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Circulating microRNAs as potential biomarkers for monitoring the response to *in vivo* treatment with Rituximab in systemic lupus erythematosus patients

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Dear Sir,

Over the last decades, huge progresses have been made in the management of systemic lupus erythematosus (SLE) patients. Among therapeutic advances, Rituximab (RTX) has been proved to be an effective treatment option, especially in those patients who are refractory or intolerant to conventional therapies [1]. The response to RTX administration is still variable, reflecting the heterogeneity and the complexity of SLE among individuals. To date, several studies have tried to identify predictors of response to RTX [2,3]. Nevertheless, the lack of reliable biomarkers for monitoring the response to B-cells depletion therapy in SLE setting still represents an unmet clinical need.

MicroRNAs (miRNAs) are a class of small non-coding RNAs able to regulate gene expression at the post-transcriptional level and implicated in the immune response, as well as in the pathogenesis and progression of several autoimmune conditions, including SLE [4,5]. Giving the wide spectrum of immune functions that have been linked to the presence of miRNAs in SLE setting, it has been postulated that these molecules might serve as effective prognostic biomarkers of response to treatment in these patients.

The aim of the present study was to identify the altered circulating miRNAs profile in a cohort of SLE patients and their modulation by the *in vivo* treatment with RTX. For this purpose, 27 healthy donors (HDs) and 15 active SLE patients were included in the study after obtaining approval from the ethics committee of the San Giovanni Bosco Hospital from Turin (Italy) and the Reina Sofia Hospital from Cordoba (Spain). All patients fulfilled the American College of Rheumatology criteria for the classification of SLE [6]. SLE patients were clinically and serologically evaluated at baseline and after 3 months of therapy with RTX (375 mg/sq. weekly for 4 weeks).

Clinical assessment of the entire cohort included changes in disease activity, using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2 K) and complete serological evaluation. All patients provided written informed consent in accordance with the Declaration of Helsinki. Demographic, clinical, and laboratory characteristics are displayed in Table 1.

Techniques for blood sample collection, miRNA isolation and expression profiling, functional classification and target gene prediction by Ingenuity Pathway Analysis (IPA) software and cytokine quantification are available and can be provided upon requested.

After 3 months of RTX therapy, active SLE patients (mean SLEDAI-2 K before treatment = 9.5) showed a significant reduction in the

Table 1
Demographic, clinical and serological characteristics of the cohort.

	HDs (n = 27)	SLE patients Before RTX (n = 15)	SLE patients After RTX (n = 15)
Demographic characteristics			
Female (n,%)	17 (62)	14 (93)	
Age, years (mean, SD)	39.5 ± 9.8	40 ± 10.9	
Caucasian (n,%)	27 (100)	15 (100)	
Clinical characteristics and CV risk factors			
SLEDAI-2 K (mean, SD)	–	9.5 ± 3	1.5 ± 1.6 *
Renal disorder (n,%)	0	13 (86)	
Thrombosis (n,%)	0	1 (6)	
Arterial hypertension (n,%)	0	7 (46)	
Dyslipidaemia (n,%)	1 (4)	6 (40)	
Diabetes (n,%)	0	0	
Obesity (n,%)	2 (7)	1 (6)	
Smoking (n,%)	2 (7)	3 (20)	
Autoimmune and laboratory profile			
Anti-dsDNA positivity (n,%)	0	14 (93)	2 (13) *
aPL positive (n,%)	0	0	
ESR, mm/h (mean, SD)	–	71.6 ± 24.7	20 ± 14 *
CRP, mg/L (mean, SD)	1.6 ± 2.3	8.2 ± 8.6	0.5 ± 0.3 *
Proteinuria 24 h, g/24 h (mean, SD)	absent	3.9 ± 3.8	0.42 ± 0.51 *
Hypocomplementemia (n,%)	0	12 (80)	5 (41) *
Treatment			
Corticosteroids (n, %)	0	14 (93)	
HCQ (n, %)	0	11 (73)	
Immunosuppressors (n, %)	0	9 (60)	

*HDs - healthy donors; SLE - Systemic Lupus Erythematosus; Cardiovascular - CV; SLEDA-2K - Systemic Lupus Erythematosus Disease Activity Index; ESR - Erythrocytes Sedimentation Rate - CRP, C-Reactive Protein; anti-dsDNA - anti-double stranded DNA; aPL - antiphospholipid antibodies; HCQ - Hydroxychloroquine. * $p < .05$.*

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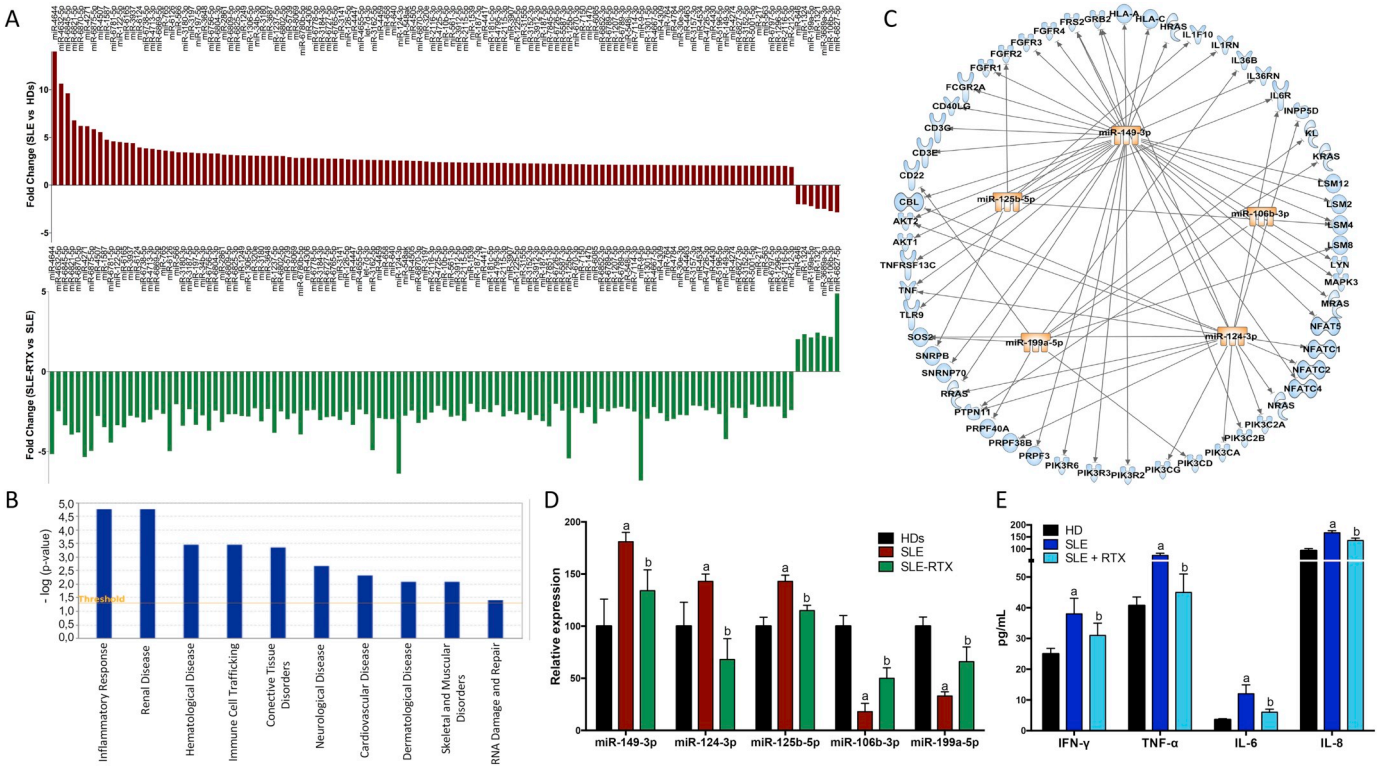

Fig. 1. Rituximab (RTX) effects on the circulating miRNA profile of SLE patients and their potential targets.
A. Circulating miRNA signature in SLE patients was obtained after the HTG EdgeSeq miRNA whole transcriptome assay. In red is shown the altered circulating miRNA signature of SLE patients as compared with HDs (cut off > 2 -fold change). In green is shown the circulating miRNA signature of SLE patients reverted by RTX therapy as compared with the baseline (cut off > 2 -fold change). B. Functional classification of the altered circulating miRNAs in SLE patients reverted after RTX therapy by using IPA software. C. Interaction network between selected miRNAs and their potential targets associated with the physiopathology of SLE by using IPA. D. RT-PCR validation studies in the entire cohort of the altered expression of selected miRNAs in SLE patients reverted by RTX. E. Altered pro-inflammatory cytokine profile of SLE patients, reverted after RTX therapy.
SLE - Systemic Lupus Erythematosus; HDs - Healthy donors; RTX - Rituximab; IPA - Ingenuity Pathway Analysis.

disease activity (mean SLEDAI-2 K after treatment = 1.5) along with the levels of acute phase reactants (C-reactive protein and erythrocyte sedimentation rate), and anti-double stranded DNA antibodies titers (Table 1).

Whole miRNome profiling of serum samples were performed in the exploratory cohort of 5 HDs and 5 SLE patients before and after RTX therapy. This analysis showed altered levels of 153 miRNAs in SLE patients at baseline when compared to HDs (cut-off: 2-fold change), of which 142 resulted to be upregulated and 11 downregulated. Among those, the expression levels of 121 out of 153 (79%) miRNAs were reverted in SLE patients after *in vivo* treatment with RTX (in detail, 114 out of 121 were downregulated and 7 out of 121 miRNAs were upregulated) (Fig. 1A).

By using the IPA software, the functional classification of these miRNAs revealed their association with clinical features of the SLE physiopathology. Thus, the altered miRNAs signature in SLE reverted by RTX was enriched for biological processes such as inflammatory response, renal and haematological disease, connective tissue disorders and neurological, cardiovascular and dermatological disease among others (Fig. 1B).

In order to validate the results in the whole cohort of SLE patients and HDs, we performed an *in-silico* analysis that allowed the identification of a set of miRNAs as potential modulators of the expression of key targets involved in the pathology of SLE. We identified a panel of 5 miRNAs including miR- 149-3p, 125b-5p, 199a-5p, 106b-3p 124-3p that showed potential targets molecules related to pivotal pro-inflammatory cytokines and critical immune receptors (TNFRSD13C, TNF, TLR9, CD3E, CD3G, CD40LG, FGFRs, HLA-A, HLA−C, IL1F10, IL1RN, IL36B, IL6R) along with a high number of molecules that control intracellular pathways associated with inflammatory and autoimmune processes (AKT1, AKT2, KRAS, LYN, MAPK3, NFAT5, NFATC1, 2 and 4, PIK3, PTPN11, RRAS) (Fig. 1C)..

The expression of the 5 selected miRNAs was further analysed by real time PCR in the entire cohort, thus showing that the relative expression of all the selected circulating miRNAs was significantly altered in serum from SLE patients at baseline when compared to HDs ($p < .05$) (Fig. 1 D). In addition, the altered expression of all selected miRNAs was reverted in serum samples from SLE patients after *in vivo* treatment with RTX ($p < .05$) thus validating the results found in the exploratory cohort (Fig. 1 D).

The effects of RTX in the reestablishment of the altered circulating miRNA signature of SLE patients, were also accompanied by a reduction of a set of key circulating pro-inflammatory cytokines upregulated in high disease activity patients. The increased levels of interferon-γ, tumor necrosis factor-α, interleukin-6 (IL-6) and IL-8 in serum of SLE patients were significantly reverted after 3 months of RTX therapy (Fig. 1E).

In the present study, the miRNA profiling has led to the identification of a set of circulating miRNAs altered in the serum samples of our cohort, allowing the distinction between HDs and SLE patients with high disease activity at baseline and after 3 months of *in vivo* therapy with RTX. Interestingly, the selected miRNAs seem to regulate mRNA targets related to several important pathologic features of SLE, including inflammation, renal damage, cardiovascular disease, and intracellular signalling pathways linked to these processes among others. These results were further demonstrated in the validation analysis performed on the serum samples of the entire cohort.

Most strikingly, we showed that after 3 months of therapy, RTX is

able to induce an early and significant global re-assessment of the expression of those miRNAs that were found to be upregulated in SLE patients before therapy, in parallel with the decrease of the disease activity, and a number of pro-inflammatory mediators. Despite the intrinsic limitation of the sample size, these results support the utility of miRNAs in the assessment of disease activity of SLE patients. Moreover, these evidences point-out the potential role of miRNAs as reliable biomarkers in monitoring the response to B-depletion treatment, allowing an early identification of those patients who could benefit more from this therapy, ultimately leading to a more personalized approach.

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