ORIGINAL ARTICLE

Expression of Autophagy Related Genes mTOR, ATG10 and P62 in The Peripheral Blood Mononuclear Cells of Systemic Lupus Erythematosus Egyptian Patients

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ABSTRACT

Background: *Autoimmune diseases are knowen to be associated with abnormalities in autophagy.* ***Objectives:*** *In our study we evaluated the expression of autophagy related genes mTOR, P62 and ATG10 in the peripheral blood mononuclear cells (PBMCs) of systemic lupus erythematosus (SLE) and assess their relationship with disease activity and immunologic features.* ***Methodology:*** *The expression of mTOR, P62and ATG10 was detected by RT-PCR in 70 SLE subjects and 30 age- and sex-matched healthy controls. Data regarding demographics and clinical parameters were collected. Disease activity of SLE was evaluated according to the SLE Disease Activity Index (SLEDAI) score. Mann-Whitney test was used to analyze the expression of mTOR, P62 and ATG10 in the two groups. Spearman's correlation was performed to analyze their relationship with disease activity and immunologic features.* ***Results:*** *The median and IQ (interquartile range) levels of P62 and ATG10 mRNA were significantly higher in SLE patients than the controls 1.11, (0.34-8.59) vs 0.25, (0.01-1.0) for P62 with P<0.002; 2.24 (0.55-19.31) vs 0.77 (0.02-1.0) for ATG10 with P<0.000). However, the mRNA relative expression level of mTOR in PBMCs was significantly very high in controls than cases of SLE 1.0, (1.0-2.14) vs0.31 ,(0.14-0.89) with P< 0.000).* ***Conclusion:*** *Correlation analysis showed that mTOR mRNA levels only correlated negatively with SLEDAI; also our research measure serum level of IL17 in the study population of cases and controls.*

INTRODUCTION

SLE is a model chronic autoimmune inflammatory illness that produces antinuclear antibodies through processes that are poorly understood and is marked by T and B cell dysfunction[1]. Atgs (autophagy-related genes) polymorphisms have been connected to SLE by genome-wide association studies (GWAS). In recent years, it has become clear that autophagy plays a crucial role in lymphocyte biology. The involvement of autophagy in SLE, however, has received little research, and the dynamic mechanism of autophagy flux in SLE had not yet been documented. As a result, the current study is very important for SLE and could have an effect on how we practise medicine[2].

By destroying unneeded cytoplasmic components and recycling nutrients, the lysosome-mediated catabolic process known as autophagy maintains cellular homeostasis [3]. (Atgs) mTOR, P62, and ATG10 are involved in the autophagy flux. In more detail, p62 is linked to the breakdown of autophagosomes[4], Becline-1 and LC3 govern the production of autophagosomes, and mTOR regulates autophagy through the signalling pathways upstream of the autophagy machinery. Autophagy has a role in almost every element of immunity, particularly in the growth and appropriate function of T and B lymphocyte populations[5]. Unexpectedly, ineffective autophagy can result in a variety of autoimmune and chronic inflammatory disorders [6].

We analyzed the expression of Atgs mTOR, P62 and ATG10 in the PBMCs of SLE patients and healthy controls at the first time. Furthermore, we also analyzed the correlations between the expression of Atgs and SLEDAI as well as immunologic features, also we measure the serum level of IL17 concentration. We hope that the present study will broaden our visions about SLE pathogenesis and provides a potentially new therapeutic avenue for SLE.

METHODOLOGY

Patients:

This case-control study was carried out in Medical Microbiology and Immunology Department and Medical Research center, Faculty of Medicine, Sohag University. This study included seventy patients with SLE; admitted to Rheumatology Department in Sohag University Hospital from July 2020 to July 2022. An informed consent was obtained from each patient to be

enrolled in the study. The study protocol was accepted by the local Ethical Committee of Faculty of Medicine, Sohag University.

All participants met at least four of the American College of Rheumatology (ACR) revised criteria for SLE. Patients were excluded in the study if they met the following criteria: (1) age <18 years old or >60 years old; (2) pregnancy or lactation; (3) coexistence of other autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, or chronic diseases affecting autophagy such as systemic infection, cancers, diabetes mellitus, neurodegeneration, and heart diseases, etc. Disease activity was assessed in accordance with the SLE Disease Activity Score (SLEDAI 2000 (SLEDAI-2K)) The SLEDAI was developed and validated as a global clinical index for the measurement of disease activity in SLE [7]. This index was modeled on clinician's global judgment. It was developed with a panel of experienced rheumatologists with expertise in SLE, using well established group of techniques and index development methodology [8]. C3, C4 were detected by immuno turbidimetry, IL17 was detected by enzyme linked immuno sorbent assay (ELISA).

In addition, 30 healthy volunteers matched for age, sex were recruited as the control group. Five milliliters of ethylenediaminetetraacetic acid (EDTA) anticoagulated venous peripheral blood were collected from the patients and healthy volunteers for the detection of mTOR, P62 and ATG10 mRNA levels in PBMCs.

Total RNA isolation and RT-PCR analysis

The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation method. Total RNA was extracted using a spin column RNA extraction kit (from Jena Bioscience (Germany) (LOT: 16200701)). RNA concentration and purity was measured by Nano Drop™ 8000 Spectrophotometer (Applied Biosystems, USA) to determine RNA concentration. A total of 0.8 µg RNA was used to synthe-size cDNA using the First Strand cDNA Synthesis Kit All reagents were supplied from Solis BioDyne (Europe) (FIREScript RT cDNA Synthesis KIT) (LOT: 00683400).

According to the manufacturer's instructions. Quantitive PCR was performed with 2 µl of cDNA, 0.4 µl of each primer (10 umol/µl) and 10 µl of SybrGreen qPCR Master Mix (SYBR green master mix (2X)) (Applied Biosystems, USA) (LOT: 00634195) which was consisted of SYBER green dye, dNTPs, MgCl2 DNA polymerase and DNase/RNase free water. Forward and reverse primers for mTOR, P62, ATG10, genes and GAPDH gene (GAPDH glyceraldehyde-3-phosphate dehydrogenase) (Invitrogen, USA) are analyzed with the LightCycler480 Software (Roche, Switzerland). The cycle threshold values were used to calculate the normalized expression of mTOR, P62 and ATG10 against GAPDH gene using the Q-Gene software .The sequences of the primer pairs are listed in table (1).

Table 1: The sequences of the primer pairs of GAPDH, mTOR, P62 AND ATG10 genes

Primer	Sequence
GAPDH	5′-ATGGCATTCCGTGTTCCTAC -3′ 5′-CCCTTCATTGACCTCAACTA-3′
mTOR	5′-TCACATTACCCCCTTCACCA-3′ 5′-TCAGCGAGTTCTTGCTATTCC-3′
P62	5′-TCCTACAGACCAAGAATTATGAC-3′ 5′-TTCTCATGCACTTTCCTACTG-3′
ATG-10	5′-CTTCCCATGGAGGAGGCTTT-3′ 5′-GGCACTTGGTAGCTACAGGAA-3′

Statistical analysis

All statistical analyses were performed using the statistical software SPSS 23.0. The normality of continuous variables was established by means of one sample K-S (Kruskal Wallis) test. Variables were summarized using the median and standard deviation (SD). Univariate comparisons between nominal variables were performed by the Chi-square test. Comparisons of continuous variables between two groups were done using the Mann-Whitney U-test. For correlations between two continuous variables, Pearson's or Spearman's correlation was used for normal or non-normal variables, respectively. $P<0.05$ was considered as statistically significant, graphs of the study were done by using SPSS and Excel program.

RESULTS

Baseline characteristics of study subjects:

Our study included 70 SLE patients included 63 (90%) were females , with median age of 33.5 The clinical manifestations of SLE patients were as follows (table 2): 46 patients (65.7%) with alopecia , 43 patients (61.4%) with arthritis, 40 patients (57.1%) with photosensitivity, 39 patients (55.7%) with oral ulcers , 36 patients (51.4%) with malar rash ,29 patients (41.4%) with skin rash , 29 patients (41.4%) with lower limb edema, 25 patients (35.7%) with cough , and 20 patients (28.6%) with lupus nephritis.,For comparison, 30 healthy persons were recruited to this study, of them, 24 (80%) were females with median age of (32.7 years) (p=0.427) compared with SLE patients.

Table 2: Clinical Characteristics of SLE patients

Parameter	No =70	Percent 100%
Female	63	90%
Skin rash	29	41.4%
Photosensitivity	40	57.1%
Alopecia	46	65.7%
Malar rash	36	51.4%
Oral ulcers	39	55.7%
Arthritis	43	61.4%
Cough	25	35.7%
Lower limb edema	29	41.4
Positive urinary albumin	41	58.6%
Leucopenia	32	45.7%
Anemia	27	38.6%
Low MCV	18	25.7%
Low platelets	21	30%
Elevated Pcratio	23	32.9
Lupus nephritis	20	28.6%
Immunological features		
Decrease C 3 (<60 mg/dl)	21	30%
Decrease C4 (<15mg/dl)	19	27.1
ANA IF (+ve)	70	100%
ANA PROFILE) (+ve)	70	100%
SLEDAI score		
No activity (SLEDAI=0)	1	1.4%
Mild activity (SLEDAI=1-5)	10	14.3%
Moderate activity (SLEDAI=6-12)	20	28.6%
High activity (SLEDAI=13-20)	28	40%
Very high activity (SLEDAI>20)	11	15.7%
Disease duration **mean±SD**	4.9±3.3	
median and IQ	4 (3-6) years	

There was a very highly significant difference of ElISA IL17 concentration between cases and controls group by using non parametric Mann Whitney test (fig 1), IL17 concentration was significantly higher in control than cases.

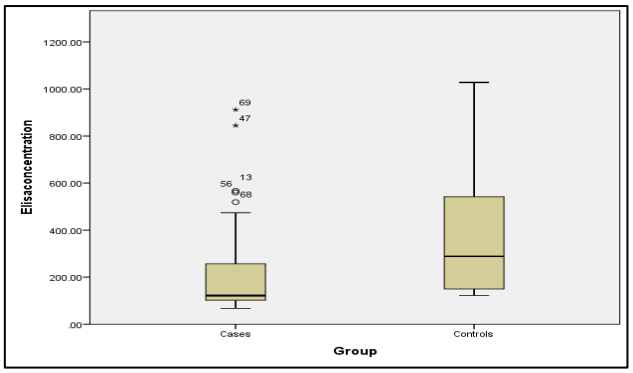

Fig. 1: ELISA IL17 Concentration

The median and IQ levels of P62 and ATG10 mRNA were significantly higher in SLE patients than the controls 1.11, (0.34-8.59) vs 0.25, (0.01-1.0) for P62 with *P*<0.002; (2.24) (0.55-19.31) vs (0.77) (0.02-1.0) for ATG10 with *P*<0.000). However, the levels of mRNA relative expression level of mTOR in PBMCs was significantly very high in controls than cases (1.0), (1.0-2.14) vs (0.31), (0.14-0.89) with *P* 0.000 as shown in table 3

Table (3) Expression of ATG10, mTOR and P62 in cases and controls.

Parameter	Cases n=70	Controls N=30	P value by Mann Whitney
`mTOR (median and IQ)	0.31 (0.14-0.89)	1.0 (1.0-2.14)	0.000***
P62 (median and IQ)	1.11 (0.34-8.59)	0.25 (0.01-1.0)	0.002**
ATG10 (median and IQ)	2.24 (0.55-19.31)	0.77 (0.02-1.0)	0.000***

There were mild negative correlations between mTOR mRNA levels and SLEDAI (Systemic lupus erythematosus disease activity index) (r=-0.37 p=0.002), while no significant correlation between mRNA levels of P62, ATG10 and SLEDAI ;also no significant correlation was found between mTOR, P62 and, ATG10 mRNA levels and C4,C3 (Figure 2). (Table 4).

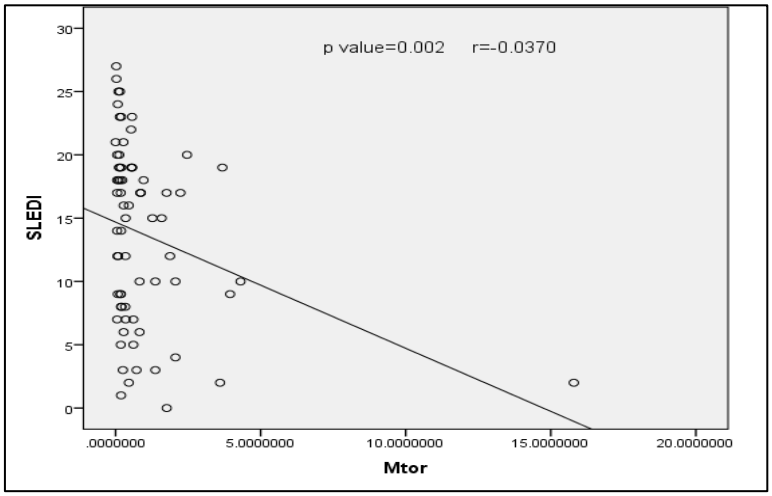

Fig. 2: Correlation between motor gene expression and SLEDAI

Table 4: Correlation between mTOR, P62 and ATG10 with SLDAI, complement 3, and complement 4 respectively

Parameter	mTOR	P62	ATG10
SLDAI	r=-0.370 p=0.002**	r=0.019 p=0.879	r=0.084 p=0.488
Complement 3	r=-0.009 p=0.940	r=-0.112 p=0.355	r=-0.101 p=0.404
Complement 4	r=-0.039 p=0.750	r=0.001 p=0.995	r=-0.101 p=0.406

There was significant correlation between expression of mTOR and ELISA IL17 concentration with mild positive correlation between them as r=-0.2 and highly significant mild positive correlation between ElIZA IL17 conc. and SLEDAI as shown in table 5 and fig.3.

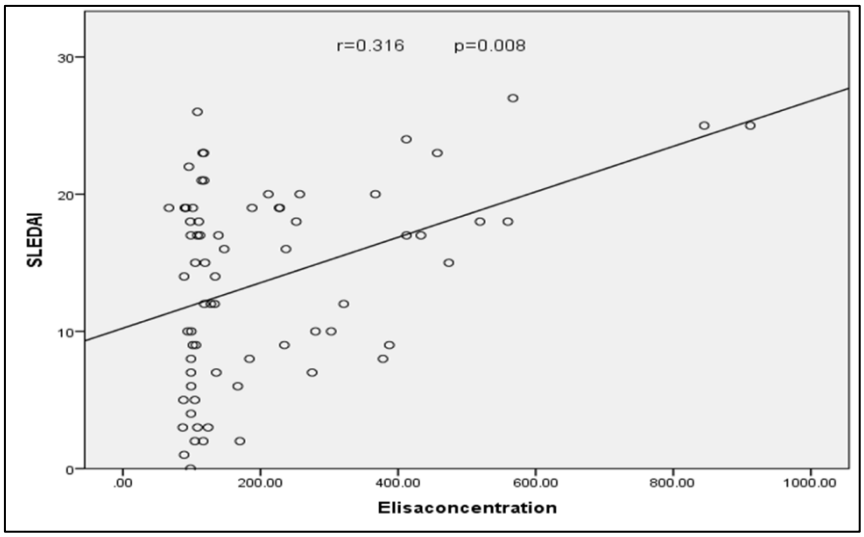

Fig. 3: Correlation between IL17 concentration and SLEDAI

Table 5: Correlation between mTOR, P62 and ATG10 with Elisa absorption and Elisa concentration

Parameter	mTOR	P62	ATG10	SLEDAI
ELISA Conc.	r=0.206 p=0.039*	r=0.00 p=0.99	r=-0.123 p=0.22	r=0.316 p=0.008**

Our study results by using non-parametric Kruskal Wallis test to see the significant difference between gene expression in between lupus nephritis patients, non-lupus patients and control found that, there were very highly significant difference in mTOR expression in the three groups ;higher in control than cases and higher in cases without LN than cases with LN ,also there were very highly significant difference in ATG10 expression in the three groups; higher in cases than control and higher in cases with LN than cases without LN while there was highly significant difference in P62 expression between the three groups; higher in cases than control; higher in cases with LN than cases without LN as shown in table 6.

Table 6: Difference between lupus nephritis patients, non-lupus patients and control according to gene expression and IL-17:

Parameter of	Groups (median and IQ)			P value by Kruskal wallis
	Control N=30	Lupus nephritis Patients N=20	Patients without nephritis N=50	
mTOR	1.0(1.0-2.1)	0.199(0.12-0.86)	0.34(0.15-0.97)	< 0.001***
P62	0.25(0.009-1.0)	1.71(0.36-7.94)	0.98(0.29-8.59)	0.009**
ATG10	0.76(0.020-1.0)	2.30(0.90-34.17)	1.67(0.54-8.4)	<0.001***
ELISA Conc.	288.5(145.0-542.2)	150.5(99.0-400.7)	118.85(103.85-240.7)	<0.001***

DISCUSSION

Defects in the process of autophagy, which is crucial for numerous immunological functions, are linked to a number of autoimmune and neurodegenerative diseases[9].

The development of phagophores, autophagosomes, the fusion of autophagosomes with lysosomes, and the formation of autolysosomes, which are necessary for the destruction of faulty proteins, are all stages in this process. The genes beclin 1, mtor, p62, atg5, atg7, lc3, atg12, atg16l1, and atg 10 govern the production of autophagosomes. [9]

The entire autophagic process may be broken down into a number of steps, such as the start, elongation, closure, maturation of autophagosomes, fusion with lysosomes, as well as breakdown and release of macromolecules back into the cytosol.

The present study was conducted to determine expression of autophagy related genes, mTOR, P62 and ATG10 in the PBMCs, their relation with SLEDAI and immunologic features (C3, C4) , and to determine level of IL17 in serum of patients and control. According to the present study SLE is more common in females (90%) which agreed with Robert G. Lahita et al[10] and *Leong*, PY et al. [11]

In the present study we detected that the mRNA relative expression level of mTOR in PBMCs was significantly high in patients than controls (P=0.000),but The mRNA relative expression level of P62 in PBMCs was significantly high in cases than control (P=0.002), these results were similar to those reported by Zhen-Zhen Wu et al.[12] and Ting peng and Hua pei[13] and disagreed with S Chen et al.[14]

mTOR is a master regulator of cellular metabolism and is essential for controlling autophagy. There is mounting evidence that the activation of mTOR and the induction of autophagy were tightly coupled. Pattingre S. and his colleagues [15] showed that autophagy was increased to recycle nutrients when energy sources were scarce and mTOR activity was low. mTOR was active and autophagy was downregulated when energy and nutrients were abundant. Meijer AJ[16] also made note of how active mTOR prevented the development of autophagosomes. Though the cause of this occurrence is yet unknown, there are a number of theories that should be taken into account. First off, several signalling pathways, such as the mTOR signalling system, the Ras/cAMP dependent protein kinase pathway, the LKB1-AMPK signal circuit, etc., control autophagy[17]. Other signalling pathways may be more crucial in controlling autophagy in SLE than mTOR. To determine which signalling pathway is the main regulator of autophagy in SLE, more mechanistic studies will be required in the future. In addition, mTOR comprises the two separate signalling complexes known as mTORC2 and mTOR-RC1. While mTORC2 does not directly regulate autophagy and the molecular mechanism of mTORC2 control by upstream effectors is largely unclear, activation of mTORC1 results in the suppression of autophagy. The activity of mTOR is also influenced by a variety of regulators, including nutrients, growth factors, energy, and stress, etc.[18]

The creation of autophagosomes and the ability for lysosomal degradation are out of equilibrium, which leads to an accumulation of autophagic vacuoles. A key indicator of autophagic degradation is p62. When Atgs or other components necessary for the fusion of autophagosomes with lysosomes are lost, p62-positive aggregates noticeably rise[19]. Our current research revealed that p62 expression was higher in SLE patients than in controls, which may indicate that autophagy

flux's downstream effects were blocked. Additionally, p62 contributes to the activation of autophagy. A recent study discovered that p62 expression was necessary for TLR7-induced autophagy[20]. Additionally, in order to prevent excessive autophagy, p62 can convey the signal from amino acids to the mTORC1 pathway. This may also help to explain why the expression of mTOR was not decreased in SLE. In consideration of the high expression of Becline-1, LC3 and p62, we speculate that autophagic vacuoles were accumulated in SLE [21].

In the present study we found that ATG10 was highly expressed in cases than control , our study is one of the first studies that search on ATG10 in SLE; many studies have been done on ATG10 in many other diseases; most of them related to colorectal cancer and a fewer studies on other autoimmune diseases. A study on Vogt-Koyanagi-Harada (VKH) disease which is a rare autoimmune disease showed that variants in genes ATG10 did not achieve significant genome-wide association threshold[22]. In a different study on primary gouty arthritis results said that the expression level of ATG10 mRNA in the AG (acute gout) and (IG) (intermittent gout) group was lower than that of the HC (healthy controls) group[23].

Studies on ATG10 expression in colorectal cancer which showed that ATG10 expression in tumors was significantly higher than that of the adjacent normal mucosa. ATG10 was increased in 18 of the 37 cases (48%) of colorectal cancer, ATG10 expression was higher in cancer cell lines than in the normal colorectal cell line; Taken together, these results indicate that ATG10 is up regulated in colorectal cancer ;this result agree with result of Jo YK[24] while another study on Acute lymphoblastic leukemia[25] results in The majority of B-ALL (Acute lymphoblastic leukemia) patients showed a significant reduction in the ATG 10 genes compared to control group (P <0.05), also according to[26]; significant reduction in the ATG 10 genes was showen in rat model Parkinson's Disease., Another study on breast cancer reported that expression was most different in the brain metastases of patients with metastatic breast cancer as compared to primary tumors of the breast. ATG10 mRNA was present at decreased quantities in brain metastatic tissues as compared to primary tumors of the breast.[27]

In the present study we showed negative correlation between expression of mTOR and SLEDAI score while there was insignificant correlation between expression of P62 and SLEDAI which disagreed with Zhen-Zhen Wu et al[28] who said that p62 mRNA levels correlated positively with SLEDAI, also no significant correlation between expression of ATG10 and SLEDAI.

In the present study we found that there was no correlation between mTOR, P62, ATG10 expression and C3 and C4 levels, which agreed with Zhen-Zhen Wu et al.[28]

IL-17 has been associated with the pathogenesis of a range of autoimmune diseases, including rheumatoid arthritis (RA), systemic sclerosis, multiple sclerosis (MS), and SLE *29* There was very highly significant difference of ELISA IL17 concentration between cases and controls group by using non parametric Mann Whitney test, serum IL17 concentration was lower in patients of SLE than control, this result disagreed with Young Ho Lee and Gwan Gyu Song[30], Yamei Tang et al[31] and Saeed Mohammadi[32]

According to Vincent et al.[33] immunosuppressant drugs used in SLE and patient response could impact on serum IL-17 concentrations, and thereby may be a confounding factor when analyzing correlation between serum cytokines and disease activity; and this may explain our study result which disagrees with other studies that our patients were stable and good responder to treatment.,the present study results show that there was highly significant mild positive correlation between Elisa IL17 conc. and SLEDAI which agreed with Sahar M.Abdel Galil et al[34] and C K Wong, et al[35] But disagreed with Fabien B Vincent, et al [36] and Xue-Fei Zhao, et al.[37]

The present study showed that the correlation coefficient between P62 and ATG10 expression with ElISA IL17 concentration is insignificant, this agreed with Tomohiro Koga et al.[38]

Our study results by using non-parametric Kruskal Wallis test to see the significant difference between gene expression in between lupus nephritis patients, non-lupus patients and control (table 7) found that, there were very highly significant difference in mTOR expression in the three groups ;higher in control than cases and higher in cases without LN than cases with LN, also there were very highly significant difference in ATG10 expression in the three groups; higher in cases than control and higher in cases with LN than cases without LN while there was highly significant difference in P62 expression between the three groups;higher in cases than control; higher in cases with LN than cases without LN ; Which is agreed with Juan Jin et al.[39] and Linlin Wang and Helen Ka Wai Law.[40]

CONCLUSION

mTOR,P62 and ATG10 are important autophagy related genes involved in the autophagy flux by regulating autophagy autohagosomes formation.

The discovery of autophagy mechanisms, autophagy-related genes, and selective autophagy has not only played a major role in elucidating physiological processes but has also helped to reveal the pathophysiology of various diseases that had remained unclear. In addition to the suppression of autophagy, the enhancement of autophagy may also be detrimental to the maintenance of autophagic functions. Currently, various clinical trials are underway to provide better

treatments for chronic diseases and cancer by modulating autophagy.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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