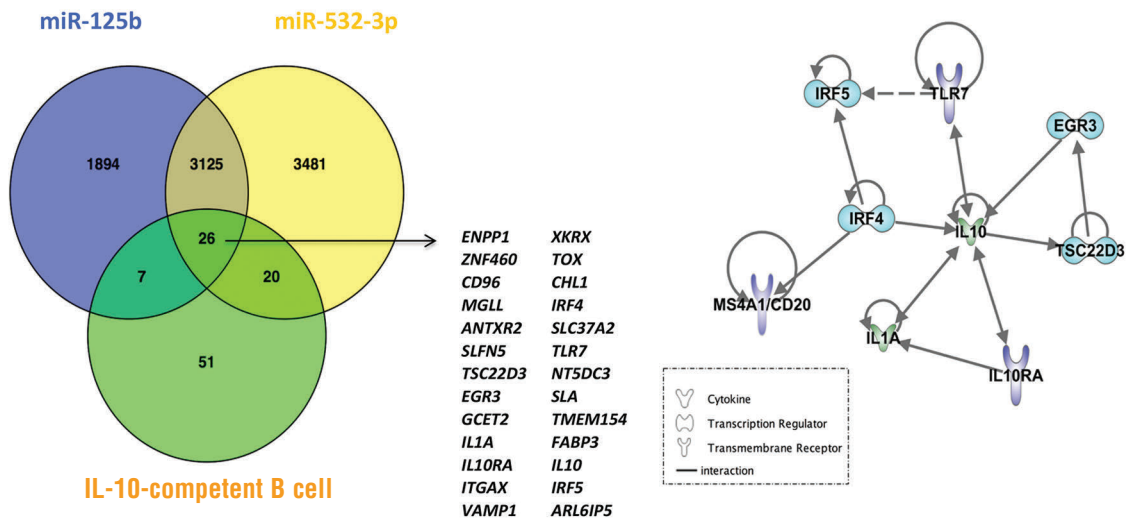


Figure 3. Target gene prediction for miR-125b and miR-532-3p. (A) To identify putative miR-125b and miR-532-3p target genes, we used miRWalk software. VENNY, an interactive tool for comparing lists with Venn Diagrams, was used to predict common genes between human regulatory B cells IL-10⁺ and IL-10⁻,³⁹ and putative miR-125b and miR-532-3p target genes. 26 genes putatively targeted by miR-125b and miR-532-3p, and specifically dysregulated in IL-10⁺ B cells transcriptome analysis are listed. (B) The layout of these 26 putative targets was built in the context of rituximab treatment (MS4A1 (CD20 gene)) using Ingenuity Pathway Analysis (IPA). 9 genes revealed a central role for the IL-10 pathway.

region, but also in the promoter, 5'UTR and coding regions as shown in Figure 5B. MS4A7 mRNA levels were positively correlated with both miR-125b and miR-532-3p expression levels, whereas MS4A3 mRNA levels were positively correlated with miR-125b expression only. Overall, these results suggest that miR-125b and miR-532-3p might differentially control the expression of the CD20 family members.

Discussion

In the study herein, we investigated whether a blood-based miRNA signature can predict the efficacy of rituximab-mediated lymphodepletion in CLL patients, and provide some clue as to the underlying molecular mechanisms. We showed that miR-125b, miR-193b, miR-652 and miR-532-3p expression levels were inversely correlated with lymphocyte counts in untreated patients, and that miR-125b and miR-532-3p negatively correlated with lymphocyte depletion after rituximab monotherapy. Finally, our data suggest that both miR-125b and miR-532-3p expression levels might provide a link between the expression level of CD20 family members and the efficacy of rituximab-mediated lymphodepletion.

Both miR-125b and miR-532-3p have been previously described in leukemia disorders. Recently, it has been shown that miR-125b was involved in specific subtypes of leukemia, either through IRF4 silencing, genetic deletion or chromosomal translocation as evidenced in B-cell leukemia or myeloid leukemia, respectively.⁴⁰⁻⁴² One of the two genes encoding for the mature form of miR-125b, namely miR-125b-1, maps at 11q24, a chromosomal region that is close to the epicenter of 11q23 deletions found in CLL, and might explain why miR-125b expression is reduced in CLL patients compared to healthy donors.²⁸ In the study herein, although the number of patients with a del11q was small, no correlation was

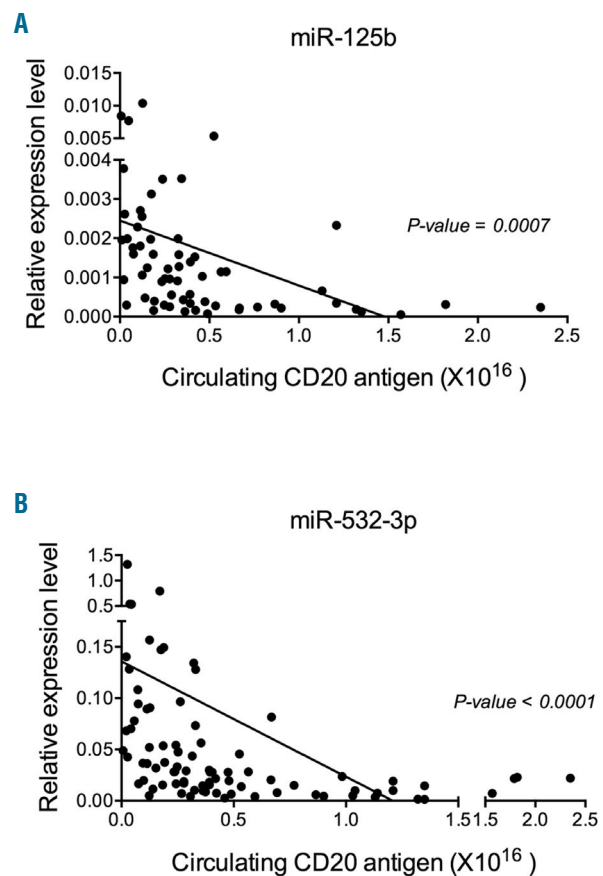


Figure 4. Inverse correlation between miR-125b and miR-532-3p expression levels and circulating CD20 surface antigen expression on CD19⁺ cells in CLL patients blood. CD20 expression levels on CD19⁺ lymphocytes were quantified using flow cytometry. PBMCs were collected and miR-125b ($r=-0.37$) (A) and miR-532-3p ($r=-0.29$) (B) were quantified using RT-qPCR. Circulating CD20 antigen was correlated to miRNA expression (Spearman's correlation test) ($n=61$).

A

	MS4A1		MS4A3		MS4A7	
	P-value	rho	P-value	rho	P-value	rho
hsa-miR-125b	0.235	-0.176	0.008	0.381	<0.0001	0.563
hsa-miR-532-3p	0.297	-0.155	0.271	0.163	<0.0001	0.633

B

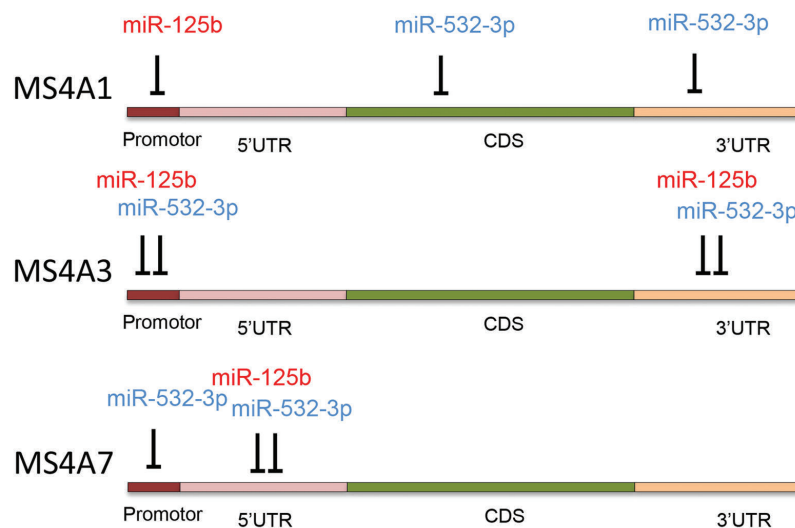


Figure 5. miR-125b and miR-532-3p expression levels correlate with the expression of three MS4A family members. (A) Spearman's correlation between miR-125b or miR-532-3p and MS4A mRNA family expression levels in CLL patients blood (n=61). (B) Putative miR-125b and miR-532-3p target binding sites on MS4A family members. CDS: coding DNA sequence; rho: correlation coefficient; UTR: untranslated region.

found with miR-125b expression levels. In a recent study investigating miRNA changes upon B-cell receptor stimulation in distinct subclasses of CLL patients, the expression of miR-532-3p was increased at 48 hours exclusively in CLL patients with stable disease.⁶ Like miR-125b, the role and implication of miR-532-3p in CLL are established, especially as it is strongly associated with progression-free survival in CLL.³⁵

Our data thus identify a novel prognostic relevance for miRNAs, and specifically for miR-125b and miR-532-3p, which are able to predict the efficacy of rituximab-mediated lymphodepletion, independently of the clinical outcome. In the study herein, logistic regression analysis showed that IL-10-competent B cells frequency and miR-532-3p were associated with lymphodepletion after rituximab pre-phase, whereas no correlation was found between miRNAs and the clinical response assessed 3 months after immunochemotherapy by FCR.

Taking advantage of the signatures of these 2 miRNAs for predicting the effect of rituximab, we searched for putative target genes according to these variables. Using available software and databases, we identified 3151 common putative target genes for miR-125b and miR-532-3p, which represent over 62% and 50% of miR-125b and miR-532-3p targets, respectively. The lack of sequence homology between miR-125b and miR-532-3p, neither for the seed sequence nor for the entire miRNA mature sequence, does not explain such a surprisingly large number of overlapping putative targets. None of the few common validated target genes reported so far for both miRNAs have been studied together in the same cellular

context. *In silico* analyses reveal that these two miRNAs rather target distinct sequences and/or regions of the same gene, suggesting a synergistic effect by collective target regulation by both miRNAs. Among the 104 genes differentially expressed between IL-10⁺ and IL-10⁻ cells,³⁹ we found over 50% of genes putatively targeted by miR-125b and/or miR-532-3p, among which 26 genes are common for both miRNAs. Pathway enrichment analysis identified 9 genes associated with the IL-10 pathway in the rituximab context. Some of these genes are already validated targets for miR-125b. Rossi *et al.* showed that miR-125b was involved in T-cell differentiation through the silencing of IL-10 receptor α (*IL10RA*). MiR-125b expression in CD4⁺ T cells could contribute to the maintenance of the naive state, while its downregulation is associated with the acquisition of an effector-memory phenotype.²⁶ MiR-125b inhibits the expression of IRF4 in B lymphocytes, diffuse large B-cell lymphomas and myeloma cell lines, and promotes myeloid and B-cell neoplasm by inducing tumorigenesis in mice hematopoietic progenitor cells.^{40,42,43} An indirect implication of miR-532-3p on TLR7 gene expression mediated by an upregulation of IL-4 was reported in peripheral blood samples from CLL patients.^{44,45} The dysregulation of miR-532-3p was also evidenced in Binet A stage CLL patients as compared with a normal B-cell subset population.³⁵ Among the miRNAs tested in relation with clinical data, miR-532-3p is part of a miRNA-based signature strongly associated with progression-free survival.³⁵

In humans, the *MS4A* gene includes CD20 and 18 other genes.⁴⁶ Rituximab is a chimeric type I monoclonal antibody

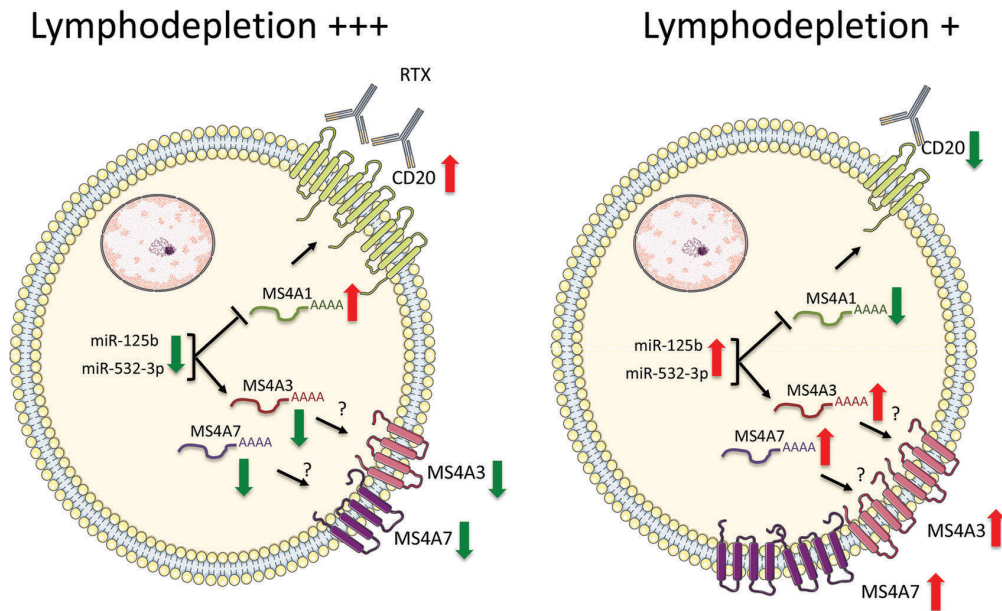


Figure 6. Schematic representation of the potential mechanisms of action of miR-125b and miR-532-3p on the modulation of rituximab activity in CLL cells. In CLL patients displaying low expression levels of miR-125b and miR-532-3p, the MS4A1 mRNA and CD20 surface receptor are upregulated, while the expression of MS4A3 and MS4A7 mRNA are downregulated (MS4A3 and MS4A7 protein expressions were unknown). Consequently, rituximab (RTX) efficacy is optimum for lymphodepletion (Lymphodepletion +++). In contrast, CLL patients with high expression levels of miR-125b and miR-532-3p display low levels of MS4A1 mRNA and CD20 surface receptor, and high levels of MS4A3 and MS4A7 mRNA (MS4A3 and MS4A7 protein expressions were unknown), which might form hetero-oligomers with CD20 and impede optimal lymphodepletion (Lymphodepletion +) by rituximab. Interrogation marks indicate data that were not experimentally confirmed in the present study.

that specifically binds to inter-tetramers of CD20.²⁹ CD20 could form homo- or hetero-oligomeric complexes with CD20 family members.⁴⁷ Herein, we show that miR-125b and miR-532-3p putatively target other members of the *MS4A* family, including *MS4A1* (coding CD20), *MS4A3* (alias Htm4) and *MS4A7*. *MS4A3* is a cell surface signaling molecule involved in the cell cycle of hematopoietic and tumor cells, whereas *MS4A7* is expressed in lymphoid tissues.^{47,48} However, their role in the mode of action of rituximab is unexplored. Herein, we showed a significant correlation between miR-125b and miR-532-3p expression levels and three *MS4A* family members in CLL patients; CD20 being negatively correlated and *MS4A3* and *MS4A7* being positively correlated.^{48,49} Negative regulation of gene expression by miRNAs through translational repression and deadenylation-dependent decay of messengers is widely described. Emerging evidence reveal that miRNAs and their associated multiprotein complexes can directly or indirectly stimulate gene expression.⁵⁰ GW182 is an essential component of the repressive miRNA complex that interacts with Ago1/2, leading to mRNA degradation. In conditions of quiescence, the absence of GW182 favors the interaction of FXR1 with Ago1/2 and induces the translation of the targeted mRNA.⁵⁰ In the context of CLL, which is a unique malignancy where quiescent B cells accumulate in the peripheral blood, we may hypothesize that miR-125b and miR-532-3p

might differently affect the expression of the 3 *MS4A* family members and thus modulate the lymphodepletion outcome upon rituximab treatment. In Figure 6, we propose a schematic explanation on how miR-125b and miR-532-3p can act in CLL patient cells to modulate rituximab efficacy on lymphodepletion.

Overall, our results suggest that miR-125b and miR-532-3p are potential non-invasive biomarkers, detectable in the peripheral blood of CLL patients before treatment, which predict rituximab-mediated lymphodepletion efficacy. These miRNAs might also play a role in the molecular mechanisms involved in the rituximab-mediated mechanism of action, through their implication in the IL-10 pathway, including IL-10-competent B cells, and through the modulation of the *MS4A* family members' expression. Further investigations in an independent cohort are needed to further explore these hypotheses.

Funding

This work is supported by a public grant overseen by the French National Research Agency (ANR) as part of the "Investissements d'Avenir" program (reference Labex MAbImprove: ANR-10-LABX-53-01), the INSERM and the university of Montpellier. This study was funded by the French Innovative Leukemia Organization (FILO) group and F. Hoffman-La Roche Ltd (Basel, Switzerland).

References

- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215-233.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006; 6(11):857-866.
- Lawrie CH. MicroRNAs in hematological malignancies. *Blood rev*. 2013;27(3):143-154.
- Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*. 2009;10(10):704-714.
- Li PP, Wang X. Role of signaling pathways and miRNAs in chronic lymphocytic leukemia. *Chin Med J (Engl)*. 2013; 126(21): 4175-4182.
- Tavolaro S, Colombo T, Chiaretti S, et al.

- Increased chronic lymphocytic leukemia proliferation upon IgM stimulation is sustained by the upregulation of miR-132 and miR-212. *Genes Chromosomes Cancer*. 2015;54(4):222-234.
7. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002; 99(24):15524-15529.
 8. Palacios F, Abreu C, Prieto D, et al. Activation of the PI3K/AKT pathway by microRNA-22 results in CLL B-cell proliferation. *Leukemia*. 2015;29(1):115-125.
 9. Rushworth SA, Murray MY, Barrera LN, Heasman SA, Zaitseva L, Macewan DJ. Understanding the role of miRNA in regulating NF-kappaB in blood cancer. *Am J Cancer Res*. 2012;2(1):65-74.
 10. Bomben R, Gobessi S, Dal Bo M, et al. The miR-17 approximately 92 family regulates the response to Toll-like receptor 9 triggering of CLL cells with unmutated IGHV genes. *Leukemia*. 2012;26(7):1584-1593.
 11. Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014; 124(4):546-554.
 12. Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124(1):84-95.
 13. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. 2005;102(39):13944-13949.
 14. Pekarsky Y, Santanam U, Cimmino A, et al. Tc1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res*. 2006;66(24):11590-11593.
 15. Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are down-regulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23(6):1159-1163.
 16. Fabbri M, Bottoni A, Shimizu M, et al. Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. *JAMA*. 2011;305(1):59-67.
 17. Zou ZJ, Fan L, Wang L, et al. miR-26a and miR-214 down-regulate expression of the PTEN gene in chronic lymphocytic leukemia, but not PTEN mutation or promoter methylation. *Oncotarget*. 2015; 6(2):1276-1285.
 18. Ferracin M, Zagatti B, Rizzotto L, et al. MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. *Mol Cancer*. 2010;9(123).
 19. Moussay E, Palissot V, Vallar L, et al. Determination of genes and microRNAs involved in the resistance to fludarabine in vivo in chronic lymphocytic leukemia. *Mol Cancer*. 2010;9:115.
 20. Zenz T, Habe S, Denzel T, et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood*. 2009;114(13):2589-2597.
 21. De Tullio G, De Fazio V, Sgherza N, et al. Challenges and opportunities of microRNAs in lymphomas. *Molecules*. 2014;19(9): 14723-14781.
 22. Gilad S, Meiri E, Yogev Y, et al. Serum microRNAs are promising novel biomarkers. *PLoS One*. 2008;3(9):e3148.
 23. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105(30):10513-10518.
 24. Visone R, Veronese A, Rassenti LZ, et al. miR-181b is a biomarker of disease progression in chronic lymphocytic leukemia. *Blood*. 2011;118(11):3072-3079.
 25. Duroux-Richard I, Pers YM, Fabre S, et al. Circulating miRNA-125b is a potential biomarker predicting response to rituximab in rheumatoid arthritis. *Mediators Inflamm*. 2014;2014(342524).
 26. Rossi RL, Rossetti G, Wenandy L, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol*. 2011; 12(8):796-803.
 27. Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci USA*. 2010;107(50):21505-21510.
 28. Tili E, Michaille JJ, Luo Z, et al. The down-regulation of miR-125b in chronic lymphocytic leukemias leads to metabolic adaptation of cells to a transformed state. *Blood*. 2012;120(13):2631-2638.
 29. Cartron G, Trappe RU, Solal-Celigny P, Hallek M. Interindividual variability of response to rituximab: from biological origins to individualized therapies. *Clin Cancer Res*. 2011;17(1):19-30.
 30. Lepretre S, Letestu R, Dartigeas C, et al. Results of a Phase II Randomizing Intensified Rituximab Pre-Phase Followed by Standard FCR Vs Standard FCR in Previously Untreated Patients with Active B-Chronic Lymphocytic Leukemia (B-CLL). CLL2010FMP (for fit medically patients): A Study of the french Cooperative Group on CLL and WM (FCGCLL/MW) and the "Groupe Ouest-Est d'Etudes Des Leucémies Aigües Et Autres Maladies Du sang" (GOELAMS). *Blood*. 2014;124(21): 3329.
 31. Li J, Zhi J, Wenger M, et al. Population pharmacokinetics of rituximab in patients with chronic lymphocytic leukemia. *J Clin Pharmacol*. 2012;52(12):1918-1926.
 32. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008; 111(12):5446-5456.
 33. Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood*. 2011; 117(2):530-541.
 34. Gagez AL, Tuaille E, Cezar R, et al. Response to rituximab in B-CLL patients is adversely impacted by frequency of IL-10 competent B cells and Fc gamma RIIIa polymorphism. A study of FCGCLL/WM and GOELAMS groups. *Blood Cancer J*. 2016; 6:e389.
 35. Negrini M, Cutrona G, Bassi C, et al. microRNAome expression in chronic lymphocytic leukemia: comparison with normal B-cell subsets and correlations with prognostic and clinical parameters. *Clin Cancer Res*. 2014;20(15):4141-4153.
 36. Moussay E, Wang K, Cho JH, et al. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2011;108(16):6573-6578.
 37. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods*. 2015;12(8):697.
 38. Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.
 39. Lin W, Cerny D, Chua E, et al. Human regulatory B cells combine phenotypic and genetic hallmarks with a distinct differentiation fate. *J Immunol*. 2014;193(5):2258-2266.
 40. So AY, Sookram R, Chaudhuri AA, et al. Dual mechanisms by which miR-125b represses IRF4 to induce myeloid and B-cell leukemias. *Blood*. 2014;124(9):1502-1512.
 41. Sonoki T, Iwanaga E, Mitsuya H, Asou N. Insertion of microRNA-125b-1, a human homologue of lin-4, into a rearranged immunoglobulin heavy chain locus in a patient with precursor B-cell acute lymphoblastic leukemia. *Leukemia*. 2005; 19(11):2009-2010.
 42. Malumbres R, Sarosiek KA, Cubedo E, et al. Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. *Blood*. 2009; 113(16):3754-3764.
 43. Gururajan M, Haga CL, Das S, et al. MicroRNA 125b inhibition of B cell differentiation in germinal centers. *Int Immunol*. 2010;22(7):583-592.
 44. Ruiz-Lafuente N, Alcaraz-Garcia MJ, Sebastian-Ruiz S, et al. The gene expression response of chronic lymphocytic leukemia cells to IL-4 is specific, depends on ZAP-70 status and is differentially affected by an NFkappaB inhibitor. *PLoS One*. 2014; 9(10):e109533.
 45. Ruiz-Lafuente N, Alcaraz-Garcia MJ, Sebastian-Ruiz S, et al. IL-4 up-regulates MiR-21 and the MiRNAs hosted in the CLCN5 gene in chronic lymphocytic leukemia. *PLoS One*. 2015;10(4):e0124936.
 46. Clark EA, Ledbetter JA. How does B cell depletion therapy work, and how can it be improved? *Ann Rheum Dis*. 2005;64 Suppl 4:iv77-80.
 47. Liang Y, Tedder TF. Identification of a CD20-, Fc epsilon R1beta-, and HTM4-related gene family: sixteen new MS4A family members expressed in human and mouse. *Genomics*. 2001;72(2):119-127.
 48. Kutok JL, Yang X, Folkert R, Adra CN. Characterization of the expression of HTM4 (MS4A3), a cell cycle regulator, in human peripheral blood cells and normal and malignant tissues. *J Cell Mol Med*. 2011;15(1):86-93.
 49. Gingras MC, Lapillonne H, Margolin JF. CFFM4: a new member of the CD20/Fc epsilon R1beta family. *Immunogenetics*. 2001;53(6):468-476.
 50. Banzhaf-Strathmann J, Edbauer D. Good guy or bad guy: the opposing roles of microRNA 125b in cancer. *Cell Commun Signal*. 2014;12:30.