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Association of TLR4 Gene Polymorphism (rs4986790) of SARS-CoV-2 Patients with Cognitive Impairment

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Abstract: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) which gave rise to coronavirus infectious disease (COVID-19) in late 2019 as a respiratory illness, has significantly impacted global health and society. Recently, there has been a link between SARS-CoV-2 and Toll-like receptor4 (TLR4). Thus, the current study aimed to shed light on how TLR4 single-nucleotide polymorphism (SNP) (rs4986790) possibly link to the severity of disease in Iraqi COVID-19 patients and establish their role in recovered individuals with cognitive impairment. A case-control study (60 patients, 30 recovered and 50 controls) investigates the A/G genotype of rs4986790 in all studied groups, using a conventional polymerase chain reaction with an allele-specific primer method. Results showed a significant increase in the median age accompanied by severe cases (p =0.003) and more than 50% of the moderate infections in the youth category. Regarding the recovered individual's occurrence six cases along with ages median of 49.5 (45.8-57) males and females with a history of mild to moderate infection. In clinical severity, allele and genotype frequencies of A/G rs4986790 TLR4 polymorphism showed no significant difference between COVID-19 patients' group (OR = 0.58; 95% CI = 0.28–1.20; p = 0.200). In recovered individuals with cognitive impairment, despite 50% cognitive impairment in the AG genotype, there is no significant difference compared to intact recovered individuals (OR = 2.62; 95% CI = 0.39– 17.78; p = 0.370). On the other hand, there were statistically significant variations (p < 0.001) in the concentrations of ferritin, D. dimer, and LDH. These inflammatory markers demonstrate their major role in COVID-19 illness and its harshness and aggravation. They were significantly greater in severe-critical cases but returned to normal in the recovered groups. In conclusion, recovered individuals with cognitive impairment are significantly associated with age older. On the other hand, despite 50% cognitive impairment in the AG rs4986790 genotype, there are no statistically significant differences between cognitive impairment and studied *TLR4* SNPs. A/G rs4986790 of *TLR4* required more information to explore their role in cognitive impairment and infection severity.

Keywords: Iraqi population, polymorphism, allele-specific primer, cognitive impairment, TLR4.

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Introduction

The virus that is causing the COVID-19 pandemic, known as severe acute respiratory syndrome coronavirus 2 has infected over 108 million people globally, with varying reported mortality rates ranging from 0.5 to 10% in different nations. China is where the novel coronavirus known as SARS-CoV-2 was first identified. How precisely it infects people and impacts their health is not entirely known. While severe infections can cause bilateral pneumonia and life-threatening acute

respiratory distress syndrome (ARDS), COVID-19 typically manifests clinically as fever, exhaustion, dry cough, and dyspnea. Young, healthy people may also experience serious side effects from the disease that necessitates critical care, even though severe complications typically appear in older patients with concurrent chronic diseases (such as diabetes, high blood pressure, etc.). The great variation in disease susceptibility, particularly in younger patients, raises the possibility that variations in each person's genetic background may be a factor in these changes (1).

Multiple infectious diseases have been linked to single nucleotide polymorphisms (SNP) in TLR4, The TLR4 SNP that has been studied the most, rs4986790, has been linked to a variety of infections, such as severe respiratory syncytial virus disease, clinical malaria, recurrent cystitis, chronic cavitary pulmonary aspergillosis, HCV infection, and the prognosis of HBV-infected individuals(2).

Single nucleotide polymorphisms, or SNPs, are the most common mutations in the human genome. They can be crucial biological markers for developing preventative, diagnostic, and therapeutic approaches to illnesses, including infectious ones (3).

Following their recovery from COVID-19, neuropsychological testing performed on a few patients after discharge indicated cognitive abnormalities, including a decrease in working memory, language expression, and executive function. According to these results, COVID-19 may hurt particular cognitive domains, including working memory and executive control. COVID–Up to seven months after infection, 19 recoveries had decreased general cognitive ability. Linguistic and computational skills were largely unaffected, but visuospatial and executive functions were primarily affected (4).

The COVID-19 virus may directly harm the structure and function of the brain, resulting in cognitive impairment, because it can enter the central nervous system by piercing the blood-brain barrier and nasal mucosa and infecting neural cells, such as neurons, astrocytes, and microglia, that aid in their transport. While most COVID-19 patients recover their cognitive function independently over time, some people, particularly older adults and those with underlying medical conditions may experience long-term cognitive impairment (5).

Material and Methods

Subjects and sample collection

This study focused on COVID-19 patients previously diagnosed in Baghdad Teaching Hospital, SARS-CoV-2 patients (patients group) from 18 to 55 years, and the age of healthy individuals range from 22 to 52 years. Case-control research was conducted on 90 patients and recovered individuals as well as 50 healthy controls during the period starting from September 2023 to December 2023 for testing SARS-CoV-2 infection. WHO Interim Guidance Recognized Criteria. There are two groups of patients (60) moderate and severe (30 cases in each group), and recovered individuals (30) after two to three weeks recovering from infection including 6 cases with cognitive impairments signs like the decline in working memory, language expression,

and executive function, and attention and sleep disorder(6).

The present research procedure was authorized by the local ethics committee of the University of Baghdad College of Science (Ref: CSEC/0923/0059). The following items are required: an EDTA tube, three milliliters of venous blood collected from patients, Additionally, until it is needed, the blood should be kept frozen at -20 °C. The DNA was extracted from the blood containing EDTA using a gSYNC DNA extraction kit (Geneaid, Taiwan) in accordance with the manufacturer's instructions.

Detection of TLR4 gene polymorphism

The PCR alleles-specific primer was used to detect the SNP rs4986790 of *TLR4* gene. The primer was designed in a recent study by NCBI primer-BLAST. In a 20µL as a total reaction, the following components were used: 2µL of template DNA, 6µL of nuclease-free water, 10µL of PCR PreMix (Pioneer, Korea), 1µL of forward primer (5′-GCATACTTAGACTACTACCTCGATGA/G-3′), and 1µL of reverse primer (5′-TTGTTCTAAGCCCAAGAAGTTTG -3′). The tube was placed in a Bio-Rad thermos cycler (Germany): a preliminary denaturation cycle at 95°C for 3 minutes; 35 cycles of denaturation at 95°C for 30 seconds; annealing at 54°C for 45 seconds; elongation at 72°C for 30 seconds; and finally, a final elongation cycle at 72°C for 5 minutes. The PCR result (5 µl) was subjected to electrophoresis in a 1.5% agarose gel in TBE buffer (1x) at 100 volts for 45 minutes to examine the migration of the specified bands. The particular forward primer in a PCR tube was labeled using gel electrophoresis, which showed three genotypes (AA, AG, and GG) associated with two alleles (A and G).

Detection of inflammatory markers

D. dimer Determination (Standard F D-dimer FIA)

The Standard F D-dimer FIA is based on the immunofluorescence technology used for elevated D. dimer. The specimen from humans should be processed for the preparation using the Standard F D-dimer FIA components. After applying the sample mixture to the test device, the complex is formed on the membrane due to the antigen-antibody reaction. The intensity of the fluorescent light produced on the membrane is proportional to the D-dimer concentration. Standard F analyzers can analyze the D-dimer concentration of a clinical sample based on a pre-programmed algorithm and display the test result on the screen.

Specimens preparation

Blood specimens in sodium citrate tubes could be used in this test. Samples and cartridges should be brought to room temperature 15-30 minutes before the test.

A-Procedure

1. The cartridge's sample ID (chip) was recorded in the designated place.
2. The plasma specimen of 50 µl was added to a sample dilution tube and mixed.
3. The mixed well sample in the dilution (50 µl) was dropped into the sample inlet on the cartridge.
4. The cartridge was entered into the cartridge slot using the arrow as a guide.

5. When the reaction in the cartridge is completed, the system automatically begins the reading process.
6. When the measurements were completed, the cartridge was automatically expelled and the results were displayed. The concentration of D-Dimer was automatically calculated.

Ferritin determination

Serum Ferritin was evaluated using a miniVIDAS analyzer for the fluorescent enzymatic detection technique Enzyme-Linked Fluorescent Assay (ELFA) (BioMerieux). Serum samples from all patients were applied to the instrument, and the concentration of Ferritin was automatically calculated.

Lactate dehydrogenase determination

Lactate dehydrogenase (LDH) in serum was quantitatively determined in vitro using the Roche Cobas Integra 400 plus C systems' Electro-Chemiluminescence Immunoassay method (free from hemolysis). The plasma samples containing precipitates were centrifuged at (6000 rpm for 10 minutes) to avoid contamination with platelets containing high lactate dehydrogenase concentrations.

Statistical Analysis

The median and interquartile range were provided for the continuous non-parametric variables that did not follow a normally distributed distribution. A Mann-Whitney U test was used to see whether there were significant differences. Number and % were used as categorical variables to describe categorical data, and Pearson's Chi-square test or the two-tailed Fisher exact test were used to find statistical significance. Utilizing https://www.had2know.org/academics/hardy-weinberg-equilibrium-calculator-2-alleles, the Hardy-Weinberg equilibrium (HWE) was assessed. We used logistic regression to get the odds ratio (OR) and 95% confidence interval (CI). Statistics were deemed significant when the probability (p) was less than or equal to 0.05. The statistical research used GraphPad Prism version 8.0.0 (San Diego, California USA) and IBM SPSS Statistics 25.0 (Armc onk, NY: IBM Corp.).

Results and discussion

As shown in Table (1), the baseline characteristics of the COVID-19 patients stratified to infection severity, and median of ages showed significant a raising in age accompanied by severe cases, (p =0.003). Age, in general, was divided into three age groups (18-25), (26-30) and (31- >35) years, with differences between the first two groups of about 5 years, while the third group was the largest because of the few scattered numbers, we had to collect age groups older than 5 years, in the cases of moderate showed a significant increase (p = 0.045) about more than 50% of the infections in the first age group (18-25) youth category. Although there is no significant appearance of infection between age groups in severe cases, the study data recorded the highest infections (80%) in ages over 25 years. Regarding the sex, it was observed that males had more infections acquired moderate (70%) and severe (63.7%). Significant differences were also observed in ferritin levels (304 ng/ml *vs* 780 ng/ml; p=<0.001), LDH levels (201 IU/L vs 252 IU/L; p=0.002), and D-dimer levels (382 mg/L *vs* 590 mg/L; p<0.001) between moderate and severe COVID-19 patients.

Table (1): Baseline characteristics of COVID-19 patients stratified to the severity of infection.

Characteristic		COVID-19 Patients		*p*-value
		Moderate (no.30)	Severe (no.30)	
Age; year		25 (22- 28)	28 (25-30)	***p* = 0.003**
Age group	**18-25**	16 (53.3)	6 (20)	***p* = 0.015**
	26-30	9 (30)	13 (43.3)	
	31- >35	5 (16.7)	11 (36.7)	
	***p*-value**	***p* = 0.045**	*p* = 0.273	
Sex	**Male**	21 (70)	19 (63.3)	*p* = 0.087
	Female	9 (30)	11 (36.7)	
	***p*-value**	***p* = 0.028**	*p* = 0.144	
Ct value	**19 – 25**	7 (23.3)	11 (36.7)	*p* = 0.102
	26 – 30	10 (33.3)	14 (46.6)	
	31 – 35	13 (43.4)	5 (16.7)	
	***p*-value**	*p* = 0.407	*p* = 0.122	
Ferritin ng/ml		304 (280-419)	780 (514-946)	**p < 0.001**
LDH IU/L		201(158-229)	252 (198-285)	**p = 0.002**
D. dimer mg/L		382(242-530)	590(446-800)	**p < 0.001**

For continuous variables, the median with interquartile range is provided; for categorical variables, the number and percentage are used; for test statistics, the probability of a Mann-bWhitney U test (for comparisons with continuous variables), a two-tailed Fisher exact test, or a Pearson Chi-square test is given; and for non-applicable variables, NA is used. Emphasized for emphasis.

Regarding the recovered individual's occurrence of six cases along with ages median of 49.5 (45.8-57) male and female with a history of mild to moderate COVID-19 infection after one to three months of infection.

Figure (1) shows the results allele-specific primer Conventional PCR was used to detect SNPs in the gene of TLR4. The outcomes of gel electrophoresis exhibited three genotypes for each SNP rs4986790 (AA, AG, GG), as 397 bp.

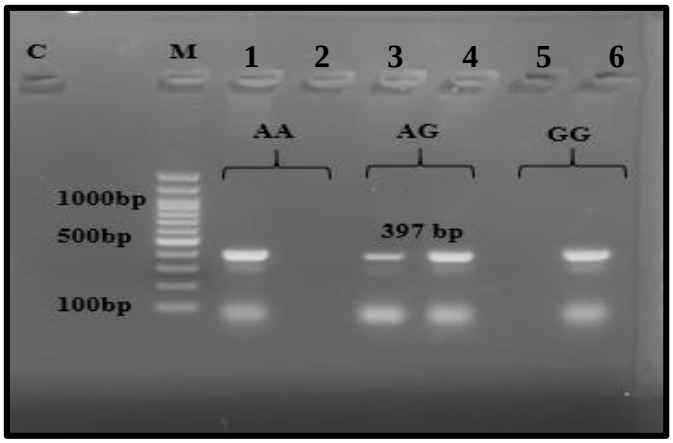

Figure (1): Agarose gel electrophoresis of PCR product of TLR4 gene using (1.5% agarose; 100 V for 45 minutes) of DNA–PCR products 397 bp for *TLR4 gene* SNP rs4986790 (A/G). C: Negative control, M: DNA ladder (100bp), 1 and 2 represents genotype (AA), 3 and 4 represents genotype (AG), 5 and 6 represents genotype (GG).

Table (2) shows that genotype frequencies of SNP rs4986790 in COVID-19 patients, recovered individuals, and control groups all corresponded with the Hardy-Weinberg equilibrium (HWE) with no significant differences.

Table (2): Hardy-Weinberg and Logistic regression analyses of *TLR4* gene SNPs in COVID-19 patients, recovered and healthy controls.

SNP	Allele/ genotype	Patient and recovered		Healthy control		OR	95% CI	*p*-value
		N=90	%	N=50	%			
rs4986790 A/G	A	99	55	67	67	Reference		
	G	81	45	33	33	1.66	1.0 – 2.76	0.057
	AA	26	30.3	22	44.9	Reference		
	AG	47	49.5	23	44.2	1.73	0.82 – 3.66	0.179
	GG	17	20.2	5	10.9	2.88	0.93 – 8.86	0.111
HWE-*p*-value		0.602		0.776				

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; OR: Odds ratio; CI: Confidence interval; p: the probability of Two-tailed Fisher's exact.

In this investigation, there was no significant difference in the frequency of the rs4986790 TLR4 polymorphism across the groups of COVID-19 patients based on clinical severity.

Table (3): Allele and genotype frequencies of *TLR4* gene SNP stratified by clinical severity in COVID-19 patients.

SNP	Allele/ genotype	Clinical severity				OR	95% CI	*p*-value
		Mild-moderate		Severe				
		N=30	%	N=30	%			
rs4986790 A/G	A	32	53.3	24	40	Reference		
	G	28	46.7	36	60	0.58	0.28 – 1.20	0.200
	AA	8	28.4	2	6.7	Reference		
	AG	16	49.8	20	66.7	0.2	0.04 – 1.00	0.074
	GG	6	21.8	8	26.9	0.19	0.03 – 1.12	0.104

SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval; *p*: Two-tailed Fisher's exact probability.

In recovered individuals with cognitive impairment, despite the occurrence of 50% cognitive impairment in the AG genotype, there is no significant difference compared to intact recovered individuals, as shown in Table (4).

Table (4): Allele and genotype frequencies of *TLR4* gene SNP stratified cognitive impairment in recovered individuals.

SNP	Allele/ genotype	Cognitive impairment				OR	95% CI	*p*-value
		Present (N=6)		Absence (N=24)				
		N	%	N	%			
rs4986790 A/G	A	7	58.3	36	75	Reference		
	G	5	41.7	12	25	2.14	0.59-7.79	0.293
	AA	2	33.3	14	58.3	Reference		
	AG	3	50	8	33.4	2.62	0.39-17.78	0.370
	GG	1	16.7	2	8.3	3.50	0.31–39.7	0.422

SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval; p: Two-tailed Fisher's exact probability; *p*: probability.

As a result, we found a statistically significant correlation (p=0.015) between age and severity, with people 50 years of age and older having a higher likelihood of developing severity. There is evidence linking age to the severity of COVID-19, but no significant difference(p=0.087) between males and females. According to another study, men typically had far more severe cases than women. Furthermore, a higher proportion of patients who passed away were older than those who lived. Nevertheless, several studies revealed that men and women were equally susceptible to SARS-CoV-2(7).

By this research, patients over 65 accounted for about 60% of COVID-19 deaths, even though 70% of suspected and confirmed cases involved people between the ages of 25 and 64. Additionally, patients in ICUs and CCUs accounted for about 50% of all deaths. Our findings showed that diabetes mellitus and cardiovascular diseases were associated with nearly half of the underlying non-communicable diseases (NCDs) and deaths related to them. Approximately 60% of deaths in a study by Hongdou et al. on 5139 infected patients occurred in those over 64 years old, although 80% of patients were between the ages of 25 and 64(8).

The course of the disease was found to be significantly predicted by the inflammatory markers ferritin, lactate dehydrogenase (LDH), and D-dimer level. Stress on the body causes systemic inflammation, which is indicated by an increased concentration of this inflammatory biomarker. In circumstances where there is more stress, like in critical and severe patient states, stress levels rise even more. This inflammatory biomarker thus indirectly represents the stress level of the body due to the severity of the disease. P<0.001 indicated that the differences were statistically significant. Ferritin levels rose from 304 ng/ml in the moderate state to 780 ng/ml in the severe state. comparing those who tested positive for COVID-19 with RT-PCR to those who tested negative we found significant increases in CRP, LDH, ferritin, and D-dimer levels. An earlier Italian study (9) found that people who tested positive for COVID-19 had significantly higher levels of CRP and LDH; these tests could be used in place of RT-PCR to identify COVID-19-positive patients. According to another study, the severity of the disease was assessed using CRP, LDH, ferritin, and D-dimer, with higher levels being linked to worse outcomes and mortality(10).

This study investigates the function of the TLR4 SNP (rs4986790) gene in patients with cognitive impairment, particularly in those who have recovered from SARS-CoV-2. The TLR4 SNP rs4986790 allele and genotype frequencies of the patient groups under study recovered, and a Hardy-Weinberg equilibrium (HWE) analysis revealed no significant differences in healthy control. Everyone has a chance of getting While the COVID-19 genotype does not prevent the disease from occurring, immunological factors like high TLR4 levels can, in certain circumstances, prevent the illness from occurring.

TLR4 rs4986790 as a protective factor against the severe course of COVID-19, Overall, the observed genotypes for *TLR4* rs4986790 were

consistent with HWE in patients with 'mild' (p = 0.20), 'hospitalized' (p = 0.16), 'severe' (p = 0.37) SARS-CoV-2 infection. The genotype distribution for all patients according to the severity of SARS-CoV-2 infection. Notably, we observed very similar rs4986790 G-allele frequencies (4.0 - 6.0%) in all groups except in patients with 'mild' SARS-CoV-2 infection (8.0%). We assessed whether carriers of the G-allele or the GG genotype might be better protected against the need for hospitalization or against severe or fatal disease outcomes. We found a significant association for protection in rs4986790 AG or GG genotype carriers comparing all patients ('hospitalized', 'severe', and 'fatal') with COVID-19 with those with 'mild' or asymptomatic SARS-CoV-2 infection (OR: 0.51, 95% CI: 0.34 - 0.77; p = 0.001)(11).

Regarding the AA genetic pattern, while no statistically significant differences exist among the groups under investigation, it should be noted that these are recessive patterns that increase susceptibility to infection, thereby lowering immunity. Of the two groups, the AA patients recovered 20% and the healthy control 13.7%. It might play a major role in promoting the likelihood that the infections will persist and the potential for cognitive impairment, and GG might be thought of as a protective factor if we examine more cases. Additionally, there were no discernible differences between COVID-19 patients in terms of clinical severity, although the proportions seemed similar. Perhaps if we collected more data, we could determine the disease's severity and look at age groups other than 45, as this could indicate a major role for this gene's heterogeneity in addition to other factors like age and chronic illness(12).

Our results are limited by the number of patients and SNPs examined, but our work has potential translation. This emphasizes the necessity of extending these trials to a larger group of people with varying degrees of cognitive impairment to inform future studies.

The current study found no statistically significant variation in genotype among recovered individuals with cognitive impairment, despite the AG genotype being present in 50% of the sample (six instances). A meta-analysis discovered a strong correlation between infectious diseases and the TLR4 polymorphism (rs4986790) (13). According to our research, there was a significant risk factor (OR = 1.79) for COVID-19 and ARDS associated with the G allele. A high risk of currently developing a severe disease was linked to the GG genotype (OR = 2.58), a finding that is confirmed by the recessive model (P = 0.012, OR = 2.60) (14).

While various aspects of COVID-19 have been covered in numerous local studies, no study has addressed this subject as thoroughly as (15, 16). Yet, this research is regarded as the initial one in the area.

Understanding the multi-organ dysfunction linked to post-COVID-19 complications is crucial, especially as the number of patients recuperating from COVID-19 increases. Notably, neurological symptoms in COVID-19 patients have been documented, indicating a strong association between COVID-19 and the eventual emergence of neurodegenerative illnesses. It has also been demonstrated that the severity of the SARS-CoV-2 infection and the

consequent rises, in particular, circulating inflammatory mediators and biomarkers are related to the risk of developing COVID-19-associated cognitive impairment and the degree of these deficits. Alternatively, some research has shown that cognitive dysfunction can occur regardless of the severity of the illness, with cognitive-associated post-COVID-19 symptoms developing in even non-hospitalized COVID-19 patients(17).

The heightened severity of COVID-19 lung disease in the elderly population may be due to age-dependent increases in SARS2-CoV-2 receptors in the respiratory epithelium. According to research reports, when comparing older children (10 to 17 years old) and adults (over 18) to younger children (less than 10 years old), the expression of ACE2 in the nasal epithelium was significantly higher in the former group (18).

Observing the IL-1βelevated level in the individuals who had previously contracted SARS-CoV-2, they examined the correlation between the IL-1βlevel and the post-COVID-19 syndrome, finding that it was unaffected by age or sex. Contrarily, regardless of the disease's severity, studies on the post-COVID-19 syndrome associated with cytokine elevation level estimation have shown that older age groups and female sex are more likely to develop post-COVID-19 syndrome (19).

Patients with COVID-19 were more likely to experience common symptoms like fever, coughing, and shortness of breath, and older patients were more likely to experience altered clinical symptoms. Fever (100%) and headache (100%) were the most common symptoms across the board. Other symptoms included lethargy (75%), hypoxia (85%), fatigue (90%), dry cough (45%), loss of appetite (30%), pneumonia (25%) and nausea (20%).In contrast to other patients, the oldest patients with the most severe conditions also had additional systemic diseases (20). In Iraqi society and during the coronavirus pandemic, we noticed the emergence of some cases after the COVID-19 infection, they suffer from cognitive impairment, as well as the lack of studies on polymorphism on the risk of COVID-19. Because the SARS-CoV-2 infection is decreasing in our population during the study period, the data in this study are limited in the numbering of samples. They may not provide clear knowledge about the TLR4 SNP rs4986790 role with and SARS-CoV-2 severity, although this study perhaps initially proven the correlation between cognitive impairment after SARS-CoV-2 infection with TLR4 SNP rs4986790.

Conclusion

Older people are more likely to experience more severe SARS-CoV-2 illness, those with chronic medical conditions, and those who have high viral loads (low Ct values) are risk factors that are positively associated with high D-Dimer, LDH, and ferritin concentration.

Also the results showed that in recovered individuals with cognitive impairment significantly associated with age older. On the other hand, despite the occurrence of 50% cognitive impairment in the AG rs4986790 genotype, there are no statistically significant between cognitive impairment and studied TLR4 SNPs.A/G rs4986790 of *TLR4* required more information to explore their role in cognitive impairment and infection severity

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Ethical responsibilities of authors

If you publish an article and there are reasons to believe that you could have used animals but did not, then you should write: "This article does not contain any studies involving animals performed by any of the authors."

Disclosure and conflict of interest

Conflict of Interest: The authors declare that they have no conflicts of interest.

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