Association of complete blood count parameters with liver histology and atherosclerosis in non-alcoholic fatty liver disease

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ABSTRACT

Aims: We aimed to evaluate the relationship between complete blood count parameters, liver histology and atherosclerosis parameters in patients with non-alcoholic fatty liver disease (NAFLD).

Methods: We included the data of biopsy-proven 66 nonalcoholic steatohepatitis, 34 simple steatosis patients and 49 healthy controls to the study. Metabolic syndrome parameters data collected from all participants. Carotid intima media thickness, flow mediated dilatation, pulse wave velocity (PWV) were measured for atherosclerosis.

Results: There was no significant difference among groups in terms of red blood cell distribution width, mean platelet volume, and neutrophil to lymphocyte ratio parameters. Hemoglobin levels in control subjects were lower than NAFLD subgroups, but no significant difference was found between NAFLD subgroups. In correlation analysis, there was only positive association between hemoglobin and PWV.

Conclusions: High hemoglobin concentration may represent an increased risk of the development of NAFLD.

Key words: Steatohepatitis, Atherosclerosis, Hemoglobin

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to the fatty infiltration of liver parenchyma in the absence of significant alcohol intake. NAFLD is a term that contains both the situations of nonalcoholic steatohepatitis (NASH) and simple steatosis (SS). NASH briefly is an inflammatory dysmetabolic status. Increased hepatic insulin resistance leads to hepatic steatosis and then subclinical inflammation progresses from simple steatosis to hepatic inflammation and steatohepatitis (1). Attention of the clinicians is focused on distinguishing simple steatosis from NASH, because NASH can lead to liver degeneration and finally progressing to liver cirrhosis. Liver biopsy is still accepted as the gold standard test for diagnosis of NAFLD and assessment of fibrosis (2). The clinicians are spending great efforts to find a noninvasive indicator of fibrosis in patients with NASH because of the risk of procedure-related complications such as bleeding and patient discomfort (3).

Although several methods have been studied to detect the presence of fibrosis in NAFLD, there is no established non-invasive test instead of liver biopsy. In recent years, researchers have focused on the relationship between complete blood count (CBC) parameters and liver histology because they are cheap, non-invasive, and widely used laboratory markers that are easily accessible. The red blood cell distribution width (RDW) is one of the CBC parameters to represent the variability of the erythrocytes size, and used for classification of anemia in combination with mean corpuscular volume (MCV). In recent studies, RDW has been reported to be increased in myocardial infarction (4), heart failure, chronic kidney disease (5), liver fibrosis and liver diseases. Kim at al. also showed a graded association between high RDW and advanced liver fibrosis in patients with NAFLD (6).

The neutrophil to lymphocyte ratio (NLR) is an increasingly popular parameter in the literature (7). The absolute lymphocyte and neutrophil counts are also CBC parameters like RDW. Miscellaneous studies have been associated high NLR with severity and prognosis of hepatocellular carcinoma, pancreatic ductal adenoma, gastric cancer, non-small cell lung cancer, coronary artery disease and chronic kidney disease. However the results of the reports regarding NASH and NLR are conflicting (8–11). Another CBC parameter; mean platelet volume
(MPV) which is an indicator of the activated platelets, has been shown to increase in the initiation of cardiovascular events (12). This relationship is considered to depend on the increase in size and expansion of activated platelets. Several clinical studies have also examined the relationship between MPV and liver diseases. Although MPV was presented to predict the histological severity of NASH and other liver diseases, the results of these studies are still controversial (13,14).

It is well known that the presence of NAFLD is a risk factor for atherosclerosis (15, 16). There are a few non-invasive screening tests for the assessment of subclinical atherosclerosis, such as measurements of carotid intima media thickness (CIMT), flow-mediated dilatation (FMD), and arterial stiffness (AS). However, the measurement of these vascular parameters cannot be useful in clinical practice. In addition, above-mentioned CBC parameters were also used for the assessment of cardiovascular disease (CVD) risk in the previous studies.

In the present study, we aimed to evaluate the relationship between CBC parameters and liver histology in patients with biopsy-proven NAFLD. We also aimed to investigate whether there is a relationship between subclinical atherosclerosis indicators and CBC parameters in our population.

Methods

Study population

We designed a cross-sectional observational cohort study including the data of patients admitted to our inpatient and outpatient clinics between September 2014 and October 2015. We included 66 patients with NASH, 34 patients with SS and 49 healthy controls in the study. The diagnosis of NASH and SS were established by liver biopsy in all patients. Measurements of vascular parameters were performed to all participants. The following criteria were used to recruit patients with NAFLD in the study: ages between 20-40 with both sex, aminotransferase elevation for > 6 months, bright liver at ultrasonography examination with no other biliary tract pathology, and NAFLD findings on the liver biopsy. The characteristics of the exclusion criteria in the study were the presence of cardiovascular risk factors such diabetes mellitus, hypertension, chronic kidney disease, CVD, morbid obesity (BMI > 40 kg/m2), hyperlipidemia (a), alcohol consumption > 140g/week (b), and inflammatory causes of liver and other organs (c), exposure of hepatotoxins or hepatotoxic drugs (d).

Healthy control subjects were selected from the hospital staff and their acquaintances. Written informed consent was obtained from all participants before entering the study, and the study was approved by the local Ethical Committee.

Laboratory and clinical assessments

Medical history, smoking status, and drug use of participants were recorded. All participants’ height and weight measurements were done using the same weighing machine without shoes. Body mass indexes (BMI) were calculated (kg/m2). The measurement of waist circumference (WC) was performed at the mid-point between the lower rib and the iliac crest at the end of normal expiration. Blood pressure was measured according to the Royal College suggestions. All participants received a standard 75-g oral glucose tolerance test (OGTT). The serum glucose measurements were made at 0 and 120 min. Venous blood samples were taken for laboratory tests including aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), fasting plasma glucose (FPG), fasting insulin, uric acid, high density lipoprotein (HDL), triglycerides (TG), creatinine, and high sensitive c-reactive protein (hs-CRP). The HOMA index rate (HOMA-IR) was calculated by the formula: fasting glucose (mg/dl) x fasting insulin (mU/ml)/405.

CBC was performed using an automated blood cell counter (ABX Pentra 120, Horiba, Japan). If all the participants did not have a disease such as infection, inflammatory disorders, and bleeding within the last 6 months, the mean of 3 measurements of CBC was used within 3 months from the date of liver biopsy. However, the mean of 2 measurements was used in the same patients. NLR was calculated using the formula: absolute neutrophil count divided by the absolute lymphocyte count.

Atherosclerosis parameters

The intima-media thickness measurements were performed on all patients using B-mode ultrasonography (Siemens, Acuson S3000, Germany) utilizing a high-resolution, 18-MHz linear-array transducer. The measurements were carried out, while the subject was in a supine position and with the head rotated contralateral to the examination in a dark, quiet room by the same radiologist blinded to the participants’ clinical characteristics. The region for the measurement of IMT for common carotid arteries (CCA) was selected as 1 cm proximal to the CCA bifurcation. Longitudinal static images were analyzed using semi-automated software (Synyo Arterial Health Package). The transducer was manually placed on a 1 cm segment of the region and then IMT was automatically measured by calculating the distance between the lumen-intima and the media-adventitia interfaces in the far wall of the region. If the operator decided that the measurement was not appropriate, the procedure was repeated. The CIMT value was defined as the average of the left and right common CIMT.

FMD measurements were made with 18-MHz linear-array transducer on M mode (Siemens, Acuson S3000) by the same physician who was blind to patients’ clinical status. The measurements were made in a controlled environment in supine position after minimum 4-h fasting. After the cuff was placed at the right upper arm, the first baseline arterial diameter was measured. A 3-minute period of forearm blood flow occlusion was provided by inflating a cuff 200 mmHg. Arterial diameter was measured again 1 minute, after the cuff was deflated. FMD at 1 minute post ischemia was calculated (100 x Diameter(1 min) - Diameter(basal)/Diameter(basal)). The limit for endothelial dysfunction was defined as FMD < %10.

Pulse wave velocity measurements (PWV) were performed for each subject in a reserved quiet room with an automatic arteriography device (TensioMed Ltd., Budapest, Hungary) by the same experienced internist who was blind to participants’ clinical characteristics. Measurements were made after resting for 5 min in supine position. Participants were not allowed to consume any food or drink and did not smoke for at least the 30 min before the measurement process. The distance between the jugular notch and symphysis pubis was measured. The data was entered to the device before the process.

Histopathology and Fibrosis Scores

A gastroenterologist performed all the liver biopsies with an ultrasonography guide. Specimens’ lengths were minimum 2 cm and contained at least 8 portal tracts. All the biopsy samples were examined in detail and scored by the same experi-
enced pathologist who was blind to patients. The classification of Kleiner et al. was used for scoring the degree of liver injury, inflammation and fibrosis (steatosis 0-3; lobular inflammation 0-3; ballooning hepatocyte degeneration 0-2) (17). The NAFLD activity score (NAS) was used for grading the histopathology features and ≥5 was defined as NASH. The stage of fibrosis was graded using a 6-point scale as: 0 no fibrosis, 1a, b mild (1a)/moderate (1b) zone 3 and perisinusoidal fibrosis; 1c portal fibrosis only; 2 zone 3 and portal/periportal fibrosis; 3 bridging fibrosis; 4 cirrhosis.

Statistical analysis

SPSS (Statistical Package for the Social Sciences ver. 17.0, SPSS Inc, Chicago, IL, USA) computer program was used for all statistical calculations. Results were reported as the mean±standard deviation (SD), frequency and percentage. The Kolmogorov-Smirnov test was used to determine the distribution characteristics of continuous variables. The normally distributed variables were compared with one-way ANOVA for multiple group comparisons and a Tukey’s post hoc test. The Kruskal-Wallis test was used for multiple-group comparisons of variables without normal distribution, and a Bonferroni-adjusted Mann-Whitney U test was used for post hoc analysis. Categorical variables were compared by chi-square or Fisher’s exact test. Student’s t test was used for binary comparisons of continuous variables. Pearson correlation analysis was used to evaluate the relationship between continuous variables. Statistical significance was defined as p<0.05.

Results

The main clinical and laboratory data of our study were summarized in Table 1. Among 100 patients; 66 patients had NASH, 34 patients had only SS. While gender and smoking status were similar among groups, the mean age in controls was lower than both the NASH and SS groups. The parameters which are also the part of metabolic syndrome criteria; WC, BMI, SBP, DBP, TG, LDL, OGTT, HOMA-IR were significantly lower in the control group (p<0.001). There was no significant difference between the NASH and SS groups in these parameters. CRP levels were not different between all groups.

The PWV, which was one of our arterial stiffness parameters, was lower in the control group, but it was not statistically different between NAFLD subgroups. While CIMT levels in patients with NASH were higher than both SS patients and controls, no significant difference was observed between patients with SS and control subjects. FMD levels in patients with NASH were lower than control subjects, but no significant difference was found in the other comparisons of FMD.

While white blood cell counts and lymphocyte counts in the controls were lower than the patients with NASH and SS, there was no significant difference among groups in terms of RDW, MPV, NLR parameters. And also when NASH patients were compared with SS patients, no significant difference was observed in these parameters. Hemoglobin levels in control subjects were lower than NAFLD subgroups, but no significant difference was found between NAFLD subgroups. In addition, NAFLD patients were divided into two groups according to the presence of fibrosis. No significant difference was observed between liver histology and CBC parameters.

In correlation analyses, hemoglobin levels were positively correlated with BMI, SBP, DBP, AST, ALT, GGT, uric acid, and LDL. Hemoglobin levels were negatively correlated with HDL levels. There was also a positive correlation between hemoglobin and PWV (Figure 1), but not with FMD and CIMT. While NLR was correlated with DBP, no significant correlation was found between other parameters (Table 2). In multiple regression analysis, the association between cf-PWV and hemoglobin did not remain significant after adjusting for the confounding variables such as BMI, blood pressure, FPG, and lipids.

Discussion

To date several clinical studies have investigated whether any of CBC parameters was associated with various disease and may be an indicator in different pathological conditions. Among parameters such as RDW, MPV and NLR has come to the forefront. We think that the main reason for the popularity of these parameters are due to being cheap, easily accessible, and widespread use of CBC. However, authors used only one of the one of these parameters in their study. In this context, we think that the present study is one of the most comprehensive studies because of examining the association with all the parameters of CBC. Small but important differences can be observed in each measurement of CBC parameters. The parameter levels can vary even if we measure the same sample again. Therefore we took the mean of 2 or 3 measurements of CBC within 3 months from the date of liver biopsy in the absence of any infection and inflammatory disorder within last 6 months. We think that this attitude makes our study more valuable in the literature (18).

In the present study, we detected significantly higher CIMT, cf-PWV levels and lower FMD levels in NAFLD group than the controls. This results are compatible with the previous studies (19). This once again shows that NAFLD patients have higher atherosclerosis and cardiovascular event risk compared with healthy population. However, there was no difference between NASH and SS groups in the subclinical atherosclerosis parameters. Indeed, several clinical studies and our previous studies showed that patients with NASH have higher atherosclerosis risk compared to patients with SS. This can be explained by the younger study population in the present study.

Although many studies have reported the relationship between NAFLD and CBC parameters, the results are still debated. Cengiz et al. reported significantly higher RDW levels in biopsy proven NASH than both the control subjects and the patients with SS unlike MPV and NLR (20). They suggested
Table 1. The clinical, biochemical and radiological characteristics of study population

<table>
<thead>
<tr>
<th>Variables</th>
<th>NASH (n=66)</th>
<th>SS (n=34)</th>
<th>Controls (n=49)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>33.5±6.3</td>
<td>34.9±6</td>
<td>30.1±5.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>38.7</td>
<td>38.2</td>
<td>24.5</td>
<td>0.062</td>
</tr>
<tr>
<td>Male (%)</td>
<td>92.4</td>
<td>88.2</td>
<td>81.6</td>
<td>0.239</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>102.6±7.2</td>
<td>99.1±9.1</td>
<td>85.5±10.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.8±2.9</td>
<td>28.8±3.3</td>
<td>23.3±2.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132.6±12.5</td>
<td>127.4±9.4</td>
<td>115.4±10.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.3±8.2</td>
<td>78.7±6.9</td>
<td>68.9±7.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-CRP (nmol/L)</td>
<td>42.8±52.4</td>
<td>48.6±43.8</td>
<td>43.8±48.6</td>
<td>0.937</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>66.5±44.1</td>
<td>47.5±18.7</td>
<td>20.8±4.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>118.1±52.7</td>
<td>93.5±42.3</td>
<td>19.9±8.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>71±63</td>
<td>74.5±59.2</td>
<td>21.1±9.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FPG (mmol/L)</td>
<td>5.67±1.26</td>
<td>5.52±0.54</td>
<td>5.01±0.41</td>
<td>0.001</td>
</tr>
<tr>
<td>2-h OGTT (mmol/L)</td>
<td>6.5±2.02</td>
<td>5.6±2.08</td>
<td>5±0.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>77.7±7.63</td>
<td>79.3±7.63</td>
<td>73.2±7.63</td>
<td>0.012</td>
</tr>
<tr>
<td>Uric acid (µmol/L)</td>
<td>404.5±71.4</td>
<td>398.5±71.4</td>
<td>309.3±71.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.23±0.78</td>
<td>3.45±1.02</td>
<td>2.66±0.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.02±0.21</td>
<td>1.17±0.27</td>
<td>1.25±0.33</td>
<td>0.01</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>2.2±1.22</td>
<td>2.33±1.33</td>
<td>1.35±0.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>151.4±134.7</td>
<td>131.3±84.7</td>
<td>70.1±38.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.5±5.6</td>
<td>4.7±3.5</td>
<td>2.2±1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NAS (0-8)</td>
<td>5.4±0.8</td>
<td>3.2±0.6</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrosis (0-4)</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cf-PWV (m/s)</td>
<td>8.6±1.4</td>
<td>8.5±1.2</td>
<td>7.2±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CIMT (mm)</td>
<td>0.464±0.08</td>
<td>0.412±0.07</td>
<td>0.410±0.07</td>
<td>0.001</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>10.6±7</td>
<td>12.7±7.3</td>
<td>15.1±10.1</td>
<td>0.039</td>
</tr>
<tr>
<td>HBG (gr/L)</td>
<td>159±9</td>
<td>158±7</td>
<td>147±13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.5±2.5</td>
<td>15.3±2.6</td>
<td>15.1±1.7</td>
<td>0.218</td>
</tr>
<tr>
<td>PLT (10⁹/L)</td>
<td>251.9±46.5</td>
<td>252.8±43.1</td>
<td>244.8±55.9</td>
<td>0.688</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>9.4±1.1</td>
<td>9.5±1.3</td>
<td>9.1±1</td>
<td>0.236</td>
</tr>
<tr>
<td>WBC (10⁹/L)</td>
<td>6.5±1.2</td>
<td>6.9±1.4</td>
<td>6.1±1.3</td>
<td>0.004</td>
</tr>
<tr>
<td>Lymphocyte (10⁹/L)</td>
<td>2.3±0.5</td>
<td>2.4±0.5</td>
<td>2±0.5</td>
<td>0.014</td>
</tr>
<tr>
<td>NLR</td>
<td>1.7±0.5</td>
<td>1.7±0.5</td>
<td>1.8±0.5</td>
<td>0.768</td>
</tr>
</tbody>
</table>

SS simple steatosis, NASH steatohepatitis, WC waist circumference, BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, hs-CRP high sensitive c reactive protein, AST aspartat aminotransferase, ALT alanine aminotransferase, GGT γ-glutamyltransferase, FPG fasting plasma glucose, OGGT oral glucose tolerance testing, LDL-C low density lipoprotein cholesterol, HDL-C high density lipoprotein cholesterol, TG triglyceride, HOMA-IR homeostasis model assessment of insulin resistance, NAS NAFLD activity score, cf-PWV carotid femoral pulse wave velocity, CIMT carotid intima-media thickness, FMD flow mediated dilatation, HBG hemoglobin, RDW red cell distribution width, MPV mean platelet volume, WBC White blood cells, NLR neutrophil-lymphocyte ratio.
that RDW can be used to demonstrate the presence of NASH and to indicate advanced fibrosis. Alkhour et al. presented NLR as a novel marker for predicting steatohapatitis in patients with NAFLD, but there was no data about RDW or MPV in their study (8). In another study, Abdel-Razik et al. reported a multicenter study and they claim MPV, NLR as a novel marker of steatohapatitis in NAFLD patients (11). Yilmaz et al. found significantly higher NLR in NASH patients than the controls. They pronounce that NLR is a better predictor than CRP for liver fibrosis (9), whereas a previous study reported that NLR is not a predictor for liver fibrosis (10). In our study, we found that RDW, MPV, and NLR are not associated with liver histology findings including fibrosis and NAS in patients with biopsy-proven NAFLD. Additionally, we also investigated the relationship between CBC parameters and vascular parameters in our study. We have demonstrated that RDW, MPV, and NLR parameters are not correlated with atherosclerosis parameters in patients with NAFLD. Our results have some important strengths such as presence of liver histology findings, measurement of three different measurements of CBC parameters, measurement of vascular parameters, and exclusion of CVD and other confounding diseases. Therefore, we think that our findings can be more valuable than the recent studies.

In the present study, hemoglobin levels in the NAFLD group were significantly higher than the healthy controls. Nevertheless, no statistically difference was found between NASH and SS in the hemoglobin levels. This finding suggest that while hemoglobin levels can play a role in development of NAFLD, it is not associated with progression to steatohapatitis. Indeed, these findings are not surprising when compared to previous studies. Recently, population-based studies reported that subjects with higher baseline hemoglobin levels were associated with a higher incidence of NAFLD (21,22). In a previous study that analyzed serum samples using SELDI-TOF-MS and bioinformatics tools and established a serum protein finger print diagnostic model for NAFLD, Yu et al reported that serum hemoglobin level independently predicts the development of NAFLD. They suggested that it may serve as a biomarker for the disease (23). Moreover, we reported that hemoglobin levels are associated with metabolic syndrome parameters and arterial stiffness. A novel finding in this study is demonstrating a relationship between hemoglobin concentration and arterial stiffness in general population and chronic renal disease (24,25). However, the underlying mechanism that leads to increased arterial stiffness in individuals with elevated hemoglobin levels are not fully understood. Nevertheless, some authors claimed that this association will be explained with blood viscosity (26), decreased adiponectin levels in elevated hemoglobin levels (27), LDL oxidation by hemoglobin derived iron (28), and the effect of hemoglobin concentration on insulin resistance (29). In fact, the association between arterial stiffness and hemoglobin did not remain significant after adjusting for metabolic syndrome parameters in our study. It is well known that metabolic syndrome is a risk factor for atherosclerosis. Moreover, it can be also common pathologic pathway for both development of NAFLD and elevated hemoglobin levels. Chung et al. newly reported in accordance with our hypothesis that the risk of developing either metabolic syndrome or NAFLD was significantly associated with serum high hemoglobin levels in men (30). Therefore, the lack of association between arterial stiffness and hemoglobin levels after multivariate analysis can be explained with metabolic syndrome as a common pathologic pathway. On the other hand, researchers have focused that phlebotomy will improve the liver histology in patients with NAFLD. Unfortunately, in a recently published study, Adams et al reported that reduction in ferritin by phlebotomy dose not improve liver enzymes, hepatic fatty, or metabolic syndrome parameters in subjects with NAFLD (31). Therefore, furt-
ther prospective studies are warranted to clarify the underlying pathological mechanisms.

In conclusion, our data demonstrated that NLR, MPV, and RDW is not associated with liver histology and atherosclerosis in patients with NAFLD. In addition, high hemoglobin concentration can be a risk factor for the development of NAFLD. Hemoglobin concentration is associated with arterial stiffness, but this association depends on metabolic syndrome parameters.

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KO designed and directed the study. OK collected the data and contributed the writing of the manuscript. HD contributed the acquisition of data. TD performed the pulse wave velocity measurements. AO performed the carotid intima media and flow mediated dilatation measurements. MC performed the pulse wave velocity measurements. YSS contributed the acquisition of data. GT contributed the acquisition of data. MK analysed the study results. GK contributed the revision of manuscript. AU reviewed the study before the submission. This research did not receive any specific grant from funding agencies.

**Conflict of Interest**

The authors do not have anything to disclose regarding conflict of interest with respect to this manuscript.

**References**


