

Spring 5-5-2018

MicroRNA expression and disease correlation in a Hispanic Lupus cohort

Thandiwe Jere

University of Nebraska Medical Center

Follow this and additional works at: <https://digitalcommons.unmc.edu/etd>

 Part of the [Medical Immunology Commons](#)

Recommended Citation

Jere, Thandiwe, "MicroRNA expression and disease correlation in a Hispanic Lupus cohort" (2018). *Theses & Dissertations*. 284.
<https://digitalcommons.unmc.edu/etd/284>

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.

**MICRO-RNA EXPRESSION AND DISEASE CORRELATION IN
A HISPANIC LUPUS COHORT**

by

Thandiwe Jere

A THESIS

Presented to the Faculty of the University of Nebraska Graduate College

in partial fulfillment of the Requirements

for the Degree of Master of Science

Pathology and Microbiology Graduate Program

Under the Supervision of Dr Kaihong Su

University of Nebraska Medical Center

Omaha, Nebraska

May 2018

Advisory Committee:

Kaihong Su, PhD Geoffrey Thiele, PhD

Javeed Iqbal, PhD

ACKNOWLEDGEMENTS

This thesis became a reality with the kind support of many individuals and I would like to extend my gratitude to them.

Firstly, I would like to thank God, for the opportunity to pursue and successfully obtain this Masters' degree. For the wisdom, grace, and strength to get through the last two years and the many people He blessed me with along the way who have made this journey so rich.

I would like to thank my family, for their endless support and encouragement and many prayers that got me through long days and nights as the paper came to life. To many special friends that have walked this journey with me, I am very grateful.

Deep gratitude to my supervisor Dr Kaihong Su without whom this thesis would not have been successful. For her endless encouragement and support, from the thesis concept and throughout the process of designing the research and analyzing the results. I am very grateful to have found such a wonderful supervisor.

To my advisory committee members, Dr Geoffrey Thiele, and Dr Javeed Iqbal, for their constructive criticism and feedback that made this paper successful. I am also thankful for their approval and allowing me to complete this degree.

I am also indebted to the Pathology and Microbiology department at University of Nebraska Medical Center (UNMC) for affording me the opportunity to obtain this master's degree.

Special thanks to Dr E. Loyo from the Hospital Jose Maria Cabral y Baez in the Dominican Republic who kindly provided all the samples as well as clinical data that were used in this the.

MICRORNA EXPRESSION AND DISEASE CORRELATION IN A HISPANIC LUPUS COHORT

Thandiwe W. Jere, MBBS, M.S.

University of Nebraska Medical Center, 2018

Advisor: Kaihong Su, PhD.

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease with varying clinical presentation and severity in different ethnicities/races. Micro-RNAs (miRNAs) modulate the immune system, and there is a growing interest in their role in SLE pathogenesis and disease presentation. Studies on miRNAs expression in SLE have been done in Caucasians and Asians but no comprehensive profiling has been done in Hispanics. This cross-sectional study of a Hispanic cohort of SLE patients (n =185) and healthy controls (HC) (n =185) from the Dominican Republic determined the expression of 10 selected miRNAs and their correlation to inflammatory markers and auto-antibody production in SLE. The expression levels of miR-451a miR-16-5p, miR-126-5p, miR-146a-5p were significantly higher in SLE patients than in HC. miR-125a-3p and miR-155-5p were lower in SLE while miR-21-5p, miR-142-3p, miR-148a-3p, and miR-223-5p showed no difference between SLE and HC. MiR-16-5p correlated with anti-Sm and IFN- γ ; miR-155-5p correlated with anti-SSA/Ro, IL-1 β , and IL-12p70; and miR125a correlated with C4 and IL-1 β . We also show that miR451a and miR125a affect activation of neutrophil-like NB4 cells, pointing to their potential role in neutrophil mediated inflammation in SLE. This novel study in a Hispanic cohort shows differential expression of miRNAs in SLE and significant correlation with markers of inflammation, setting an important premise for further study on their mechanism of action.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
ABSTRACT.....	ii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES.....	vi
LIST OF TABLES.....	vii
LIST OF ABBREVIATIONS	viii
INTRODUCTION.....	1
MATERIALS AND METHODS	4
Study Participants	4
Measure of autoantibody titers and levels of complement C3 and C4.....	4
Total RNA extraction.....	4
Quantification of miRNA by qPCR.....	5
Inflammatory Cytokine/Chemokine assay	6
Cell culture	6
Plasmid preparation	7
Transfection of NB4 cells	7
Oxidative burst assay using Dihydrorhodamine (DHR)	7
RESULTS	9

Differential miRNA expression in SLE	9
Dysregulated miRNAs correlate with autoantibodies, complement and cytokines	9
Transfection with miR125a and miR451a affects oxidative response in NB4 cells	10
DISCUSSION	19
REFERENCES.....	24

LIST OF FIGURES

Figure 1: Selection and validation of internal controls for normalization.	11
Figure 2: Relative miRNA expression levels	12
Figure 3: Relative miRNAs fold change in SLE patients compared to HC	13
Figure 4: Oxidative burst measurement in NB4 cells transfected with miR125a.....	14
Figure 5: Oxidative burst measurement in NB4 cells transfected with miR451a.....	15

LIST OF TABLES

Table 1: Clinical characteristics of SLE patients and healthy controls	16
Table 2: Correlation between miRNAs and complement C3, C4, autoantibodies.....	17
Table 3: Correlation between dysregulated miRNAs and inflammatory cytokines	18

LIST OF ABBREVIATIONS

SLE	Systemic Lupus Erythematosus
miRNA	Micro-RNA
HC	Healthy controls
UNMC	University of Nebraska Medical Center
dsDNA	double stranded DNA
SSA/Ro	Sjogren's syndrome antigen A
SSB/La	Sjogren's syndrome antigen B
Sm	Smith
RNP	Ribonucleic Protein
SnRNA	Small nuclear Ribonucleic acid
RT-qPCR	Real time quantitative Polymerase Chain Reaction
DHR	Dihydrorhodamine

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease hallmarked by the production of autoantibodies against nuclear antigens, and excessive inflammatory responses that affect multiple organs (1). The disease is characterized by hyperactive T and B cells, leading to autoantibody production and formation of Immune complexes that deposit in end organs (1).

The prevalence of SLE is higher in females with a nine to one ratio compared to males. SLE is heterogeneous in nature and differences in disease development, clinical manifestation and disease activity have been seen in different individuals and ethnicities (2). Hispanics and Black/African Americans have higher prevalence, earlier onset of disease, and more frequent clinical complications than Caucasians and Asians (3, 4). It has also been seen that Hispanics have a higher prevalence and incidence of Lupus Nephritis than Caucasians. Multiple genetic and environmental factors are known to influence the development and nature of the disease and contribute to this variation and complexity (2).

Central to the organ damage that occurs in SLE is the tissue deposition of antibody-antigen immune complexes (ICs) that trigger plasmacytoid dendritic cell (pDCs), T cells, and macrophages to produce pro-inflammatory cytokines (5). ICs also activate the complement system and immune effector cells such as neutrophils, which are the key mediators of tissue damage in SLE, owing to their release of reactive oxygen species, tissue damage proteases and enhanced neutrophil extracellular traps (NETs) formation (6).

Although efforts have been made to understand the pathogenesis of SLE, there is still a lack of sufficient knowledge about the exact mechanisms underpinning the disease to develop effective therapies for SLE patients. Recent studies indicate that micro-RNAs (miRNAs) are involved in the

development of SLE, which gives new insight into the pathogenesis of SLE and might lead to the finding of new therapeutic targets (1, 7).

Micro-RNAs are small non-coding RNAs that regulate gene expression. They are known to play a critical role in the development of the immune system, as well as regulation of the innate and adaptive immune systems (8). Altered expressions of miRNAs are seen in autoimmune diseases such as SLE and rheumatoid arthritis, as well as in cancer (8). Considering this, miRNAs have become an area of interest owing to their contributory role to disease pathogenesis. In SLE, there is activation of both the innate and adaptive immune systems, and specific miRNAs are linked to some key processes involving both. Some of these processes include: interference in the Type 1 Interferon (IFN)-signaling pathway (miR-146a, miR-155) (9, 10); DNA hypo-methylation in T cells (miR-21, miR-126, miR-148a) (11, 12); aberration in inflammatory chemokine pathways (miR-125a) (13); neutrophil development and function (miR125a, miR223, miR451a) (14, 15); B-cell hyperstimulation and T-cell over-activation (miR-142-3p/5p) (16); induction of regulatory T cells (miR-16) (17); and regulation of myeloid cell development (miR-223) (18, 19).

With the arising knowledge about miRNAs involvement in SLE, studies have determined the differential expression in SLE. Most of the profiling studies have been done in Caucasian and Asian populations. However, there are no studies that have profiled miRNA expression patterns in Hispanic SLE patients. In addition, variations in these patterns have been seen in different races and this lack of consistency raises the question of whether there is race- and ethnic-specific miRNA expression signatures in SLE (7).

Therefore, we hypothesize that miRNAs are dysregulated in Hispanic SLE patients compared to healthy controls(HC) and the dysregulated miRNAs may contribute to the pathogenesis of SLE. The purpose of the study was to determine the expression of cell -free circulating miRNAs in serum in a cohort from the Dominic Republic. Although several miRNAs have been linked to SLE pathogenesis, we selected 10 miRNAs that have been dominantly implicated in inflammation and

autoimmunity and shown to be deregulated from comprehensive review articles and studies done in other populations. We further tested the correlation of miRNA profiles with serum markers of inflammation (complement C3 and C4), inflammatory cytokines, and SLE autoantibodies (anti-double stranded DNA (dsDNA), anti-Smith (Sm), anti-Ribonucleic Protein (RNP), anti-Ro/Sjogren's syndrome antigen A (SSA) and anti-La/Sjogren's syndrome antigen B (SSB). Further, we tested the functional effects of up or down-regulation of miR125a and miR451a in neutrophil-like NB4 cells.

MATERIALS AND METHODS

Study Participants

The study participants were recruited from the Hospital Jose Maria Cabral y Baez in the Dominican Republic. We selected patients diagnosed with SLE (n = 185) according to the 1997 Revised American College of Rheumatology (ACR) criteria. Age- and sex-matched HC (n = 185) were also recruited from the same community. The study was approved by the Institutional Review Board (IRB) affiliated with the hospital and the University of Nebraska Medical Center (UNMC). All participating subjects provided written informed consent according to the Declaration of Helsinki. Data on medical history, clinical manifestations, SLE Disease Activity Index (SLEDAI) score, and Systemic Lupus International Collaborating Clinics (SLICC) scores were collected from review notes. Blood was collected from SLE patients and HC. The samples were processed, and serum extracted before shipment (with dry ice at -78.5 °C) to the Su lab at UNMC where they were stored at -80 °C until analysis.

Measure of autoantibody titers and levels of complement C3 and C4

Auto-antibody (anti-dsDNA, anti-Smith, anti-U1RNP, anti-Ro/SSA and anti-La/SSB) titers in patients' serum samples were obtained using the respective FDA-cleared ELISA-based clinical tests from Euroimmun US (Mountain Lakes, NJ). The serum levels of complements (C3 and C4) were determined by ELISA kits obtained from Cell Sciences (Newburyport, MA).

Total RNA extraction

Total RNA was extracted from serum using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). A starting volume of 200 µl was maintained for all samples. Serum was lysed with QIAzol Lysis Reagent (Qiagen) at room temperature for 5 min. To control for miRNA individual

variation across samples, an exogenous spike-in control was used, namely *C. elegans* miRNA mimic. Chloroform was added to the mix, shaken vigorously, and incubated for 3 min at room temperature, followed by centrifuging for 15 min at 12,000 x g at 4 °C for phase separation. The upper aqueous phase was mixed with 80% ethanol and centrifuged at 8000 x g for 30 s at room temperature in a RNeasy MinElute spin column. This step was repeated using buffers supplied with the kit to wash the column. The final wash was performed with 80% ethanol, and centrifuged for 2 min at 8000 x g. The RNA was then eluted in RNase-free water provided with the kit. To test for RNA extraction efficiency, the eluted RNA sample was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in the DNA Microarray Core Facility (UNMC, NE, USA).

Quantification of miRNA by qPCR

Generation of cDNA was done using the Mir-X miRNA First-Strand Synthesis kit (Clontech, CA, USA) as per the manufacturer's instructions and reverse transcription done using an Applied Biosystems 2720 Thermal Cycler. The miRNA-specific 5' primers for qPCR were obtained from Invitrogen, CA, USA after sequence validation from the miRbase database.

The reaction mix for qPCR was made using the Mir-X miRNA q-RT PCR SYBR kit (Clontech, CA, USA) as per the manufacturer's instructions with the following volume adjustments: 5.1 µl ddH₂O, 7 µl SYBR Advantage Premix (2X), 0.3 µl ROX dye (50X), 0.3 µl miRNA-specific 5' primer, 0.3 µl MRQ 3' primer, and 2 µl sample cDNA. The qPCR was performed with a 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA) in a 384-well clear plate with cycling conditions as per the manufacturer's protocols. All reactions were done in duplicate.

To effectively determine the expression levels of target miRNAs by qPCR, it is important to standardize to internal controls that account for differential miRNA expression that occurs due to

individual sample variation. There is no known universal reference control; therefore, we analyzed the expression of 2 miRNAs, namely miR-24-2-5p, and miR-484. U6snRNA has been widely used as a reference control in miRNA profiling; therefore, we also included it in our study to select the best stably expressed that would serve as internal control. Data were processed using the RQ Manager 1.2 software (Applied Biosystems, CA, USA).

Inflammatory Cytokine/Chemokine assay

To determine and quantify the presence of inflammation, a 13-panel bead based immuno-assay was carried out using the LegendPlex Multi-analyte flow assay kit (BioLegend Inc., CA, USA) as per the manufacturer's instructions. The assay measured Interleukins 1 β , 6, 8,10,12p70, 17A, 18, 23, and 33; Interferons α and γ ; Tumor Necrosis Factor- α , and MCP-1. Briefly, SLE (n=100) and HC (n = 100) serum samples were diluted 2-fold with the provided assay buffer and incubated with fluorescence-encoded beads and biotinylated detection antibodies on a plate shaker for 2 hours at room temperature. Streptavidin-phycoerythrin (SA-PE) was then added followed by further shaking for 30 minutes. After centrifuging for 5 minutes, the supernatant was removed, and the beads washed two times with the provided wash buffer. Samples were read on a flow cytometer in FACS tubes and the concentrations determined from standard curves generated for each cytokine/chemokine.

Cell culture

NB4 cell line was cultured and maintained in DMEM High Glucose medium (GE Lifesciences, Utah) supplemented with 10% Fetal bovine serum (FBS), 1% HEPES, free acid (GE Lifesciences, USA), 1% Sodium Pyruvate (100mM) (Mediatech Inc. USA), 1% GlutaMax(100X) (ThermoFischer, USA) and 1% Penicillin-Streptomycin (ThermoFischer, USA).

Plasmid preparation

Vectors containing precursor miR451a mimic, miR451a scrambled control, miR125a inhibitor and miR125a inhibitor scrambled control (GeneCopoeia, MD, USA) were grown overnight on Agar plates at 37 °C. Colonies were picked, followed by incubation in Luria broth (LB) at 37 °C in a shaker overnight. Following this, plasmid extraction was done using the EndoFree plasmid maxi kit (Qiagen, Germany) as per the manufacturer's instructions.

Transfection of NB4 cells

Cells were plated overnight in 2.5 ml growth medium in a 6 well plate at a density of 5×10^5 cells/ml, followed by transfection using the TransIT-X2 Dynamic Delivery System (Mirus Bio LLC, USA) as per the manufacturer's instructions. The transfected cells were incubated for 48 hours at 37 °C and 5% CO₂. This was followed by selection using Puromycin 0.1ug/ml, and further incubation for 48 hours. Transfection efficiency was determined by flow cytometry. Differentiation to terminal neutrophil-like cells was induced by administration of All-Trans retinoic acid (ATRA) 1ug/ml.

Oxidative burst assay using Dihydrorhodamine (DHR)

The differentiated cells were harvested and resuspended in FACS buffer (FBS/PBS), followed by incubation with DHR dye (Sigma-Aldrich, USA) at room temperature for 10 mins. Stimulation was done by adding 5ug/ml ovalbumin: anti-ovalbumin immune complex and incubating in a water bath at 37°C for 30 mins. The tubes were placed on ice for 10 mins to stop all reactions, followed by centrifuging and washing using PBS. Cell pellets were resuspended in 200ul FACS buffer and measurement done on a Novocyte flow cytometer (Acea biosciences, Inc, CA, USA).

Statistical Analysis

All the analyses were done using either SPSS 24.0 (IBM Corp, NY, USA) or GraphPad Prism 7.03 (GraphPad Software, Inc. CA, USA). Values were normalized to the selected internal controls and miRNA relative levels were calculated using the delta-delta C_t method. Student's t-test was used to compare the results obtained for SLE and HC. Correlation analysis was done using Spearman's non-parametric correlation to compare miRNA relative levels with levels of serum autoantibodies (anti-dsDNA, anti-Smith, anti-U1RNP, anti-Ro/SSA and anti-La/SSB), complement C3, C4, and inflammatory cytokines. *P* values <0.05 were considered statistically significant.

RESULTS

The characteristics of the SLE study subjects and the age- and sex-matched HC are shown in Table 1. Clinical characteristics obtained from the SLE patients revealed a higher prevalence of arthritis, renal disease, and muco-cutaneous disease.

Differential miRNA expression in SLE

We tested the expression of 2 miRNAs and 1 snRNA to select the best internal reference control. These included mir-24-2-5p, miR-484, and U6 (Figure 1). There was a difference in the mean Ct values for U6 snRNA ($P = 0.001$) between SLE and HC. Meanwhile, there was no difference in the mean Ct values for miR-24-2-5p ($P = 0.09$) and miR-484 ($P = 0.51$) between SLE and HC samples. Accordingly, miR-24-2-5p and miR-484 were selected as the internal controls.

Relative expression levels of the 10 miRNAs of interest in patients with SLE and HC, normalized to miR-24-2-5p and miR-484 and expressed as delta CT are shown in Figure 2. The expression levels of miR-451a ($P < 0.0001$), miR-16-5p ($P < 0.0001$), miR-146a-5p ($P = 0.008$), and miR126-3p ($P = 0.004$) were higher in SLE patients than in HC. The expression levels of miR-125a-3p ($P < 0.0001$) and miR-155-5p ($P = 0.004$) were lower in SLE than in HC. However, the serum levels of miR-21-5p, miR-142-3p, miR-148a-3p, and miR-223-5p were not different between SLE patients and HC. The corresponding relative fold changes are shown in Figure 3.

Dysregulated miRNAs correlate with autoantibodies, complement and cytokines

Spearman's correlation analyses were done between autoantibodies and serum markers of inflammation and miR16-5p, miR-125a-3p, miR126-3p, miR-146a-5p, miR-155-5p and miR-451a (Table 2). There was observed correlation between miR-16-5p and C4 ($r = 0.242$, $P = 0.003$). In addition, miR16-5p inversely correlated with anti-Sm antibody ($r = -0.12$, $P = 0.023$). miR125a

inversely correlated with C4 ($r = -0.207$, $P = 0.005$). There was observed correlation between miR155-5p and anti-SSA ($r = 0.226$, $P = 0.003$), and inversely with anti-Sm ($r = -0.163$, $P = 0.04$). There was no correlation observed between C3, anti-dsDNA, anti-SSB, anti-RNP and all the miRNAs.

As a measure of inflammation, a panel of 13 cytokines was tested in the SLE serum samples and spearman's correlation analysis done with the differentially expressed miRNAs (Table 4). There was observed correlation between miR16-5p and IFN- γ , miR125a-5p and IL-1 β . miR155-5p correlated with IL-12p70 and inversely with IFN- γ .

Transfection with miR125a and miR451a affects oxidative response in NB4 cells

Neutrophils have been shown to play an important role in end organ damage in SLE (5,6) To determine if miRNAs dysregulated in SLE patients have functional effects in neutrophils, we used neutrophil-like NB4 cell line as a model system because of the short half-life of primary neutrophils. NB4 cells were stably transfected with miR125a inhibitor and miR451a mimic to imitate the expression pattern seen in SLE. Non-targeting scrambled sponge control vectors were used as negative controls.

Upon stimulation with ovalbumin: anti ovalbumin immune complexes, NB4 cells stably transfected with miR125a inhibitor showed increased oxidative burst, compared to the negative control, and untransfected cells (Fig 4). We also observed increased oxidative burst in cells stably transfected with miR45a mimic, compared to the untransfected and negative scrambled controls (Fig 5). Collectively, these results show that inhibition of miR125a and over-expression of miR451a enhanced the oxidative response in neutrophil-like NB4 cells stimulated with immune complexes.

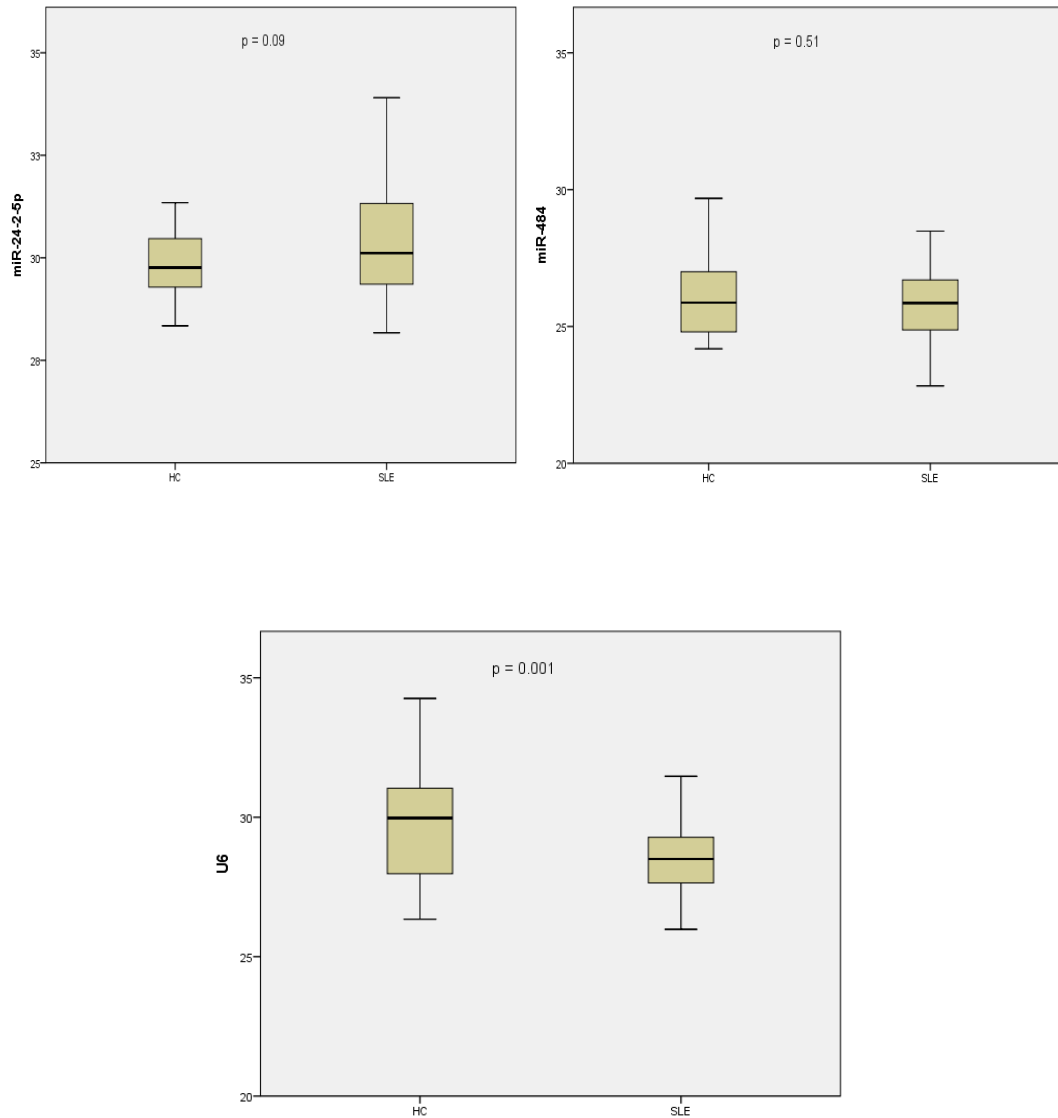


Figure 1: Selection and validation of miR-24-2-5p and miR-484 as internal controls for normalization.

Comparison of mean Ct values derived from qPCR of the 3 miRNA internal controls (U6 SnRNA, miR-24-2-5p, and miR-484) between SLE (n = 44) and HC (n = 48). The error bars represent the standard deviation from the mean. Student t-tests were used to determine mean differences between groups with level of significance $P < 0.05$. Reactions were run in duplicate.

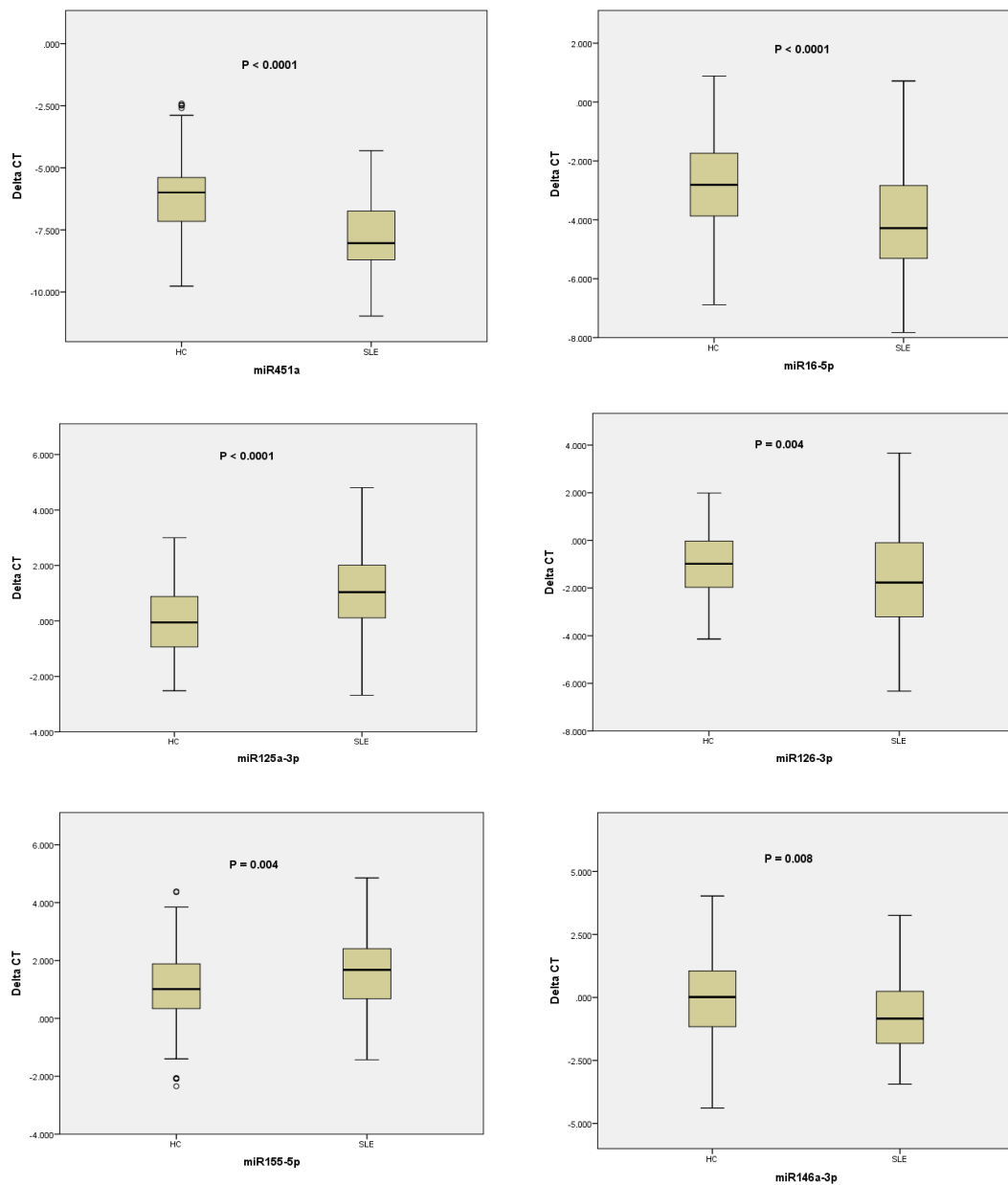


Figure 2: Relative expression levels of miR-16-5p, miR125a-3p, miR126-3p, miR146a-3p, miR155-5p and miR451a. The CT values from qPCR were normalized to miR24-2-5p and miR-484 to obtain the delta CT. The SLE group was compared to HC by Mann Whitney's two tailed test. The error bars represent the standard deviation of the mean. P < 0.05 was statistically significant.

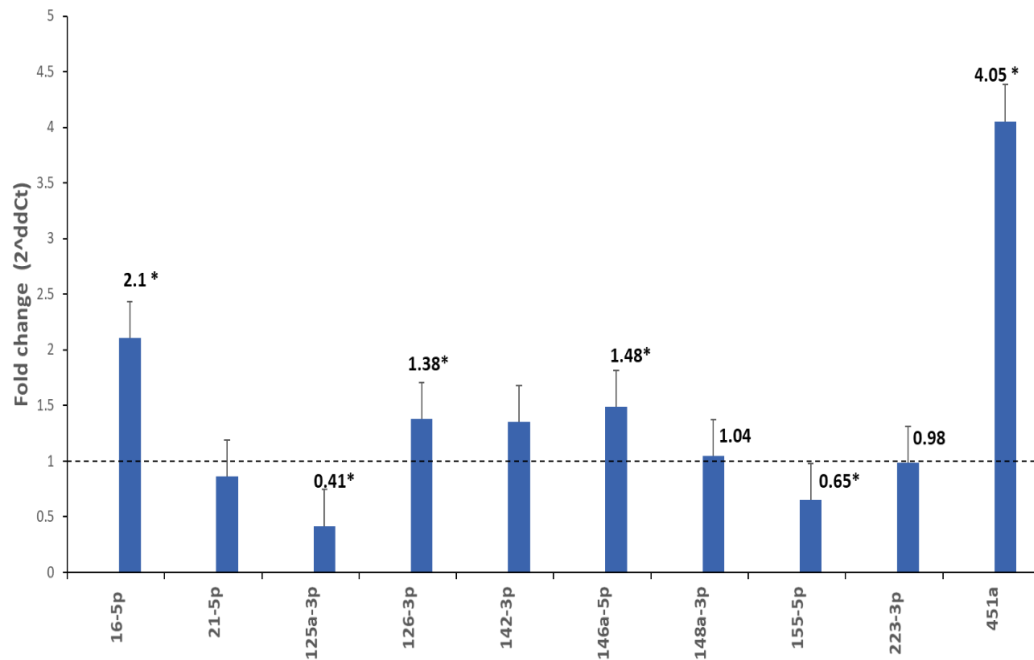


Figure 3: Relative miRNAs fold change in SLE patients compared to HC

Values for healthy control samples (n = 185) were normalized to 1 (shown by the dotted line). Fold changes of the 10 miRNAs in SLE (n = 185) were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to the endogenous controls miR-24-2-5p and miR-484. The error bars represent the standard deviation of the mean (SD). The numbers represent the relative fold changes. All qPCR assays were run in duplicate for each miRNA. Student t-tests were used to compare means with level of significance $P < 0.05^*$.

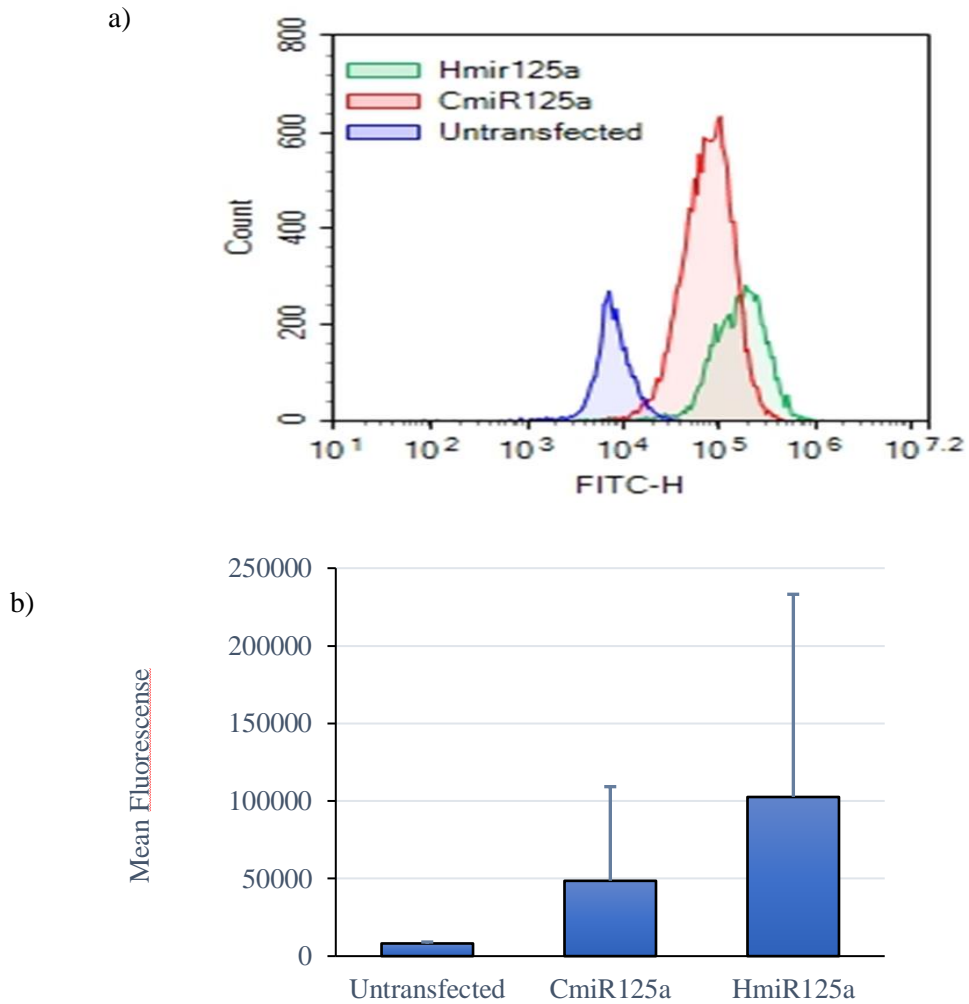


Figure 4: Oxidative burst measurement of NB4 cells transfected with miR125a by flow cytometry using DHR.

(a) Fluorescence histogram of the metabolic response in NB4 cells stably transfected with miR125a inhibitor, miR-125a inhibitor scrambled control and untransfected cells stimulated with 5ug/ml ovalbumin: anti-ovalbumin immune complex.

(b) Quantitative representation of the mean fluorescence in the three groups. Three independent transfection and activation assays were done, and the error bars represent the standard deviation of the mean. CmiR125a miR125a scrambled control, HmiR125a miR125a inhibitor.

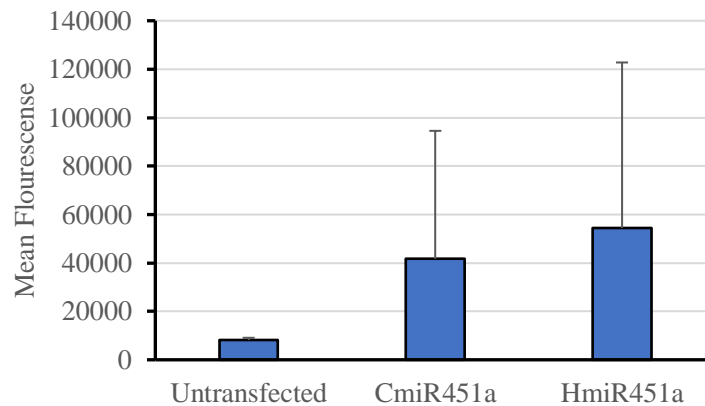
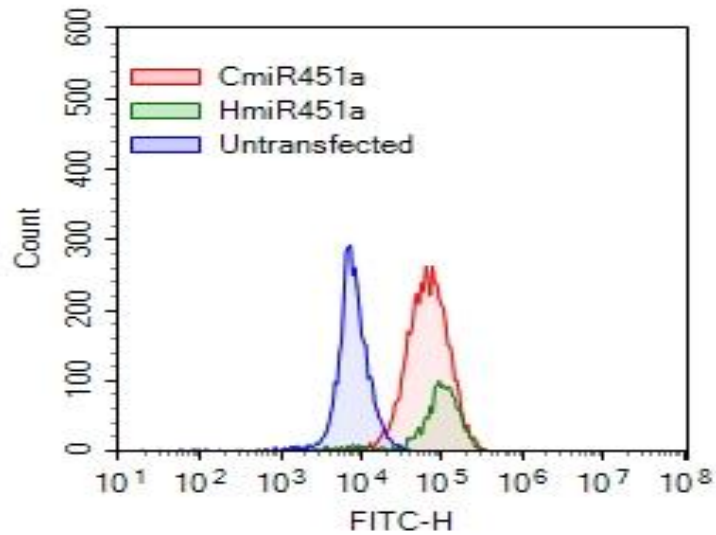


Figure 5: Oxidative burst measurement of NB4 cells transfected with miR451a by flow cytometry using DHR.

(a) Fluorescence histogram of the metabolic response in NB4 cells stably transfected with miR451a mimic, miR451a scrambled control and untransfected cells stimulated with 5ug/ml ovalbumin: anti-ovalbumin immune complex.

(b) Quantitative representation of the mean fluorescence in the three groups. Three independent transfection and activation assays were done, and the error bars represent the standard deviation of the mean. CmiR451a mi451a scrambled control HmiR451a miR451a mimic.

Table 1: Clinical characteristics of SLE patients and healthy controls

Characteristics	SLE	HC
Age mean \pm SD years	33 \pm 12.3	32 \pm 8.73
SLEDAI score, mean \pm SD	5 \pm 4.17	N/A
Sex, % (n)	Female 94 (174) Male 6 (11)	96 (144) ; Male 4 (6)
Disease Manifestation % (n)*		N/A
Hematological	36.4 (56)	
Muco-cutaneous	41.6 (65)	
Renal disease	55.2 (86)	
Neuro-psychiatric	16.2 (25)	
Cardiopulmonary	24 (37)	
Arthritis	66.9 (104)	
Autoantibodies, %		N/A
Anti-dsDNA	57	
Anti-Sm	34	
Anti-RNP	44.2	
Anti-SSA/Ro	48	
Anti-SSB/La	6.4	

dsDNA, double stranded DNA; Sm, Smith; RNP, ribonucleic protein; SSA, Sjogren's Syndrome-related antigen A; SSB, Sjogren's Syndrome Antigen B; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

*Disease manifestation is shown as a percentage of those that had the clinical features against the total N.

Table 2: Correlation between Complement C3, C4, autoantibodies, and the dysregulated miRNAs.

	C3 (mg/dl)	C4 (mg/dl)	Anti- dsDNA (IU/ml)	Anti- SSA (RU/ml)	Anti- Sm (RU/ml)	Anti- RNP (RU/ml)	Anti- SSB (RU/ml)
miR-16-5p	-0.03 0.60	0.242 0.003*	0.059 0.483	-0.012 0.134	-0.12 0.023*	-0.120 0.143	-0.034 0.675
miR-125a-3p	0.014 0.861	-0.207 0.005*	0.006 0.942	0.143 0.079	0.03 0.717	0.021 0.799	-0.048 0.557
miR-146a-5p	0.090 0.268	0.023 0.780	0.091 0.26	-0.063 0.442	-0.064 0.435	0.067 0.412	-0.125 0.123
miR-155-5p	0.002 0.977	0.15 0.855	-0.10 0.903	0.226 0.003*	-0.163 0.044*	-0.042 0.613	-0.05 0.536
miR451a	0.065 0.426	0.042 0.608	-0.09 0.269	-0.013 0.873	-0.09 0.271	-0.055 0.500	0.008 0.923

r Spearman's non- parametric correlation; $P < 0.05$ *

Table 3: Correlation between dysregulated miRNAs and inflammatory cytokines in SLE patients

	IFN-gamma	IL-1β	IL-12p70
miR-16-5p	r = 0.311 <i>P</i> = 0.03*	r = - 0.23 <i>P</i> = 0.10	r = 0.07 <i>P</i> = 0.34
miR-125a-3p	r = - 0.072 <i>P</i> = 0.497	r = -0.285 <i>P</i> = 0.006*	r = -0.54 <i>P</i> = 0.01
miR-155-5p	r = -0.255 <i>P</i> = 0.014*	r = -0.01 <i>P</i> = 0.47	r = 0.248 <i>P</i> = 0.017*

IL-1 β Interleukin-1Beta, IFN- γ Interferon Gamma, IL-12p70 Interleukin 12-p70. *P* < 0.05*

DISCUSSION

Quantitative PCR analysis of 10 selected serum miRNAs in this study cohort showed a difference in the expression of miR-16-5p, miR-125a-3p, miR126-3p, miR-146a-5p, miR-155-5p, and miR-451a in SLE patients compared to the age- and sex-matched healthy controls. Whilst miR-16-5p, miR-125a-3p, miR126-3p and miR-451a were overexpressed in the SLE group; there was reduced expression of miR-155-5p and miR-146a-5p. Further correlation analyses of these 6 miRNAs with autoantibodies and serum inflammatory markers (complement C3 and C4) revealed that C4 correlated with miR-16-5p and inversely with miR125a-5p, miR155-5p correlated with anti-SSA and anti-Sm, whilst miR16-5p inversely correlated with anti-Sm antibody. Despite differences in the expression of 5 miRNAs being observed between SLE patients and HC, there were no observed correlation with anti-dsDNA, anti-SSB/Ro, and anti-RNP. In addition, Overall, we found a differential expression pattern of miRNAs in a large Hispanic cohort that has not been studied before.

The varied nature of SLE has been attributed partly to the genetics involved, with different ethnicities portraying different forms of the disease. Disease progression and organ involvement have been reported to be different, with black and Hispanic populations having a higher incidence rate and more severe lupus nephritis compared with Caucasians (2, 20). Array studies on human T cells have shown changes in gene expression in different ethnicities that relate to disease activity and clinical presentation (21). Therefore, population specific studies are an important approach to characterize SLE in different cohorts because of the heterogeneous nature of SLE.

The Type I IFN pathway is a major contributing pathway implicated in the persistent inflammation that occurs in SLE. It has been shown that miR-146a negatively regulates the Type I IFN pathway by direct interference with two IFN-related transcription factors, IRF5 and STAT1 (9). Therefore, reduced expression of miR-146a has been implicated in SLE pathogenesis, and mice deficient in miR-146a show severe autoimmune phenotypes (22). We found the levels of miR-

146a-5p to be higher in SLE patients than HC in this study, and this was consistent with results from a study in Danish and Swedish cohorts (23). Studies by Tang *et al* (9) and Wang *et al* (22) in Chinese cohorts reported decreased levels of miR-146a l in peripheral blood mononuclear cells (PBMCs) as well as serum. Dai *et al* also reported decreased levels in splenic T cells and splenocytes in three murine lupus models (24). These overall divergent results point to the possibility of variations in the expression of miRNAs in SLE in different ethnicities. Of note is that these studies were done on different tissue types and this could explain in part the contrasting results from different studies as it has been shown that miRNA levels vary in different cell compartments, subtypes, and tissue types.

Some miRNAs have multiple effects on the type 1 IFN pathway and Toll-Like Receptor signaling, resulting in defective B and T cell interaction. These are central pathways in the pathogenesis of SLE (25). It has been reported that miR-155 promotes inflammation and type1 IFN signaling, as well as negatively regulates the expression of CD1d, a class-1 like MHC molecule, on the B cells of SLE patients (25, 26). CD1d deficiency was reported to aggravate disease symptoms and further impair the antigen presenting ability of B cells. Our study revealed reduced expression of miR-155 in the SLE group, and this corroborates results from Wang *et al* (22) and is in keeping with the proposed reduced serum expression of miR-155 in SLE.

The production of inflammatory cytokines and chemokines is central in SLE and miR-125a has been implicated in the activity of RANTES, an inflammatory chemokine. A study by Zhao *et al* reported that miR-125a negatively regulates RANTES; therefore, reduced expression of miR-125a contributes to the elevated expression of RANTES in SLE (13). Our study revealed decreased expression of miR-125a, consistent with these findings. In contrast, a study on a Caucasian cohort reported elevated levels of miR-125a (23) and could be due to differential miRNA expression patterns in different races or ethnic cohorts.

Although miR-16 and miR-451a have been implicated in the pathogenesis of SLE, the exact mechanisms have not been well studied. In addition, little data exist on the expression patterns in SLE, as well as their correlation to disease activity. A recent study by Cheng *et al* reported that the over expression of miR-451a was associated with increased levels of CD4+CD69+, CD4+/CD8+ T cells, and the levels of the serum cytokines IL-17a and IL-4 (27). Both promoted the inflammatory process in SLE. The inflammatory regulatory factor 8 (IRF8) was identified as a possible target of miR-451a. Our study results showed the over expression of miR-451a and miR-16 in the SLE group. This sets an important platform to further study their role in immune cell activity in SLE.

Measures of complement activation are part of the standard laboratory tests done on most SLE patients on a regular basis. These include complement C3 and C4, which may serve as an indication of active disease because they are acute phase proteins (28). The deficiency of early complement proteins, mainly C1 and C4, is strongly associated with development of SLE (29). We observed a correlation between miR16-5p, miR125a-3p and complement C4. There was no observed correlation between the differentially expressed miRNAs and complement C3.

A central characteristic of SLE is the production of autoantibodies and several are implicated in the diagnosis of disease. We observed an inverse correlation between miR16-5p, miR155-5p and anti-Sm antibody; miR-155-5p and anti-SSA antibody. Of interest is that anti-Sm antibody has been linked with lupus nephritis (30) and anti-SSA antibody has been characterized in a group of patients that present with cutaneous SLE and photosensitivity (31). Our population cohort displayed renal and cutaneous disease as the highest prevalence. This suggests a role of miR16-5p and miR155-5p in the production of these autoantibodies and their possible contribution to these clinical manifestations in SLE. Further characterization in these clinical phenotypes would provide better insight.

There were no observed correlations between anti-dsDNA, anti-SSB, and anti-RNP with the differentially expressed miRNAs. While autoantibodies serve as important diagnostic criteria for SLE and other autoimmune diseases, the presence of autoantibodies alone is not a distinct indicator of disease activity, and multiple factors should be considered.

Whilst B and T cells are the key players in autoimmunity and autoantibody production in SLE, tissue damage occurs due to chronic inflammation after immune complex deposition in end organs such as kidneys, lungs, or vasculature. Abnormalities in neutrophils (increased activation and apoptosis) (5, 32, 33) and the identification of an abnormal subset of low density granulocytes provides support that these cells are central in driving inflammation and resulting in tissue damage in SLE (34, 35). Using neutrophil-like NB4 cells, we have shown that immune complex-induced activation of neutrophils (oxidative burst) are enhanced by overexpression of SLE-upregulated miR451a or inhibition of SLE-downregulated miR125a. Our results support the hypothesis that dysregulated miRNAs in SLE may play a role in SLE pathogenesis and tissue damage.

There were a few limitations in this study. By design, the current study was restricted to 10 miRNAs chosen for analysis. Whilst a global profiling tool such as microarrays offers a more comprehensive initial screening method for miRNA expression patterns, use of this technology in our analysis was prohibitively expensive. Therefore, we selected the miRNAs of interest from literature and from previous studies that have been done to identify SLE- related miRNAs.

Whilst most studies have focused on PBMCs in their miRNA profiling analysis, this present study examined cell-free circulating miRNAs in serum. Although serum and plasma naturally have lower levels of miRNAs compared to PBMCs, they offer an easier tool for analysis and cast a broader net on the levels of miRNAs that are not limited to specific cell types.

Our study reports novel data in a large Hispanic cohort showing that serum miR-16-5p, miR126-5p, miR-451a and miR-146a-3p are over expressed in SLE patients, with reduced

expression of miR-155-5p and miR-125a-3p. In addition, there is an inverse correlation between miR16-5p and anti-Sm antibody, miR-146a-5p and anti-SSA antibody. Collectively, these differential expression patterns could contribute to the pathogenesis of SLE seen in this cohort and offers an important platform for future studies on the exact mechanisms of these miRNAs in SLE. We also show increased oxidative response in neutrophil-like NB4 cells stably transfected with miR125a and miR451a, pointing to the potential role of these miRNAs in neutrophil-mediated inflammation in SLE.

REFERENCES

1. Qu B, Shen N. miRNAs in the Pathogenesis of Systemic Lupus Erythematosus. *Int J Mol Sci*;16(5):9557-9572 (2015).
2. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD. Manifestations of systemic lupus erythematosus. *Maedica (Buchar)*;6(4):330-336 (2011).
3. Izmirly PM, Isabella W, Sara S, et al. The Incidence and Prevalence of Systemic Lupus Erythematosus in New York County (Manhattan), New York: The Manhattan Lupus Surveillance Program. *Arthritis & Rheumatology*;69(10):2006-2017 (2017).
4. Dall'Era Maria, Cisternas MG, Kurt S, Herrinton LJ, Caroline G, Helmick CG. The Incidence and Prevalence of Systemic Lupus Erythematosus in San Francisco County, California: The California Lupus Surveillance Project. *Arthritis & Rheumatology*;69(10):1996-2005 (2017).
5. Kaplan MJ. Neutrophils in the pathogenesis and manifestations of SLE. *Nature reviews. Rheumatology*;7(12):691-699 (2011).
6. Smith CK, Kaplan MJ. The role of neutrophils in the pathogenesis of systemic lupus erythematosus. *Curr Opin Rheumatol*;27(5)2015.
7. Chen JQ, Papp G, Poliska S, et al. MicroRNA expression profiles identify disease-specific alterations in systemic lupus erythematosus and primary Sjogren's syndrome. *PLOS ONE*;12(3) (2017).

8. Dai R, Ahmed SA. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Transl Res*;157(4):163-179 (2011).
9. Tang Y, Luo X, Cui H, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum*;60(4):1065-1075 (2009).
10. Zhou H, Huang X, Cui H, et al. miR-155 and its star-form partner miR-155* cooperatively regulate type I interferon production by human plasmacytoid dendritic cells. *Blood*;116(26):5885 (2010).
11. Sha Z, Yu W, Yunsheng L, et al. MicroRNA-126 regulates DNA methylation in CD4⁺ T cells and contributes to systemic lupus erythematosus by targeting DNA methyltransferase 1. *Arthritis & Rheumatism*;63(5):1376-1386 (2011).
12. Pan W, Zhu S, Yuan M, et al. MicroRNA-21 and MicroRNA-148a Contribute to DNA Hypomethylation in Lupus CD4⁺ T Cells by Directly and Indirectly Targeting DNA Methyltransferase 1. *J Immunol*;184(12):6773 (2010).
13. Zhao X, Tang Y, Qu B, et al. MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. *Arthritis & Rheumatism*;62(11):3425-3435 (2010).
14. Qin Y, Wu L, Ouyang Y, et al. MiR-125a Is a critical modulator for neutrophil development. *PLOS Genetics*;13(10) (2017).
15. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*; 451:1125 (2008).

16. Shu D, Yunsheng L, Ming Z, et al. Decreased microRNA-142-3p/5p expression causes CD4+ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. *Arthritis & Rheumatism*;64(9):2953-2963 (2012).
17. Singh Y, Garden OA, Lang F, Cobb BS. MicroRNA-15b/16 Enhances the Induction of Regulatory T Cells by Regulating the Expression of Rictor and mTOR. *J Immunol*;195(12):5667 (2015).
18. Gilicze AB, Wiener Z, Tãth S, et al. Myeloid-Derived microRNAs, miR-223, miR27a, and miR-652, Are Dominant Players in Myeloid Regulation. *BioMed Research International*; 2014:870267 (2014).
19. Kim B-, Jung J-, Jeon J-, Kim H-, Suh C-. Circulating hsa-miR-30e-5p, hsa-miR-92a-3p, and hsa-miR-223-3p may be novel biomarkers in systemic lupus erythematosus. *HLA*;88(4):187-193 (2016).
20. Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. Understanding the epidemiology and progression of systemic lupus erythematosus. *Semin Arthritis Rheum*;39(4):257-268 (2010).
21. Sharma S, Jin Z, Rosenzweig E, Rao S, Ko K, Niewold TB. Widely divergent transcriptional patterns between SLE patients of different ancestral backgrounds in sorted immune cell populations. *Journal of Autoimmunity*. 60(Supplement C):51-58 (2015).
22. Wang G, Tam LS, Li EK, et al. Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus. *J Rheumatol*;37(12):2516-2522 (2010).
23. Carlsen AL, Schetter AJ, Nielsen CT, et al. Circulating microRNA expression profiles associated with systemic lupus erythematosus. *Arthritis Rheum*;65(5):1324-1334 (2013).

24. Dai R, Zhang Y, Khan D, et al. Identification of a common lupus disease-associated microRNA expression pattern in three different murine models of lupus. *PLoS One*;5(12) (2010).
25. Tsokos GC, Lo MS, Costa Reis P, Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat Rev Rheumatol*;12(12):716-730 (2016).
26. Liu F, Fan H, Ren D, et al. TLR9-induced miR-155 and Ets-1 decrease expression of CD1d on B cells in SLE. *Eur J Immunol*;45(7):1934-1945 (2015).
27. Cheng J, Wu R, Long L, et al. miRNA-451a Targets IFN Regulatory Factor 8 for the Progression of Systemic Lupus Erythematosus. *Inflammation*;40(2):676-687 (2017).
28. Walport M, J. Complement and systemic lupus erythematosus. *Arthritis Res*; 4 (Suppl 3): 279-293 (2002).
29. Macedo ACL, Isaac L. Systemic Lupus Erythematosus and Deficiencies of Early Components of the Complement Classical Pathway. *Frontiers in Immunology*; 7:55 (2016).
30. Ahn SS, Yoo Byung-Woo, Song JJ, Park Yong-Beom, Lee Soo-Kon, Lee Sang-Won. Anti-Sm is associated with the early poor outcome of lupus nephritis. *Int J Rheum Dis*;19(9):897-902 (2016).
31. Menendez A, Gomez J, Caminal-Montero L, Diaz-Lopez JB, Cabezas-Rodriguez I, Mozo L. Common and Specific Associations of Anti-SSA/Ro60 and Anti-Ro52/TRIM21 Antibodies in Systemic Lupus Erythematosus. *The Scientific World Journal*; 2013:832789 (2013).
32. Keeling DM, Isenberg DA. Hematological manifestations of systemic lupus erythematosus. *Blood Reviews*. 7(4):199-207 (1993).

33. Yu Y, Su K. Neutrophil Extracellular Traps and Systemic Lupus Erythematosus. *Journal of clinical & cellular immunology*; 4:139 (2013).
34. Bennett L, Palucka AK, Arce E, et al. Interferon and Granulopoiesis Signatures in Systemic Lupus Erythematosus Blood. *J Exp Med*;197(6):711-723 (2002).
35. Carmona-Rivera C, Kaplan MJ. Low density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Seminars in immunopathology*;35(4):455-463 (2013).

