Original Article

QUANTIFICATION AND METHYLATION STATUS OF FREE CIRCULATING DNA BASED BIOMARKER IN DIABETIC PATIENTS FOR THE EARLY DIAGNOSIS OF DIABETES MELLITUS THROUGH LIQUID BIOPSY

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ABSTRACT

Background: The prevalence rate of diabetes mellitus (DM) is becoming higher not only in developing countries but also worldwide. The commonly used tests for the diagnosis of DM lack standardization and sensitivity. DNA based free-circulating biomarkers are gaining attention as promising biomarkers for the diagnosis, prognosis, and progression of different diseases. In DM, β-cell destruction results in the release of free DNA into the plasma which can be quantified by different approaches. In our study, we have quantified and compared the level of total free circulating DNA and methylation status of preproinsulin (INS) DNA fragments levels in the plasma of diabetic patients and healthy controls.

Material and Methods: Our study included 45 diabetics and 45 age and sex-matched control individuals. We used Quantitative real-time PCR as a more reliable tool due to high specificity and sensitivity to determine the circulating copies of demethylated *INS*. The ROC curve was used to determine the sensitivity and specificity of the assay used for diagnosis purposes.

Results: In the plasma of patients, a relatively higher amount of free circulating DNA was observed as compared to the normal healthy person. ROC curve analysis showed strong discrimination potential of fcDNA concentrations for diabetic patient diagnosis with the area under the curve to be 0.794 (95% CI: 0.698-0.889; $P < 0.05$).

Conclusion: These assays may be used to detect the extent of destruction of β-cell death in DM and can give insights into progression and responses to the treatment used.

Key Words: DNA, Diabetes mellitus, Methylation, Preproinsulin

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INTRODUCTION

Diabetes mellitus has the highest rate of incidence and mortality globally and it will be the 7th dominant cause of death by the year 2030 as reported by the World Health Organization. It is reported that about 347 million people are suffering from diabetes around the globe.[1]

The prevalence rate of diabetes mellitus is the not highest in developing countries, but in the upcoming 25 years, they would encounter an elevation in the prevalence rate.[2] Asia has the leading prevalence rate.[3] In Pakistan, the incidence rate of glucose intolerance is approximately 22 % in urban areas and 17.1 % in rural areas.[4] The risk of diabetes onset increases two to six times in individuals with a family history of diabetes.[5] Various risk factors of type 2 diabetes include a family history of diabetes, overweight or obesity, unhealthy diet, high blood pressure, increasing age, physical inactivity, impaired glucose tolerance, and poor nutrition during pregnancy (International Diabetes

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Federation, 2001). Individuals who have stopped smoking possess the highest incidence rate to develop diabetes.[6] This is due to various factors such as lifestyle changes, genetics and environmental exposures. Growth hormones, cortisol, glucagon, epinephrine and some other hormones act in opposition to insulin activity.[7] diabetes mellitus is commonly diagnosed by different tests such as plasma glucose in fasting state, hemoglobin glycation and oral glucose tolerance test (OGTT).[8] OGTT possess some pitfalls such as complexity, lower reproducibility and high cost. It points out more individuals with diabetes than fasting glucose tests.[9] Tests employed for the determination of the type of diabetes or the assessment of the demand for insulin depend on the β-cell activity. These include C peptide levels and immune mediated β-cell devastation markers such as autoantibodies against pancreatic islet cells, glutamic acid decarboxylase, insulin levels and tyrosine phosphatase.[10] Antibody testing is limited due to availability, prognostic value and cost.[11] Cell free circulating DNA has become an attractive strategy for the detection of β-cell destruction in-vivo due to its ability to provide accurate and timely information about the health of cells. This strategy is gaining attention from scientists because it can be used to detect β-cell destruction without having to directly access the cells. It also offers a non-invasive approach, which makes it ideal for use in clinical settings. Furthermore, it can provide real-time data about the health of cells that can be used to inform treatment decisions and monitor disease progression.[12] Usually, free circulating DNA is secreted from the degraded cells and subsequently cleaved by endonucleases but the definite mechanism of release of free-circulating DNA is not obvious.[13] Endonucleases cut down the chromatin into smaller nucleosomal units.[14] Various clinical assays have been evolved for the detection of the destruction of cells in vivo which depend upon the identification of the nucleic acids, secreted by the lysed cells into the circulation.[15] Specific PCR dependent assays can be done to detect the presence of these molecules. Circulating DNA that is differentiated by the methylation pattern is used as a biomarker to identify the disease.[16] Then methylated and unmethylated positions can easily be differentiated. These positions can be quantified by PCR based assays and DNA sequencing.[17] A specific, sensitive and quantitative methylation specific PCR based assays are necessary for the detection of free circulating β-cell DNA.[15] Unmethylated circulating preproinsulin (*INS*) may be considered as a biomarker for β-cell death (15-16).[18] With the increasing prevalence of Diabetes mellitus, quantification of fcDNA and determination of methylation status of INS has become an attractive tool for screening and managing this disease. This method is important because it not only helps in early diagnosis but also provides valuable insight into the underlying causes and mechanisms of this chronic condition. Furthermore, it also helps in developing better strategies for treatment and monitoring. Thus, quantification of fcDNA and determination of methylation status of INS has become a crucial tool for screening and managing Diabetes mellitus.

MATERIAL AND METHODS

The cross sectional study was conducted in the year 2022 in the tertiary care teaching hospital in district Lahore. The population comprised 45 patients and 45 healthy controls with ages≥ 18 years and were permanent residents of Pakistan. The inclusion criteria for the patient series was a cytological or histological substantiated diagnosis of Type1 and Type2 Diabetes Mellitus as defined per WHO criteria.[19] The exclusion criteria were diagnosis of AIDS, pregnancy and cancer. A total of 45 control samples were selected by characteristics like the patients but having no clinical history of any type of diabetes mellitus. A 3ml peripheral blood sample from each patient and control in series was collected and immediately plasma was separated. DNA was isolated from the blood plasma using Abcam DNA Isolation Kit-Plasma/Serum

(ab156893) by following the instructions of the manufacturer.

The total concentration of fcDNA in both patients and healthy controls was determined by nanodrop spectrophotometer and the INS gene was used as a reference gene to measure the level of fcDNA in patients suffering from diabetes and healthy controls using real time PCR. After that, plasma fcDNA of all samples were subjected to bisulfite conversion by using EpiMark® Bisulfite Conversion Kit (# E3318S). This modified fcDNA was used as a template in quantitative PCR using primers reported in the literature. The conditions employed for thermal cycling were as follows: denaturation steps at 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15-sec denaturation, annealing at 55 º C for 30 sec and extension for 30 sec at 72 ºC. All samples along with positive controls (*in-vitro* methylated leukocyte DNA) and negative controls (normal leukocyte DNA) were used. The leukocyte DNA from healthy controls was methylated using M. SssI methyltransferase (Thermo Scientific, USA) to generate the positive control.

SPSS for windows (version 21.0, USA) was used for the statistical analysis of data. A 2-tailed *P* value < 0.05 was considered significant statistically and box plots were generated to represent results. The diagnostic performance of selected makers was assessed by the receiver operating characteristic (ROC) curve and the respective area under the curve (AUC). The characteristics like specificity and sensitivity values having 95 % confidence intervals (CIs) were evaluated for biomarkers.

RESULTS

The plasma concentrations of fcDNA in the patient, as well as control subjects, were determined spectrophotometrically at 260 nm by using nanodrop. It is noticeable from figure 1 that four-fold higher median concentrations of fcDNA of diabetics as compared to control subjects were found. This is statistically significant ($P < 0.001$). Significantly higher concentrations of free circulating DNA were observed in the patient series as compared to the levels in the control series but there are some individuals in the patient series in which the levels are comparatively low. In the patient series greater variability of free circulating DNA was perceived. In the majority of them, higher levels were observed. Whereas there are some subjects in the patient series who possess low levels of fcDNA as shown in figure 1.

Table-1. Clinicopathological characteristics of patients and healthy controls

	Patients (45)	**Control (45)**
Gender		
Male	24	24
Female	21	21
Type of Diabetes		
Type 1	26	-
Type 2	19	-
Age (Years)		
20 or less	1	1
21-30	2	2
31-40	8	8
41-50	14	14
51-60	10	10
60 or above	10	10
BMI		
1-18.5	2	2
18.5-22.9	8	8
23-24.9	8	8
Above 25	27	27

The diagnostic performance of concentrations of fcDNA was evaluated by ROC (Receiver Operating Characteristic) curve analysis. The competence of this assay to differentiate patients from controls was determined by AUC. Closer the value to 1, the greater the diagnostic power. The respective area under the curve was found to be 0.864 (95% CI, 0.786-0.942, $p<0.05$), as shown in figure 2. It depicts the strong discrimination potential of fcDNA concentrations for diabetic patient diagnosis. It has been observed that a lower cut-off value enhances the specificity of the assay but at the cost of the sensitivity and vice versa. So, to favor the sensitivity of the assay, we select 640 ng/ml. as this test is 1st level test so it further needs confirmation, this

value let us minimize the risk of false negatives.

Free-circulating DNA and INS concentration was significantly correlated with BMI (γ = 0.206;
$p < 0.05$). The amount of fcDNA increased as the BMI increases. A significant correlation also present with age of the subject (γ = 0.311; $p < 0.05$). As the age grows the fcDNA levels may elevate. But no significant relation was observed between the onset of disease and gender.

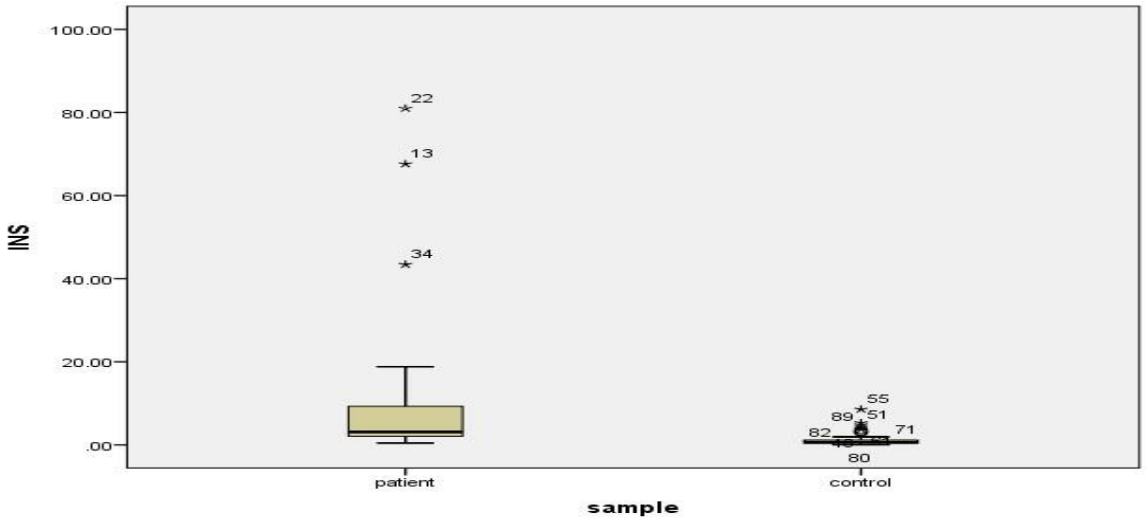

Figure-1: Box-Plot representation of fcDNA measured in ng/ml in diabetic patients and corresponding healthy controls by using *INS* as a marker. The upper and lower error bars show 90% of the values. The median is the solid line in the box

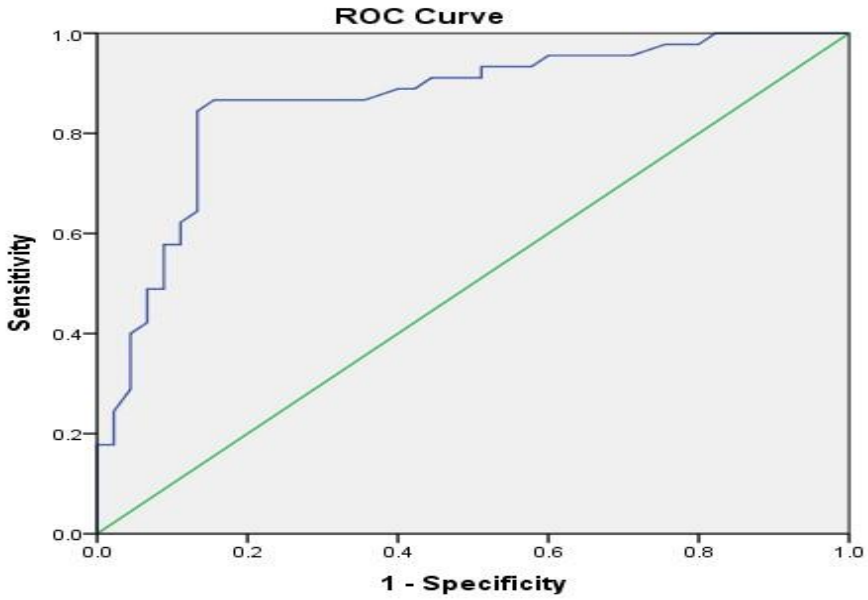

Figure-2: ROC curve for the diagnosis of Diabetes Mellitus using Plasma fcDNA concentrations

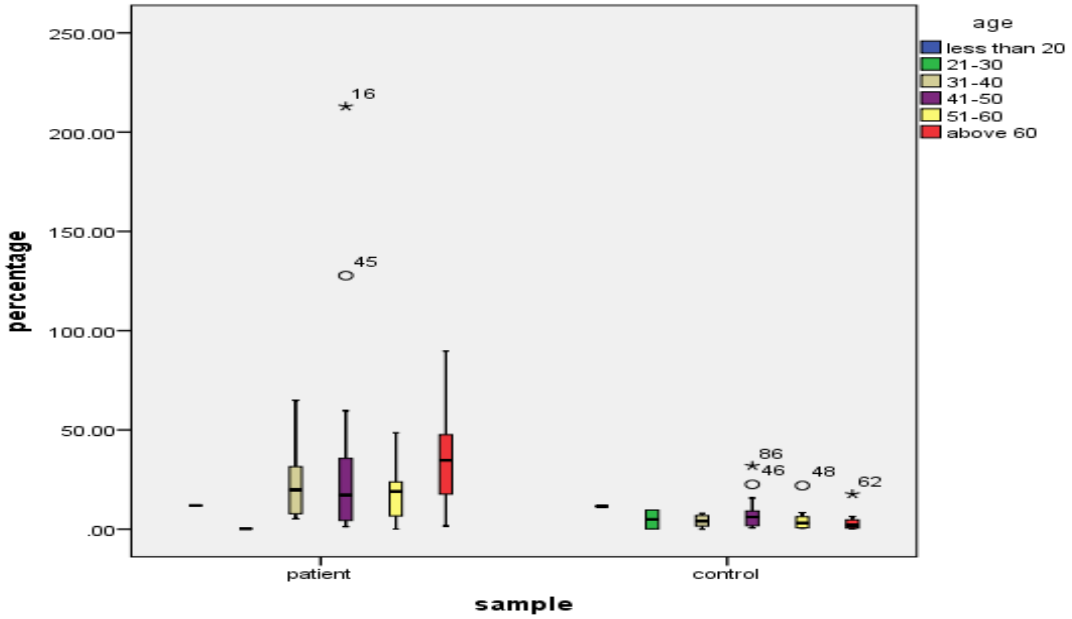

Figure-3. A) Correlation of fcDNA level in ng/mL with age in patients and healthy controls

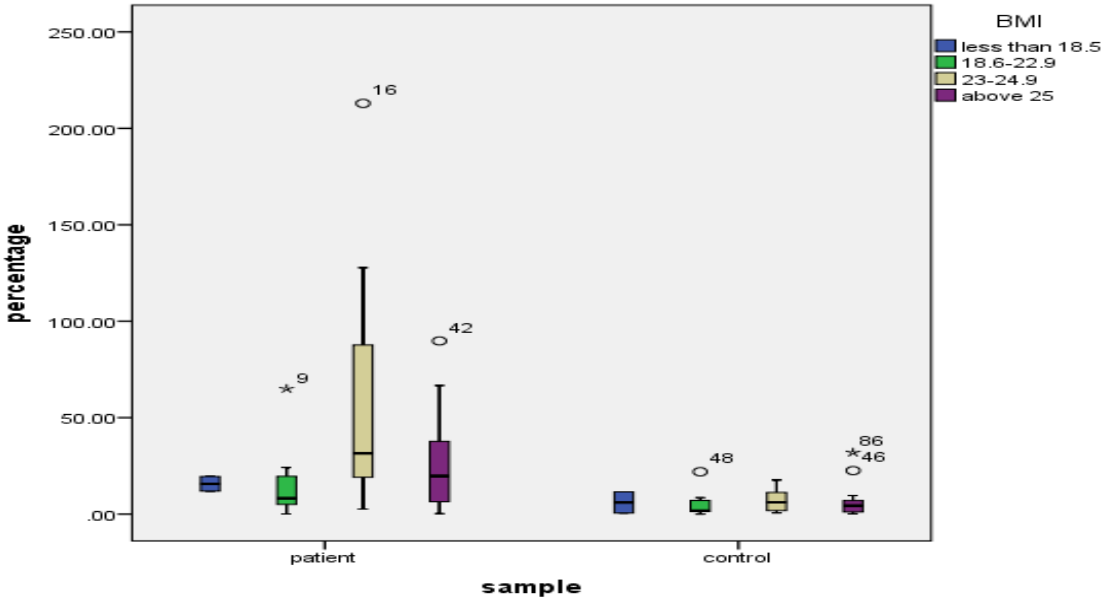

Figure-3. B) Correlation of fcDNA levels in ng/ml with BMI in patients and healthy controls.

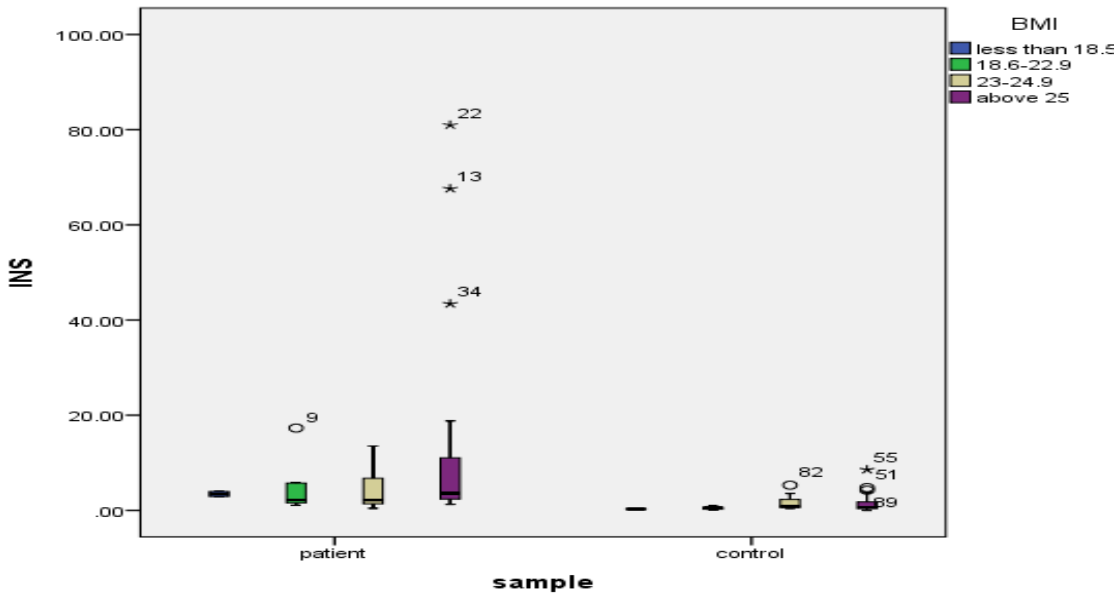

Figure-4: A) Correlation of INS DNA fragments levels in ng/ml with age in healthy controls and patients.

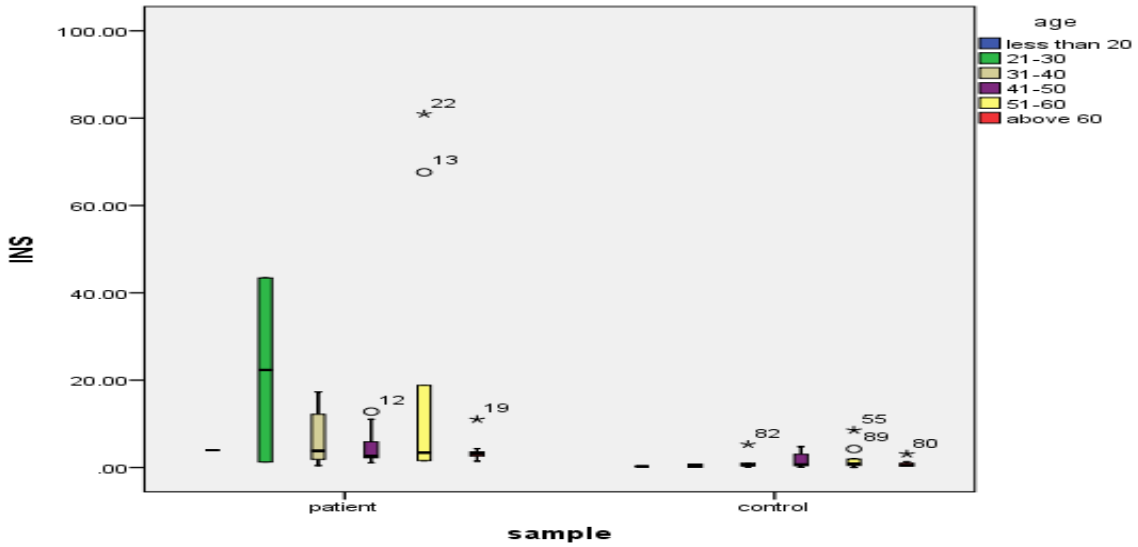

Figure-4: B) Correlation of INS DNA fragments levels in ng/ml with BMI in patients and controls

DISCUSSION

Diabetes is a growing health issue, and early diagnosis is key to managing it. Cell-free DNA based biomarkers are playing an increasingly important role in the early detection of diabetes through liquid biopsy. With quantification and methylation status analysis of cell-free DNA, it is possible to identify the genetic markers associated with diabetes in a patient's blood sample. This can help doctors detect the disease before it becomes more serious and provide more personalized treatments for each patient.[20] It is found that fcDNA levels in those with Type 2 Diabetes (T2DM) were significantly higher than those without, regardless of if they had any complications or not. This suggests that fcDNA can be a useful tool for personalizing the management of T2DM patients. Accordingly, fcDNA levels are positively correlated with adverse metabolic outcomes in T2DM patients with or without complications, suggesting that it is

appropriate for use as a biomarker for monitoring the effect of therapeutic interventions on reducing cardiovascular events in these patients. Different approaches have been employed for the quantitative analysis of fcDNA but the quantitative real time PCR assay has been the most reliable and promising due to its high specificity and sensitivity. We used Quantitative real-time PCR as a more reliable tool due to high specificity and sensitivity to determine the circulating copies of demethylated INS. The ROC curve was used to determine the sensitivity and specificity of the assay used for diagnosis purposes. In the plasma of patients, a relatively higher amount of free circulating DNA was observed as compared to the normal healthy person. Normal values of the ROC curve in this assay are indicated in the figure-2.

We chose to stick with the established methods of using serum samples for fcDNA isolation in our study since it is a popular approach for liquid biopsy. This gave us the ability to generate results that could be compared with other previous experiments. Moreover, we have successfully managed to reproduce quality results using relatively small serum volumes - something that has been a challenge in previous experiments with similar assays, indeed, the cancer field where these assays are most highly advanced is in many cases moving toward sample volumes an order of magnitude larger than those used here. Our findings from direct & indirect measurements showed that the quantity of the sample used was enough to carry out further analysis. Methylation is a cell-specific phenomenon, with genetic material being unmethylated in certain tissues. This profile will be preserved in the release of cfDNA from those tissues, making it possible to identify β-cells through this marker.

CONCLUSION

In our study, a four-fold higher concentration of fcDNA has been found in diabetic patients as compared to the control. A significant correlation of fcDNA was observed with body mass index. As the BMI increases, the amount of fcDNA also increases in diabetes mellitus. Similarly, a significant correlation of fcDNA was observed with age as age grows the level of fcDNA also elevated. So, the methylation-specific PCR assay for the INS gene can be helpful to detect beta cell death in diabetes and may provide an understanding of the progression of the disease.

Conflict of Interest

The author declares that there is no conflict of interest

AUTHOR'S CONTRIBUTION

ZK: Conceptualization and formal analysis
ZA: Data collection, formal analysis, methodology
NMA: Methodology writing
FA: Data collection and drafting of the article
ZK: Design of study and revision of the article
SF: Critical revision of the article

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