Original Article

Association between active pulmonary tuberculosis and miRNA-146a: A preliminary study from Serbia

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Abstract

Introduction: Tuberculosis (TB) continues to be a significant public health problem. The role of small non-coding RNAs, such as microRNAs (miRNAs), was investigated extensively in *Mycobacterium tuberculosis* (MTB) infection as well as in a variety of other pathophysiological processes in recent years. It was found that miRNAs act as regulators of both early reaction to MTB infection and in process of adaptation of the host immune cells during latent course of the disease. Molecule miRNA-146a is expressed exclusively in immune cells and it has the most prominent role in modulation of innate immunity.

Methodology: We investigated the level of expression of miRNA-146a using an RT-qPCR technique in peripheral blood mononuclear cells of 44 patients with active pulmonary TB and 17 healthy individuals. We also analyzed the significance of *miRNA-146a* rs2910164 SNV for expression profile of miRNA-146a, in order to investigate potential usage of miRNA-146a as a biomarker for TB.

Results: There was statistically significant decrease of expression of miRNA-146a in TB group compared to control group. When gender cohorts were analyzed, the expression levels in TB male and TB female subgroup were significantly lower than the expression levels in the same gender control subgroups.

Conclusions: Our results indicate that miRNA-146a plays a significant role in the pathogenesis of TB, suggesting that miRNA-146a could be used as a biomarker for active pulmonary TB.

Key words: miRNA-146a gene expression; biomarker; active pulmonary tuberculosis.

Introduction

The role of small non-coding RNAs, such as microRNAs (miRNAs), was investigated extensively in a variety of physiological and pathophysiological processes in recent years. Studies showed that the fate of cells during fundamental biological processes such as proliferation, differentiation, and apoptosis is tightly regulated by these specific molecules [1]. Growing data reveal their importance in immune response [2], embryogenesis [3], and tumorigenesis [4]. The regulatory role of various miRNAs in the immune system appears to be significant in both infectious and autoimmune diseases [5,6]. In this respect, they act as susceptibility factors as well as a regulatory component of innate and adaptive immunity [7] and they play a crucial role in bacterial or viral infectious diseases [8,9].

One-third of the human population is infected by *Mycobacterium tuberculosis*, but due to differences in host immunity, only about 10% of infected individuals actually develop active tuberculosis, while the remaining 90% exhibit latent infection [10]. Immunity in all of its complexity has been extensively investigated in respect to TB. It was found that miRNAs act as regulators of early reaction to MTB infection, but also in the process of host immune cells adaptation during the latent course of infection [7]. Several miRNAs are proven to regulate T cell differentiation and function, and play a critical role in regulating the innate function of macrophages, dendritic cells, and NK cells [7]. Macrophages are the first line of defense against MTB infection, using phagocytosis for the destruction of mycobacteria and initiating an

inflammatory response. On the other hand, MTB has adopted several mechanisms to survive within the host cells and attenuate the efficacy of the phagolysosome compartment. Experimental studies showed that MTB is able to control the expression of miRNAs and to use miRNAs for modulation of inflammation in host tissues, thus ensuring its own survival within the host cells [11].

The role of miRNA-146a in TB

miRNA-146a is one of the most investigated immunity-related miRNAs. It is exclusively expressed in immune cells, with the most prominent role in the modulation of the innate immunity [12]. It belongs to the group of early-response factors after exposition to various microbial components and proinflammatory mediators [9]. In TB, the expression of miRNA-146a is triggered by MTB-induced inflammation, followed by an escalation of proinflammatory mediators [11]. Early expression of miRNA-146a can be explained by activation of the proinflammatory TLR-TRAF6-IRAK1-NF-κB signaling pathway by MTB [13]. NF-κB binding site has been found in the promoter region of miRNA-146a gene, while the expression of *IRAK1* gene is directly negatively regulated by miRNA-146a [9]. The significant role of *IRAK1* gene in TB was previously shown [14].

So far, the role of miRNAs in TB was mostly investigated using *in vitro* model system. Recently, the assessment of circulating miRNAs has emerged as an option for their research. They are represented in sputum, blood, serum, plasma, and other body fluids, which enables their utilization as noninvasive diagnostic and prognostic biomarkers for a plethora of diseases - cancer, autoimmune and infective diseases, including TB [11,15].

To investigate the possibility of using miRNA-146a as a biomarker for TB, here we analyzed its expression level in patients who are in the acute phase of the disease. We also investigated the significance of the miRNA-146a rs2910164 SNV for its expression.

Methodology

Patients and control subjects

This study was performed on 44 TB patients (21 females, 23 males), with an average age of 58.4 ± 18.2 years. Patients who had diabetes, cancer, and other pulmonary diseases or co-infection with other pathogens were excluded. Study also included 17 healthy individuals (8 females and 9 males, 59.1 ± 8.5 years) free of active or latent TB infection and who did not display any clinical symptoms of any other

Table 1. Demographic characteristics of TB patients and control subjects.

	TB (N = 44)	Control (N = 17)	*p*
Gender			
Male, n (%)	23 (52.3)	9 (52.9)	0.963
Female, n (%)	21 (47.7)	8 (47.1)	
Age			
Years, mean ± SD	58.4 ± 18.2	59.1 ± 8.5	0.849

infectious or noninfectious diseases. They were recruited as controls. Demographic characteristics of TB patients and control group are shown in Table 1.

Informed consent was obtained from all participants and the protocol for the research project was approved by the Ethics Committee of the Clinical Centre of Serbia. We described 44 TB patients having the clinical form of pulmonary tuberculosis. The TB diagnoses were established between October and December 2018 in Clinic for pulmonology, Clinical Centre of Serbia and in the Special hospital for lung diseases Ozren. All patients were HIV-negative, and were receiving anti-tuberculous therapy at the time of blood sampling. All TB patients were drug responders to the first-line treatment (isoniazid, rifampin, ethambutol, pyrazinamide). The exclusion criteria for the healthy participants also included a history of TB and a pulmonary infection. The TB diagnosis was made if acid-fast bacilli had been positive in search and/ or culture in each case.

Blood sampling, DNA preparation, and genotyping

Venous blood was collected into two 4.5-mL sodium citrate anticoagulant tubes (Vacutainer, Becton-Dickinson, Plymouth, UK). Genomic DNA was isolated from whole peripheral blood with QIAampDNA Mini Kit (Qiagen GmbH, Hilden, Germany), and stored at -20 °C until analysis. Analysis of genetic variant in *miRNA-146a* (rs2910164 C > G) was performed by direct sequencing of PCR product using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on 3130 Genetic Analyzer (Applied Biosystems, USA). The PCR of the miRNA-146a C > G SNP was performed using the following primers to generate a 233-bp DNA product: forward: 5'-GACAGGCCTGGACTGCAAG-3' and reverse 5'-CTGGAGACAGAAGGCAGAGTC-3'.

RNA extraction

Fresh peripheral blood collected on 4.5-mL sodium citrate was used for isolation of peripheral blood mononuclear cells (PBMCs) using Ficoll density-gradient centrifugation (GE Healthcare, Sweden). TRI

Reagent Solution (Ambion, USA) was used for total RNA extraction. The quantity of RNA was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, USA).

Quantification of miRNA-146a expression

Twenty nanograms of total RNA were reverse transcribed using TaqMan miRNA-146a Gene Expression Assays (ThermoFisher Scientific, USA) and miRNA-specific stem-loop primers. Normalization was performed using RNU6B (TaqMan microRNA Control Assay RNU6B, Thermo Fisher Scientific, USA) and TaqMan MicroRNA Reverse Transcription Kit, and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, UK). ABI 7900HT Real-Time PCR System (Applied Biosystems, USA) was used for all real-time PCR analysis. Relative quantification analysis was performed by a comparative ddCT method, using the median expression level of the healthy control group as the calibrator (SDS 1.3.1.21, Applied Biosystems, USA). All experiments were performed in duplicates.

Statistical analysis

Genotype frequencies in patient and control groups were tested for the Hardy–Weinberg equilibrium (HWE) using the χ^2 test. The differences in the allele and genotype distributions between patients and controls were performed by Chi-square test by Fisher exact test. The recessive model was used for comparisons between genotypes, where the frequency of homozygous for minor allele was compared to grouped homozygous major allele plus heterozygous genotype. For the relative expression analyses data are presented as medians with range, means ± SD, or as absolute numbers with percentages. Distributions of continuous data were tested with the Shapiro-Wilk and Kolmogorov-Smirnov tests. Differences in these

Table 2. Genotype distributions and summarized results of association study of miR-146a rs2910164 C > G among TB patients and controls.

Genotype	TB (N = 44)	Control (N = 17)	p
CC, n (%)	1(2.3)	1 (5.9)	
GC, n (%)	14 (31.8)	6 (35.3)	0.4831
GG, n (%)	29 (65.9)	10 (58.8)	

GG + GC vs CC. Reference sequence used for the analysis of *miRNA-146a* rs2910164 variant was NR_029701.1

variables were analyzed using a nonparametric Mann-Whitney *U* test for distribution between two groups. Correlations between variables within the group of TB patients were analyzed using Spearman's rank-order correlation coefficient (r). The statistical analyses were performed using the SPSS computer software 20.0 (SPSS, Inc, Chicago, IL, USA). For all analyses, the *p* values were 2-tailed, and *p* < 0.05 was considered statistically significant.

Results

This study included 44 patients with TB and 17 controls. Baseline characteristics of patients are presented in Table 1. Patients and controls were matched by age and gender, and there was no statistical difference in genotypes between two groups.

Association between miRNA-146a rs2910164 C > G variant and susceptibility to TB

Comparison of variant in *miRNA-146a* gene in TB patients and healthy controls was performed through recessive model. Both patient's and control group were in HWE for *miRNA-146a* rs2910164 C > G ($\chi^2 = 0.937$, $p = 0.645$ and $\chi^2 = 0.006$, $p = 0.212$, respectively). There was no statistically significant difference between TB patients and control group regarding genotype and allele distribution (Table 2).

Figure 1. A) Analysis of relative expression in PBMCs samples of miR-146a in TB patients and healthy controls; **B)** Relative expression of miR-146a of male TB patients vs male controls; **C)** Relative expression of miR-146a of female TB patients vs female controls.
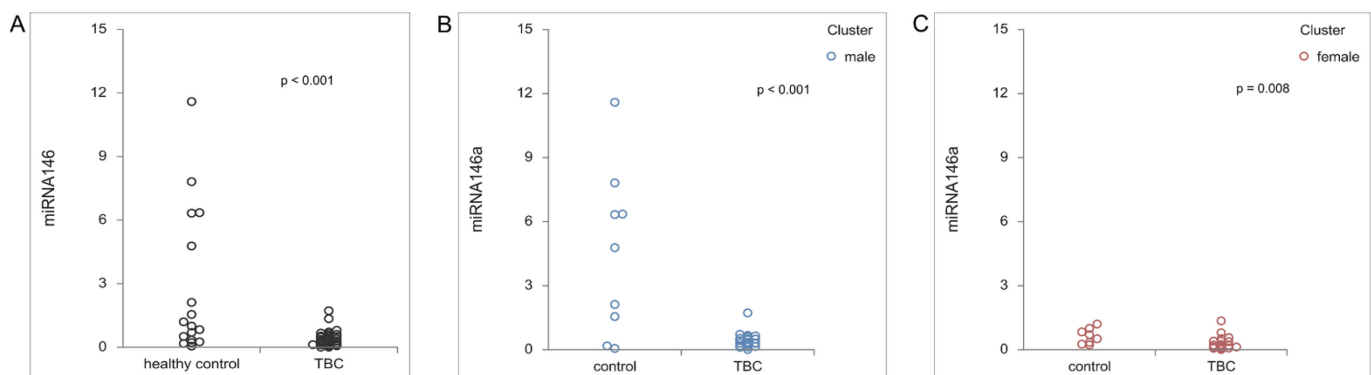
Expression analyses of miRNA-146a

The expression level of miRNA-146a in TB patients was significantly lower compared to control subjects (median 0.30, (0.01-1.72). vs. median 1.000 (0.06-11.59), *p* < 0.001) (Figure 1a).

Next, the correlation between miRNA146a and age was examined in the whole study cohort (patients and controls, n = 61). No significant relationship between these two variables was observed (r = 0.077, *p* = 0.575).

The correlation of miRNA-146a expression in relation to gender was examined in the entire sample (patients and controls, n = 61). Expression level of miRNA-146a was higher in male (median 0.46 (0.01-11.59)) than female (median 0.24 (0.01-1.3)), but statistical significance was not observed (*p* = 0.061). However, significantly decreased expression was found in TB male group (median value 0.31(0.01-1.72)) compared to males in control group (median value 4.78 (0.06-11.59), *p* < 0.001) (Figure 1b). When group of females were analyzed, miRNA-146a levels were significantly lower in TB female group (median 0.19 (0.01-1.35) (n = 8) compared to control female group (median 0.61 (0.22-1.20)), (*p* = 0.008) (Figure 1c).

Association of genotype variants with miRNA-146a expression level

To investigate the influence of the C and G allele of rs2910164 on the production of the mature form of miRNA-146a, carriers of C allele were compared to carriers of G allele in both TB and healthy control groups. No significant difference was observed between groups.

Discussion

Specific miRNAs have been shown to be associated with pulmonary TB and the first study that identified SNPs in precursor miRNAs was conducted in 2005. New and more effective biomarkers are needed to establish early diagnosis of TB and thus enhance its treatment and prevention.

It has been observed that miRNA-146a plays an important role in MTB infection and in the pathogenesis of pulmonary TB. Several experimental studies have provided evidence that C allele in rs2910164 of *miRNA-146a* may reduce the production of mature miRNA-146a, which results in modification of the inflammatory process [16]. Although the role of *miRNA-146a* rs2910164 C > G SNV in the pathogenesis of pulmonary TB is unclear, this SNV may have an impact on the immune response in MTB infection, thereby increasing the incidence of pulmonary TB. miRNA-146a has previously been described as a negative

regulator of the immune response and its systemic down-regulation may be associated with an escalated inflammatory response in TB patients [17,18]. SNV rs2910164 C > G , is encoded on chromosome 5q33 and located in pre-miRNA-146a in the precursor stem region, + 60 relatives to the first nucleotide pre-miRNA-146a, as opposed to the mature miRNA-146a sequence [17,19]. This transversion results in C: U mismatch in the structure of the miRNA-146a precursor, which may reduce the stability and processing efficiency of pre-miRNA, and decrease the expression of pre-miRNA-146a and mature miRNA-146a [20].

Previous studies have shown that *miRNA-146a* rs2910164 was associated with altered risk of colorectal cancer [21], breast cancer [22], or ovarian cancer [23].

Three studies described an association between rs2910164 C > G in *miRNA-146a* and TB. One study was conducted in the Kazak population [16], another in the Tibetan/Han population [17], and a third in the Chinese Han population [18].

Zhang *et al.* investigated the potential link between the four precursors of miRNA SNPs (*miRNA-146a* rs2910164 C > G, *miRNA-149* rs2292832 T > C, *miRNA-196a2* rs11614913 T > C and *miRNA-499* rs3746444 T > C) and susceptibility to pulmonary tuberculosis in Chinese Uygur, Kazak, and South Han. The frequencies of alleles and genotypes of *miRNA-146a* differed significantly between these two groups in the Kazak population. *miRNA-146a* SNP has been found to be associated with pulmonary tuberculosis using codominant, recessive, and additive models [16].

Li *et al* investigated the association of *miRNA-146a* rs2910164 G > C SNV with susceptibility to pulmonary tuberculosis in Chinese Tibetan and Han populations. An association with the risk of pulmonary TB in both the Tibetan and Han populations was found [17]. They speculated that this was the main reason for differences in different ethnic groups.

Wang Min *et al.* did not repeat previous significant discoveries in the Chinese Han population, nor did they reveal an association between miRNA-146a and pulmonary tuberculosis.

The results of these three studies suggest that susceptibility to pulmonary TB may be closely related to individual differences caused by genetic factors among different ethnic groups in China. The gene-gene interaction is often combined with genetic and environmental factors and makes the perceived difference more complicated between different ethnic groups.

Yuksen Fatma *et al.* also determined the diagnostic potential of miRNA-146a in patients with active TB. Their results suggest that miRNA-146a may distinguish active pulmonary TB from healthy controls based on data provided from normalization with miRNA-93 in Turkish population [24].

In our study, we found that there was a statistically significant difference in miRNA-146a expression level between TB and control groups, with values higher in healthy controls. MiRNA-146a levels compared between CC/GC and GG genotypes showed no significant influence on the production of the mature form of miRNA-146a.

The expression level of miRNA-146a in our study was higher in males than females, but without statistical significance. Comparison between males in TB and males in control group, as well as the same comparison between females, revealed significantly lower expression in TB subgroups, which indicates a common mechanism in both sexes [25,26].

The main limitation of this work is the small sample size. Since this is the first study in Serbia that investigated active pulmonary tuberculosis in correlation to miRNA-146a, the results presented here are a valuable and open new paradigm for using small noncoding RNAs as potential biomarkers and diagnostics, prognostics, and stratification tools in the future.

Conclusions

We believe that miRNA research will provide a new method of testing as well as understanding the pathogenesis and treatment of pulmonary TB. Despite the above limitations, this is the first preliminary study in Serbia to examine changes in miRNA-146a levels in patients with tuberculosis. We found that miRNA-146a may be a suitable candidate for further research as a potential biomarker for TB.

Authors' Contributions
IB, VŠT and MS performed clinical management of patient and conducted the case and control sample collection. MS and GA participated in clinical management of the patient and participated in design of the study. JML performed statistical analysis. MA, MV and VS carried out the molecular genetic studies and drafted the manuscript. VS and SP designed the study, coordinated the research and drafted the manuscript. All authors read and approved the final manuscript. VS take the primary responsibility for the paper.

References
1. Hwang H-W, Mendell JT (2006) MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 94: 776–780.
2. Tsitsiou E, Lindsay MA (2009) MicroRNAs and the immune response. Curr Opin Pharmacol 9: 514–520.
3. Pauli A, Rinn JL, Schier AF (2011) Non-coding RNAs as regulators of embryogenesis. Nat Rev Genet 12: 136–149.
4. Shenouda SK, Alahari SK (2009) MicroRNA function in cancer: Oncogene or a tumor suppressor? Cancer Metastasis Rev 28: 369–378.
5. Khan AA, Penny LA, Yuzefpolskiy Y, Sarkar S, Kalia V (2013) MicroRNA-17~92 regulates effector and memory CD8 T-cell fates by modulating proliferation in response to infections. Blood 121: 4473–4483.
6. Escobar T, Yu C-R, Muljo SA, Egwuagu CE (2013) STAT3 activates miR-155 in Th17 cells and acts in concert to promote experimental autoimmune uveitis. Invest Ophthalmol Vis Sci 54: 4017–4025.
7. Harapan H, Fitra F, Ichsan I, Mulyadi M, Miotto P, Hasan NA, Calado M, Cirillo DM (2013) The roles of microRNAs on tuberculosis infection: meaning or myth? Tuberculosis (Edinb) 93: 596–605.
8. Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X (2009) MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol 183: 2150–2158.
9. Taganov KD, Boldin MP, Chang K-J, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 103: 12481–12486.
10. Getahun H, Matteelli A, Chaisson RE, Raviglione M (2015) Latent *Mycobacterium tuberculosis* infection. N Engl J Med 372: 2127–2135.
11. Sinigaglia A, Peta E, Riccetti S, Venkateswaran S, Manganelli R, Barzon L (2020) Tuberculosis-associated MicroRNAs: From pathogenesis to disease biomarkers. Cells 9.
12. Li M, Wang J, Fang Y, Gong S, Li M, Wu M, Lai X, Zeng G, Wang Y, Yang K, Huang X (2016) microRNA-146a promotes mycobacterial survival in macrophages through suppressing nitric oxide production. Sci Rep 6: 23351.
13. Li S, Yue Y, Xu W, Xiong S (2013) MicroRNA-146a represses mycobacteria-induced inflammatory response and facilitates bacterial replication via targeting IRAK-1 and TRAF-6. PLoS One 8: e81438.
14. Buha I, Škodrić-Trifunović V, Adžić-Vukičević T, Ilić A, Blanka-Protić A, Stjepanovic M, Anđelković M, Vreća M, Milin-Lazović J, Spasovski V, Pavlović S (2019) Relevance of TNF-α, IL-6 and IRAK1 gene expression for assessing disease severity and therapy effects in tuberculosis patients. J Infect Dev Ctries 13: 419–425. doi: 10.3855/jidc.10949.
15. Condrat CE, Thompson DC, Barbu MG, Bugnar OL, Boboc A, Cretoiu D, Suciu N, Cretoiu SM, Voinea SC (2020) miRNAs as biomarkers in disease: Latest findings regarding their role in diagnosis and prognosis. Cells 9.
16. Zhang X, Li Y, Li X, Zhang W, Pan Z, Wu F, Wang C, Chen Z, Jiang T, Xu D, Ping Z, Liu J, Liu C, Li Z, Li J-C (2015) Association of the miR-146a, miR-149, miR-196a2 and miR-499 polymorphisms with susceptibility to pulmonary

tuberculosis in the Chinese Uygur, Kazak and Southern Han populations. BMC Infect Dis 15: 41.

17. Li D, Wang T, Song X, Qucuo M, Yang B, Zhang J, Wang J, Ying B, Tao C, Wang L (2011) Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population. Hum Immunol 72: 598–602.

18. Wang M, Xu G, Lü L, Xu K, Chen Y, Pan H, Burstrom B, Burstrom K, Wang J (2016) Genetic polymorphisms of IL-17A, IL-17F, TLR4 and miR-146a in association with the risk of pulmonary tuberculosis. Sci Rep 6: 28586.

19. Spinelli S V, Diaz A, D'Attilio L, Marchesini MM, Bogue C, Bay ML, Bottasso OA (2013) Altered microRNA expression levels in mononuclear cells of patients with pulmonary and pleural tuberculosis and their relation with components of the immune response. Mol Immunol 53: 265–269.

20. Zhang X, Gu Y, Liu X, Yu Y, Shi J, Yu Q, Sun H, Kanu JS, Zhan S, Liu Y (2015) Association of Pre-miR-146a rs2910164 polymorphism with papillary thyroid cancer. Int J Endocrinol 2015: 802562.

21. Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A (2008) Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. Proc Natl Acad Sci U S A 105: 7269–7274.

22. Mashayekhi S, Saeidi Saedi H, Salehi Z, Soltanipour S, Mirzajani E (2018) Effects of miR-27a, miR-196a2 and miR-146a polymorphisms on the risk of breast cancer. Br J Biomed Sci 75: 76–81.

23. Chae YS, Kim JG, Lee SJ, Kang BW, Lee YJ, Park JY, Jeon H-S, Park JS, Choi GS (2013) A miR-146a polymorphism (rs2910164) predicts risk of and survival from colorectal cancer. Anticancer Res 33: 3233–3239

24. Kalaycı Yüksek F, Kığ C, Ortaköylü G, Uzun M (2021) Association between active pulmonary tuberculosis and circulating microRNAs: a preliminary study from Turkey. Turkish J Med Sci 51: 1894–1904.

25. Kangas R, Pöllänen E, Rippo MR, Lanzarini C, Prattichizzo F, Niskala P, Jylhävä J, Sipilä S, Kaprio J, Procopio AD, Capri M, Franceschi C, Olivieri F, Kovanen V (2014) Circulating miR-21, miR-146a and Fas ligand respond to postmenopausal estrogen-based hormone replacement therapy--a study with monozygotic twin pairs. Mech Ageing Dev 143–144: 1–8.

26. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA (2008) Suppression of LPS-induced Interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. Blood 112: 4591–4597.

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