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MicroRNA-126 and 146a as potential biomarkers in systemic lupus erythematosus patients with secondary antiphospholipid syndrome

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ABSTRACT

Background: MicroRNAs (miRs) are noncoding gene regulators that may have a role as diagnostic or prognostic biomarkers in systemic lupus erythematosus (SLE) and its complications. SLE is an autoimmune disease that may be associated with secondary antiphospholipid syndrome (APS).
Aim of the work: To evaluate the plasma levels of both miR-146a and miR-126 as well as serum alpha interferon (α IFN) in Egyptian SLE patients with and without secondary APS and to investigate their potential role in disease pathogenesis and their utility as biomarkers for APS.
Patients and methods: 88 SLE patients including 30 cases with secondary APS and 40 matched healthy individuals were enrolled in this study. SLE disease activity index (SLEDAI) was assessed. The plasma levels of miR-146a and miR-126 were determined by Realtime polymerase chain reaction (PCR) in all participants.
Results: The mean age of the patients was 31.3 ± 9.6 years with disease duration 1–17 years. Plasma miR-146a was significantly lower and miR-126 significantly higher in SLE compared to controls. MiR126 was also higher in secondary APS patients compared to patients without. Serum IFN-α ws significantly higher in patients (71.2 ± 19.7 pg/ml) compared to control (43.2 ± 9.7 pg/ml) ($p < 0.001$). MiR-126 at a cut off of 2.66 can discriminate between SLE patients with and without secondary APS with a sensitivity of 76.67% and specificity of 81.01% ((95% CI 0.685–0.902, $P < 0.001$).
Conclusion: Circulating miR-126 could be a potential noninvasive biomarker in SLE associated with secondary APS. Further studies are needed in view of the limited data on the expressions of microRNA in APS.

1. Introduction

The micro-ribonucleic acids (MiRNAs or MiR) are small endogenous non coding molecules which act as post-transcriptional gene regulators and play essential roles in immune cell differentiation and function [1] Circulating miRNAs are encapsulated within exosomes and thus are protected from endogenous RNAses, rendering them stable and suitable for non-invasive analysis in patients [2]. Patients with systemic lupus erythematosus (SLE) have distinctive miRNA profiles and their dysfunction has been related to the disease development and activity [3]. Thisprovides new insights into the pathogenesis of SLE and a unique opportunity to adopt novel diagnostic, monitoring or therapeutic targets [4].

Antiphospholipid syndrome (APS) is an autoimmune disorder that occurs clinically in the form of recurrent venous or arterial thrombosis and/or pregnancy morbidity and fetal loss and associated with antiphospholipid antibodies (aPLs), [5] Although challenging, the differentiation between SLE patients complicated by secondary APS and those not is important, since the diagnosis of APS involves serious sequelae as long term anti-coagulation [6]. The pathogenesis of APS is unfortunately still not well established. It is unclear whether aPLs are involved in pathogenesis or represent an epiphenomenon, since about 5 percent of healthy people have aPL. [7]. Recently, miRs expressed in many types of diseased tissues were claimed to be involved in the pathological progression of APS and hence may become a useful biomarker in diagnosis and follow up [8].

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Both MiR-126 and miR-146a are important regulators that aim at a group of genes involved in immune response. MiR-126 gene is located in humans on chromosome 9 within intron 7 of the epidermal growth factor (EGF)-like-domain, multiple 7 (EGFL7) gene [9]. MiR-126 has been involved in the pathogenesis of many diseases including cardiovascular system [10], autoimmune diseases [11] and cancer [12]. Meanwhile miR-146a gene is located on the long arm of chromosome 5 [13]. It modulates innate immunity through regulation of Toll-like receptor (TLR) signaling and cytokine response. Dysregulation of miR-146a has been demonstrated in several chronic inflammatory diseases, such as psoriasis [14], rheumatoid arthritis (RA) [15], osteoarthritis [16] and SLE [17].

The aim of the present study was to evaluate the plasma levels of miR-146a and miR-126 and serum interferon in Egyptian SLE cases with and without secondary APS and to investigate the potential role of these miRNAs in disease pathogenesis and their utility as potential disease biomarkers for APS.

2. Patients and methods

This cross sectional observational study was conducted on 88 adult Egyptian SLE patients recruited from the Rheumatology and Obstetrics and Gynecology Departments of Kasr Al-Ainy Medical School, Cairo University Hospitals who fulfilled the SLE International Collaborating Clinics (SLICC) classification criteria [18]. 30 patients met the clinical and laboratory International criteria for the classification of APS: persistently elevated aPLs namely lupus anticoagulant (LAC), anticardiolipin (ACL) and β2-glycoprotein 1 antibodies associated with thrombosis and/or pregnancy-related morbidity [19]. Forty age and sex matched healthy subjects were included as controls. The study was approved by the Clinical Pathology Department ethical committee, Faculty of Medicine, Cairo University. Consent was obtained from all the patients included the study.

Clinical and demographic data such as age, gender, medical and obstetric history as well as laboratory data including assay of serum autoantibodies consisting of antinuclear antibodies (ANA), anti-double stranded deoxyribonucleic acid (anti-dsDNA), LAC, ACL and anti-β2 glycoprotein), serum complement C3 and C4 levels, erythrocyte sedimentation rate (ESR), serum creatinine levels and the amount of proteinuria in 24 h were collected at the time of sampling. Patients were classified into active (score > 5) and inactive (score ≤4) according to the SLE Disease Activity Index (SLEDAI) score [20].

Plasma levels of miR-126 and miR-146a and of α IFN were measured in all subjects.

2.1. Measurement of miR-146a and miR-126

Anticoagulated (EDTA) peripheral blood (5 cc) specimens were obtained by phlebotomy and centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was transferred to Eppendorf tubes. These samples were re-centrifuged at 15,000 rpm for 10 min to precipitate cell debris and the supernatants were stored at −80 °C until RNA extraction.RNA for miRNA expression profiling was isolated and purified from the plasma using Qiagen®miRNeasy Serum/Plasma Kit (Catalog no. 217184) according to manufacturer instructions.

RNA was reverse transcribed using the TaqMan® MicroRNA RT Kit in combination with the stem loop RT primer, allowing simultaneous reverse transcription of target miRNAs and control. Real time PCR was performed using TaqMan microRNA assays specific to the corresponding mature sequence miR-146a and miR-126. The quantitative polymerase chain reaction (qPCR) reactions were run in StepOne real time PCR system (Applied Biosystems). The level of miRNA expression was measured using the C_T. The expression for each miRNA was calculated as the difference between its C_T value and the average C_T value of the reference gene. RNU6B was used as the reference gene. Relative expression (fold change) was calculated for each target miRNA within each group using the equation $2^{-\Delta\Delta CT}$ [21].

Measurement of serum levels of α IFN was performed using commercial Elisa kits according to manufacturer's instructions (EIAab®Science Co.Ltd., China, Catalog #P0033Rb-h).

2.2. Statistical analysis

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test. Pearson coefficient was used to test correlation between numerical variables. The Receiver Operating Characteristic curve was used for prediction of cut off values of miR0146a and miR126 as potential diagnostic markers for SLE. All tests were two-tailed. A p-value < 0.05 was considered significant.

3. Results

The 88 SLE patients were 78 females and 10 males (F:M 7.8:1) with a mean age of 31.3 ± 9.6 years and disease duration of 1–17 years. 30 patients had secondary APS; 10 SLE patients suffered from recurrent abortions >3 times. The 40 control subjects were of matched age (32.6 ± 7.4 years; p > 0.05) and sex (34 females and 6 males; F:M 5.7:1; p > 0.05). 64 patients were receiving corticosteroids alone or in combination with hydroxychloroquine and/or immunosuppressives while 24 were newly diagnosed and not receiving any treatment at the time of sampling. The patients were active in 47 and inactive in 41. 30 patients had secondary APS; 13 were active and 17 inactive.

The demographic and laboratory data of the patients are presented in Table 1. The clinical characteristics of patients were as follows: mucocutaneous manifestations in 42 (48%), arthritis in 53 (60%), serositis in 14 (16%), neurologic in 35 (31%) and renal affection in 28 (32%). There was no significant difference in clinical features between SLE patients with and without secondary APS (p > 0.05). There was a significant difference between active and inactive SLE patients with secondary APS regarding C3, C4, Hb, platelet count and 24 h urinary protein.

In SLE patients, the MiR-146a expression level was significantly lower compared to the control (p < 0.001) and tended to be lower in the active *vs* inactive patients (p = 0.47). The expression of miR-126 was significantly higher in patients in comparison to the control (p = 0.007). The expression was insignificantly higher in the active versus the inactive patients (Table 2). miR-146a expression was similar between treated and untreated patients. Serum IFN-α levels were significantly higher in patients (71.2 ± 19.7 pg/ml) compared to control (43.2 ± 9.7 pg/ml) (p < 0.001).

In patients with secondary APS, a significant up-regulation of plasma miR126 level was noticed compared to SLE patients without (p = 0.004) and to controls (p < 0.001). MiR-126 expression tended to be higher in the SLE patients without APS compared to the control. MiR-146a expression was comparable among SLE patients with and without APS (Table 3).

The diagnostic value of miR146a in SLE patients and of miR-126 in SLE patients with APS are presented in Table 4 and graphically

Table 1
Demographic and laboratory data of systemic lupus erythematosus patients with and without antiphospholipid syndrome.

Parameter mean ± SD	SLE patients (n = 88)			Secondary APS (n = 30)		
	with APS (n = 30)	Without (n = 58)	*p*	Active (n = 13)	Inactive (n = 17)	*p*
Age (years)	29.5 ± 6.7	32.1 ± 10.6	0.21	26.9 ± 7.5	31.4 ± 5.5	0.07
SLEDAI	7.6 ± 8.5	6.95 ± 6.1	0.79	15.7 ± 6.9	1.5 ± 1.6	**<0.001**
C3 (mg/dl)	77 ± 34.4	78.6 ± 34.3	0.95	50.5 ± 28.5	97.3 ± 23	**0.001**
C4 (mg/dl)	14.7 ± 8.5	17.3 ± 10.5	0.35	10.8 ± 6.9	17.6 ± 8.6	**0.03**
Hb (g/dl)	10.5 ± 2	10.5 ± 2.4	0.9	9.5 ± 1.7	11.2 ± 1.9	**0.02**
WBC ($x10^3/\mu l$)	6.7 ± 3.1	8.2 ± 6.3	0.22	6.1 ± 3.6	7.1 ± 2.6	0.1
PLT ($x10^3/\mu l$)	255.3 ± 88	286.5 ± 112	0.29	282.1 ± 84.9	234.8 ± 88.9	**0.046**
Proteinuria (g/24 h)	1.6 ± 1.7	1.3 ± 2.6	0.13	2.4 ± 1.4	1.04 ± 1.7	**0.007**
Creatinine (mg/dl)	0.99 ± 0.7	1 ± 0.92	0.54	1.1 ± 0.83	0.92 ± 0.61	0.49

SLE: systemic lupus erythematosus; APS: antiphospholipid syndrome; SLEDAI: SLE disease activity index; C: complement; Hb: hemoglobin; WBC: white blood cells; PLT: platelets. Bold values are significant at p < 0.05.

Table 2
miR 146a and miR126 in all systemic lupus erythematosus patients.

Fold Changes Median (range)	SLE patients (n = 88)	Control (n = 40)	p
miR-146a	0.41 (0.16–0.85)	1.14 (0.33–2.44)	**<0.001**
miR-126	3.03 (0.77–5.47)	1.34 (0.56–2.33)	**0.007**
	Active SLE (n = 47)	Inactive SLE (n = 41)	
miR-146a	0.3 (0.16–0.66)	0.56 (0.17–0.85)	0.47
miR-126	2.16 (0.81–5.47)	1.48 (0.77–4.82)	0.07
	Treated SLE (n = 64)	Untreated SLE (n = 24)	
miR-146a	0.46 (0.16–0.82)	0.3 (0.17–0.85)	0.93
miR-126	2.13 (0.83–5.47)	1.92 (0.77–4.48)	0.25

SLE: systemic lupus erythematosus. Bold values are significant at p < 0.05.

Table 3
miR 146a and miR126 in systemic lupus erythematosus patients with antiphospholipid syndrome.

Fold Changes Median (range)	APS patients (n = 30)	Control (n = 40)	p
miR-146a	0.56 (0.28–0.72)	1.14 (0.33–2.44)	**0.003**
miR-126	4.17 (3–5.47)	1.34 (0.56–2.33)	**<0.001**
	SLE + APS (n = 30)	SLE without (n = 58)	
miR-146a	0.56 (0.28–0.72)	0.4 (0.16–0.85)	0.26
miR-126	4.17 (3–5.47)	2.3 (0.77–3.2)	**0.004**
	APS active (n = 13)	APS inactive (n = 17)	
miR-146a	0.49 (0.28–0.65)	0.71 (0.26–0.72)	0.54
miR-126	3.9 (3–5.2)	3.6 (3.22–4.7)	0.74

APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus. Bold values are significant at p < 0.05.

presented in Fig. 1. Plasma miR-126 levels were strongly associated with the presence of previous fetal losses and correlated with adverse pregnancy outcome in APS patients (p = 0.006).

4. Discussion

Epigenetic factors, including DNA methylation, post-translational histone alterations and miRNAs, interplay with genetic programs to control the immune functions. Dysregulation of miRNAs, can result in aberrant immune responses, and contribute to the pathological processes of many disorders. The use of unique miRNA expression signatures in SLE could be important and cost-effective non-invasive means to monitor predisposition to disease complications as APS and lupus nephritis [22]. aPL antibodies are common in SLE patients and their association with recurrent miscarriage is variable [23]. Both aPL and anti-dsDNA induce distinctive changes in the expression of proteins related to the biogenesis and expression of miRNAs in APS and SLE as well as their target proteins [10].

Reduced plasma levels of miRNA-146a in SLE patients found in the present study is consistent with *Löfgren et al* supporting a possible role in the immune pathogenesis of the disease [24]. MiR-146a has also been found to be down regulated in CD4+ T cells from lupus patients compared to healthy controls, and it was conversely linked to disease prognosis [25]. In this study, there was no difference in miR-146a expression between treated and untreated patients. This is inconsistent with *Wang et al* who showed that the expression of miR-146a was significantly increased in patients with SLE after 6 months of treatment with calcitriol [4].

In the SLE cohort, serum IFN levels were significantly higher compared to control. Inappropriate activation of type 1 IFN is closely associated with the pathogenesis of SLE and correlates with the anti-dsDNA antibodies titre and with SLE activity scores [26]. MiR-146a was proven to be a negative regulator of the IFN pathway thus its under expression might contribute to alterations in the type I IFN levels in SLE. By targeting signaling adaptor proteins as the TNF receptor-associated factor 6 (TRAF-6) and IL-1R-associated kinase (IRAK-1), miR-146 suppresses NFkB activation and subsequent cytokine production as IL6 and IL8 which are key mediators of inflammation [27]. Targeted deletion of miR-146a in mice caused several inflammatory disorders, supporting its key

Table 4
Sensitivity and specificity of miR-146a between all systemic lupus erythematosus (SLE) patients (n = 88) or those with activity (n = 47) *vs* control (n = 40) and of miR126 patients with antiphospholipid syndrome (APS) (n = 30) versus control and *vs* cases without (n = 58).

Parameter	Cut-off	AUC	Sens. (%)	Spec. (%)	95% CI	p
miR-146a						
SLE *vs* control	0.86	0.7	75.7	62.5	0.6–0.79	**<0.001**
active *vs* control	0.83	0.73	80.5	62.5	0.61–0.84	**<0.001**
miR-126						
SLE + APS *vs* control	4.1	0.81	70	90	0.71–0.92	**<0.001**
SLE + APS *vs* without	2.66	0.79	76.7	81	0.69–0.9	**<0.001**

SLE:systemic lupus erythematosus; APS: antiphospholipid syndrome. AUC: area under the curve; Sens.: sensitivity; Spec.: specificity. Bold values are significant at p < 0.05.

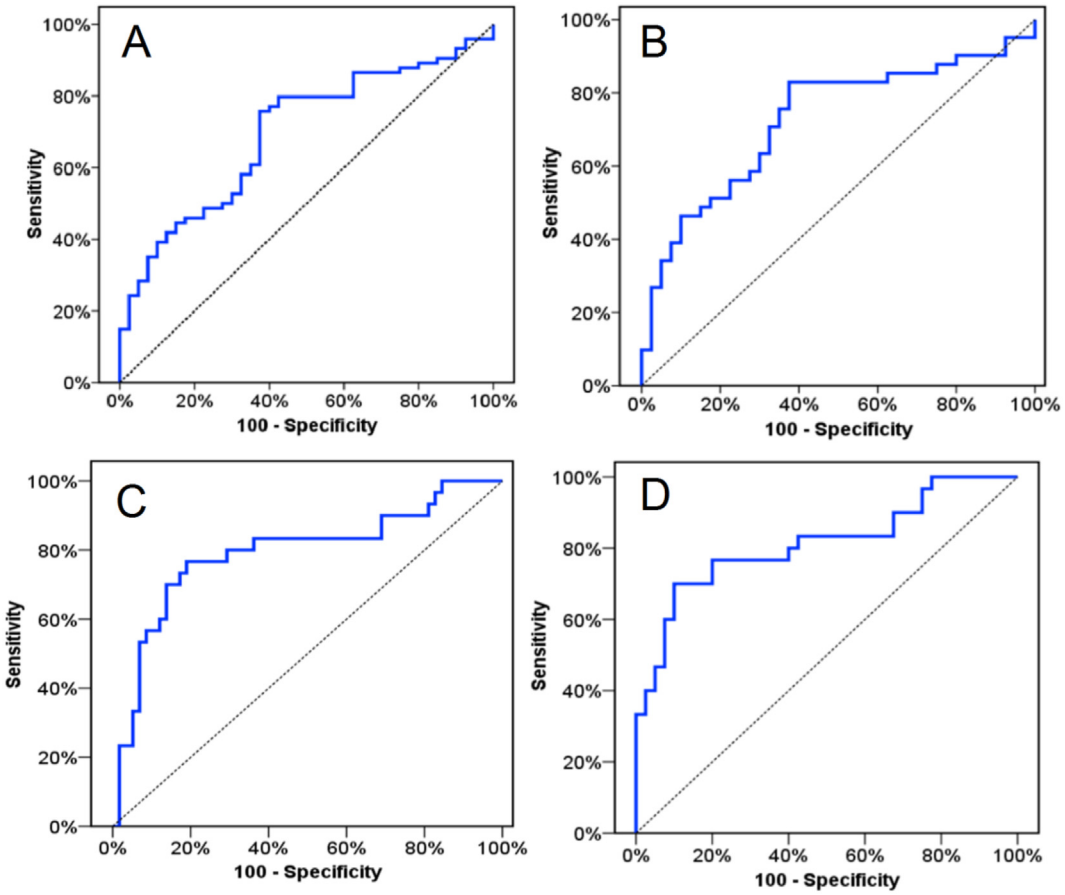

Fig. 1. Receiver operating characteristic (ROC) curve analysis of the miR-146a and miR-126 in systemic lupus erythematosus (SLE) patients versus control as a potential diagnostic marker. (A) Plasma miR-146a between all patients and control. (B)Plasma miR-146a between active patients and control. (C) Plasma miR-126 between patients with antiphospholipid syndrome (APS) and control. (D) Plasma miR-126 between patients with and without APS.

role as a molecular brake on inflammation [28]. Reduced expression and binding affinity of Ets-1 to miR-146a promoter may lead to its reduction and contributes to the up-regulated type I IFN pathway in SLE [29]. An aberrant expression of type 1 IFN-regulated genes, termed IFN signature has been reported in SLE [30].

In the present study, plasma miR-126 was significantly higher in SLE patients compared to controls. This is consistent with the findings of other studies [31,32]. MiR-126 results in DNA demethylation by directly binding to DNA methyltranferase 1 (DNMT1) and thus suppressing DNMT1 transcription activity. This block was suggested to upregulate miR-126 expression and an overproduction of CD4+ cells that enhance IgG production with a consequent worsening of the disease [33]. Recently, *Lopez et al* stated that the methylated status in a promoter region blocks the accessibility to transcriptional activators and thus inhibits the gene transcription, serving as a repressive "lock," while an unmethylated state at the promoter permits transcription [34]. In line with this, T cells from patients with active lupus were reported to display global DNA hypomethylation state [31].

The present results revealed no significant difference in miR-146a plasma expression between SLE patients with and without APS. In agreement, *Van den Hoogen et al* reported a global reduction in the expression of miRNAs in SLE patients with secondary APS [35]. In partial disagreement, *Perez et al* reported up-regulation of miR-146a in monocytes in aPL positive compared with aPL negative SLE cases. However in the same study reduced levels of miR-146 were observed in purified neutrophils and lymphocytes from both APS and SLE patients in relation to the control. miRNAs biogenesis is significantly altered in neutrophils and monocytes of APS and SLE; is related to the atherothrombotic status and can be modulated by specific autoantibodies [10]. The current work evaluated miRNA expression in plasma only. Accordingly it can be hypothesized that these apparently contradicting data might be attributed to the distinctive miRNA expression displayed by different leucocyte subsets and plasma. Eventually this could differentially influence the overall inflammatory environment in secondary APS.

In this study, SLE patients with secondary APS had significantly higher levels of plasma miR-126, compared to those without and to control. A gradient of expression in plasma miR-126 was observed among SLE patients with and without APS and controls. Altered expression of miR-126 was associated with the presence of previous fetal losses and correlated with adverse pregnancy outcome in APS patients. Consistent with these findings, it has been suggested that the altered regulation of circulating miRNAs in maternal placental blood might result in pregnancy complications, thus representing non-invasive diagnostic and prognostic biomarkers for pregnancy monitoring [36].

The influence of autoimmunity on the circulating profile of miRNAs in APS was demonstrated by the significant correlation between high titers of aPL antibodies with the aberrant expression of several miRNAs integrating the signature [37]. The inflammatory state of the vasculature can be influenced by several miRNAs, including miR-126, which effects both the expression of proinflammatory cytokines and vascular cell adhesion molecule (VCAM-1).

Although miR-126could promote atherosclerosis the increase of miR-126 could also be a result of micro particle-mediated transmission from neighboring endothelial cells [38]. aPLs were suggested to induce changes in the expression of miRNA biogenesis proteins in leucocytes of APS patients, which are then translated into an altered miRNA profile and, consequently, in the aberrant expression of their protein targets related to thrombosis and atherosclerosis [39].

Perez et al identified a signature of 10 miRNA ratios in APS related to fetal loss, atherosclerosis, and thrombosis. Differentially expressed circulating miRNAs in APS modulated partially by aPL might represent novel biomarkers of disease features and typify patients' atherothrombotic status [42]. Unfortunately, miR-146a and 126 were not among their studied miRs. A single miR may interact with hundreds of mRNAs, and thus the net effect of actions of individual microRNAs are often complex. In addition, miRNAs may be released in circulating exosomes and taken up by neighboring cells or, via the circulation, by distant cells with consequent cell phenotype changes [44]. The possibility of clinical control of angiogenesis via miRs warrants further investigations. Significantly increased levels of circulating plasma miR-126 found in secondary APS patients suggest a novel potential role as a biomarker in APS patients. There are a number of advantages favoring the use of miRNAs as disease indicators. Their expression in serum is stable, reproducible, and consistent. Moreover, compared with other biomarkers, detection of miRNAs seems to be more available with low complexity [4].

Among the study limitations are the small sample size, the defect of follow up and serial measurements of the studied miRs as well as the lack of a detailed relation to clinical manifestations. Including a larger number of miRNAs will help explore the specificity of the miRNA signature found in APS patients.

In conclusion, this work showed that plasma miR-146a was significantly lower and miR-126 significantly higher in SLE compared to controls. MiR126 was higher also in secondary APS patients. The potential of circulating miR-126 as a non-invasive biomarker in APS patients warrants further study in view of the limited data on the expressions of microRNA in APS. Characterization of microRNA profile in blood of patients with APS could be useful in identifying their spectrum in APS.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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