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Oxidative stress and metabolic parameters in hypertensive patients with/without diabetes mellitus in cardiovascular risk evaluation

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Abstract

Introduction: *Hypertension and diabetes mellitus affect a large number of patients and can significantly influence their life expectancy. Changes in metabolic and oxidative stress parameters are common in these pathologies, contributing to associated complications. The aim of the study was assessment of relationship between laboratory parameters and their role in evaluation of cardiovascular risk, and possible gender-related differences in the protective factors.* ***Material and methods****: Blood samples were collected from hypertensive patients with/without diabetes mellitus admitted to the Cardiovascular Rehabilitation Clinic in Tîrgu Mureș and controls without these pathologies. Biochemical analyses were performed on Konelab analyzer (glycemia, lipid profile, kidney function tests, zinc, hsCRP). Oxidative stress markers, such as serum malondialdehyde (MDA), oxidized (GSSG) and reduced glutathione (GSH) were evaluated using an HPLC-UV/VIS technique at GEP UMPhST. Statistical analysis was performed by GraphPad InStat3.* ***Results****: Mean age of hypertensive patients (n=131) was 69.44 ± 9.02 years, 45.8% males, 31.3% being diabetics. 74.1% of the studied patients had zinc deficiency, 19.8% presented slightly elevated hsCRP. The control group included 24 nonhypertensive/nondiabetic patients of similar age. Average GSH was significantly lower (p=0.0002) in hypertensive patients, 1.89 ± 0.82 µg/ml, compared to the control group (3.23 ± 0.49 µg/ml), and no correlation could be observed between GSH and MDA values. GSH concentration was significantly higher in males (p=0.0395) and HDL-cholesterol significantly higher in females (p=0.0132). A negative correlation was observed between serum triglyceride and HDL-cholesterol concentration.* ***Conclusions****: Gender differences are present in the level of protective factors against cardiovascular diseases, while oxidative stress is intensified in hypertensive/diabetic patients.*

Keywords*: hypertension, diabetes mellitus, oxidative stress markers, cardiovascular complications, HPLC*

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Introduction

The incidence of hypertension and diabetes mellitus is increasing worldwide, these pathologies, together with obesity, often coexisting in the same patient (1,2). According to statistics, patients older than 55 years have 90% chance to develop hypertension (3). Insulin resistance is more frequent in hypertensives and the incidence of hypertension is double in diabetic subjects compared to non-diabetic ones (4). Cardiovascular complications of these population-level diseases have negative impact on the patients' state of health, thus comprehensive risk assessment is an important part of their management (3,5). Atherosclerosis is the main pathological process in the case of macrovascular manifestations. Microvascular complications occur at the level of different organs (kidneys, eyes, brain), early diagnosis being essential, these being major causes of morbidity and mortality (4). Chronic inflammation and oxidative stress have a major contribution to the development of macrovascular and microvascular diseases in hypertensive patients with or without diabetes mellitus (6,7,8,9).

Often the evolution of microvascular complications, such as retinopathy or nephropathy, might be silent in hypertensive patients (10). Serum cystatin C is an early marker of nephropathy, useful in the investigation of hypertensive and diabetic patients (11,12), which is not used as a routine test in Romania.

Microvasculopathy is even more severe in case of hypertensive patients having diabetes mellitus as a comorbidity, carbohydrate metabolic imbalance being an essential factor in the development and progression of nephropathy and retinopathy (13). There is evidence that reactive oxygen species also have an important contribution to endothelium dysfunction in the microvascular system (14).

Antioxidant vitamins and minerals, such as magnesium, selenium, chromium, zinc have an important contribution to proper antioxidant status, being benefic in cardiovascular risk prevention, some of these minerals being involved in the metabolism of glutathione (15,16,17). Besides measurement of minerals and vitamins, several biomarkers can be used for the assessment of cardiovascular risk: high sensitive C reactive protein (hsCRP), HDL-cholesterol and oxidative stress parameters like malondialdehyde (MDA), hydroxynonenal, oxidized/reduced glutathione ratio, nitrotyrosine, oxidized LDL-cholesterol and the level of certain antioxidants (18,19).

The aim of the study was a complex laboratory investigation of hypertensive patients with/without diabetes mellitus, including parameters revealing cardiovascular risk, and the investigation of possible gender-related differences in the protective factors against cardiovascular diseases. The relationship between different laboratory findings and their role in the evaluation of cardiovascular risk have been assessed.

Material and methods

The study group consisted of hypertensive patients with/without diabetes mellitus and a control group of similar age without these pathologies, admitted to the Cardiovascular Rehabilitation Clinic in Tîrgu Mureș. The research was approved by the Ethical Committee of the Emergency Clinical County Hospital and the Ethical Committee of the ”GE Palade” UMPhST. Inclusion criteria in the study group were: age over 50 years, documented essential hypertension (at least stage 2) with or without type 2 diabetes mellitus, with good metabolic balance and willingness to participate. Exclusion criteria were: secondary hypertension, stage 1 hypertension, depression, organ failure, acute coronary syndrome, type 1 diabetes mellitus, severe metabolic imbalance (glycated hemoglobin exceeding 9%). The patients signed the informed consent document prior to the enrollment. The research was a prospective, transversal study. Demographic (age, gender) and anamnestic data were collected, systolic and dyastolic blood pressure and body mass index (BMI) values were recorded. Blood samples were taken in vacutainers containing clot activator. After centrifugation and separation, serum samples were stored at -70 ^{o}C prior to processing. The following parameters were determined from the serum samples: triglycerides, total and HDL-cholesterol (LDL cholesterol was calculated based on the previous results), glucose, creatinine, cystatin C, zinc, hsCRP. Oxidative stress markers, such as serum malondialdehyde (MDA), oxidized (GSSG) and reduced glutathione (GSH) were also measured.

Measurement of serum malondialdehyde was performed by a new, validated method of our research group, on a Merck-Hitachi HPLC system, consisting of a quaternary L-7100 pump, L-7200 injector, L-7360 column thermostat, D-7000 interface, solvent degasser L-7612 and Diode Array Detector L-7455. Stationary phase was Supelcosil ™ LC-18 (3 μm) SUPELCO 3,3 cm x 4,6 mm column. 20 mM phosphate buffer (pH=6) and acetonitrile (VWR International SAS) served as mobile phase, while 98% thiobarbituric acid (TBA) (Sigma-Aldrich) was used for complex formation with MDA, 96% H_2SO_4 (Chemical Company) and 99% 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) stock solution for preparation of MDA standards. Protein precipitation was made by adding acetonitrile to the serum samples in a 3:1 volume ratio, after

centrifugation at 5000 rpm, 300 µl supernatant was treated with 450 µl TBA (4 mg/ml) and 750 µl H_2SO_4 (50 mM). After vortexing, the mixture was heated to 100˚C for an hour followed by cooling on ice and trichloroacetic acid was added to the samples in 1:3 volume ratio. After centrifugation at 5000 rpm, the samples were transferred into HPLC vials and analyzed by the HPLC UV/VIS equipment. Measurement conditions were: isocratic elution, flow rate 1 ml/min, injection volume 100 µl (loop mode); diode array detection was made at 532 nm, retention time of MDA being 0.63 minutes and total analysis time 1 minute (20).

Oxidized (GSSG) and reduced glutathione (GSH) measurement was performed using Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid), which forms with GSH a dimer with UV absorbance. The same Merck-Hitachi HPLC system was used for this measurement. Zorbax Eclipse XDB-C8, 5 µm, 4,6x150 mm column served as stationary phase and the mobile phase was a mixture of phosphate buffer (20 mM, pH 2.5) and acetonitrile (VWR International SAS). Glutathione standards and Ellman's reagent were provided by Sigma Aldrich, and trichloroacetic acid (Chemical Company) was used for protein precipitation. Defrosted serum samples were treated with Ellman's reagent in 1:1 ratio. After deproteinization (using trichloroacetic acid and centrifugation), the supernatant was transferred into HPLC vials. Samples were boiled for 30 minutes before deproteinization in the case of GSSG measurement to reduce GSSG to GSH. Measurement conditions were: flow rate 1 ml/minute, injection volume 50 µl (cut mode), DAD range 200 - 700 nm, with the best chromatogram extracted at 330 nm, retention time of GSH-DTNB being 9.11 minutes.

Biochemical tests were performed on Konelab analyzer, using Diagnosticum LTD reagents. Lipid profile included: total cholesterol, HDL-cholesterol and triglyceride concentration measured by enzymatic methods, and LDL-cholesterol calculated using the Friedewald formula. Glycemia was measured by glucose oxidase method. High sensitive CRP (hsCRP) was determined by turbidimetry. Kidney function tests included serum creatinine (by Jaffé method) and cystatin C measurement (by turbidimetry). A photometric procedure (Sentinel Diagnostics kit) was applied for serum zinc concentration measurement. In diabetic hypertensive subjects glycated hemoglobin (HbA1c) was measured by reflectometry, using a NycoCard™ Reader II equipment (Axis-Shield) and NycoCard HbA1c kit (Abbott).

GraphPad InStat3 software was used for statistical processing of the data (unpaired two-tailed Student's t-test with and without Welch correction, Pearson correlation). Kolmogorov-Smirnov normality test was used for checking the Gaussian distribution of the data. The threshold of significance was set at $p<0.05$.

Results

The average age of the patients included in the study group was 69.44 ± 9.02 years, ranging between 50 and 89 years, the difference being insignificant ($p>0.05$) compared to the mean age of the control group, 68.50 ± 7.07 years (n=24).

The study group contained 131 hypertensive patients, 41 of them (31.3%) also having type 2 diabetes mellitus. The gender distribution was 71 females (54.2%) and 60 males (45.8%), similar to that of the control group (half females, half males).

Results regarding average blood pressure, body mass index, glycemia, triglyceridemia, serum total and LDL-cholesterol concentration in the study group are shown in **Table 1**.

Overweight and obesity were quite frequent in the study group, 74.8% of the enrolled patients having BMI exceeding 25 kg/m^2, 38.9% of the subjects being obese (having BMI over 30 kg/m^2).

Serum triglyceride concentration was higher

Table 1. Blood pressure, BMI and metabolic parameters in the study group

Parameter	SBP	DBP	BMI	SGL	STC	STG	LDL
Units	mmHg	mmHg	kg/m^2	mmol/l	mmol/l	mmol/l	mmol/l
Mean	137.89	80.78	31.02	5.93	4.79	1.54	2.14
± SD	19.11	10.05	6.66	1.42	1.39	0.80	1.56

SBP - Systolic blood pressure; DBP - Dyastolic blood pressure; SGL - Serum glucose level; STC - Serum total cholesterol; STG – Serum triglycerides

than normal (exceeding 1.7 mmol/l) in 31.3% of the hypertensive patients with/without diabetes mellitus. 33.6% of the studied patients presented hypercholesterolemia (values over 5.2 mmol/l). Fasting serum glucose was increased (exceeding 5.5 mmol/l) in 50.4% of the subjects. There is a weak, positive, non-significant correlation (r=0.2538, p=0.0724) between glycemia and BMI. No correlation was found between BMI and triglyceridemia (r=0.1835, p=0.2020) or between BMI and cholesterolemia (r=-0.1463, p=0.3058) using Pearson's test.

Diabetic hypertensive patients had significantly higher (p<0.0001) serum glucose values, 6.63 ± 1.56 mmol/l, compared to non-diabetic subjects, 5.17 ± 0.57 mmol/l, using unpaired t-test with Welch correction. The average value of glycated hemoglobin was 6.38 ± 0.80 % in the diabetic hypertensive patients, and a moderate positive correlation was found between HbA1c and fasting serum glucose values (r=0.5608, p=0.0007) in this subgroup using Pearson's test. 66% of the diabetic subjects enrolled in the study had good carbohydrate metabolic balance (HbA1c<6.5%). There was a significant difference (p=0.0013) between serum triglyceride concentration between diabetic (1.93 ± 0.99 mmol/l) and non-diabetic (1.41 ± 0.76 mmol/l) hypertensive patients using unpaired t-test with Welch correction. The difference was not significant (p=0.0646) between the serum cholesterol values of these two subgroups: the average was 5.06 ± 1.37 mmol/l in the non-diabetic subgroup, slightly higher than in the subgroup of diabetic hypertensive patients, 4.62 ± 1.29 mmol/l, using unpaired t-test. The average of HDL-cholesterol was 48.88 ± 12.08 mg/dl. HDL-cholesterol levels were significantly higher (p=0.0132) in female hypertensive patients, 51.07 ± 13.64 mg/dl, compared to male subjects included in the study group (45.55 ± 9.32 mg/dl), using unpaired Student's t-test with Welch correction. There is a weak, positive, non-significant correlation between HDL-cholesterol and BMI (r=-0.2327, p=0.1003), using Pearson's test. 30.5% of the patients had increased LDL-cholesterol level (exceeding 130 mg/dl, corresponding to 3.36 mmol/l).

A weak, negative, significant correlation was found between serum HDL-cholesterol and triglyceride concentration (r=-0.3160, p=0.0010) (**Figure 1**). A weak, positive, significant correlation could be observed between glycemia and triglyceridemia (r=0.2629, p=0.036), while there is a weak, negative, significant correlation between serum glucose and total cholesterol values (r=-0.2181, p=0.0158) using Pearson's test.

74.1% of the patients in the study group presented zinc deficiency, mean zinc concentration being 9.99 ± 1.77 µmol/l (normal range: 11.02 – 24.02 µmol/l).

20.6% of the hypertensive patients presented mild increase of hsCRP, the average being 1.94 ± 2.21 mg/l (one value over 10 mg/l, showing inflammation, was eliminated from statistics). The range between 3-10 mg/l corresponds to the interval predisposing to increased cardiovascular risk, normal values being <1 mg/l.

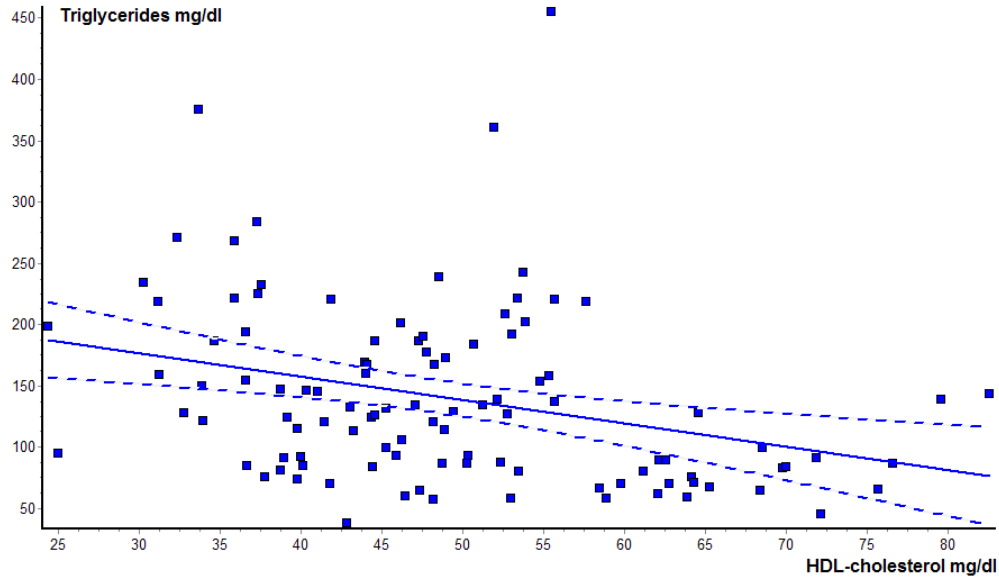

Figure 1. Correlation between serum HDL-cholesterol and triglyceride concentration

A weak, positive, significant correlation was found between serum creatinine and cystatin C values (r=0.3789, p=0.0002). The mean serum creatinine in the study group was 1.14 ± 0.37 mg/dl, the average cystatin C value was 1.11 ± 0.35 mg/l (normal range for the over 50 age group: 0.72-1.32 mg/l), 22.1% of the hypertensive patients having increased cystatin C values. Average values of serum GSH were significantly lower (p=0.0002) in the studied hypertensive patients, 1.89±0.82 µg/ml, compared to the control group (3.23 ± 0.49 µg/ml). Comparing the average MDA values of the study group (60.94 ± 25.38 ng/ml) to that of the control patients (55.79 ± 12.43 ng/ml), no significant difference could be observed using unpaired t-test with Welch correction (p=0.3863). The data followed gaussian distribution based on the Kolmogorov-Smirnov normality test.

No correlation could be found between serum GSH and MDA values (r=0.0877, p=0.6164) and no correlation could be observed between GSH and HDL-cholesterol values (r=-0.1943, p=0.2633). We further tested for relationship between GSH and hsCRP values, but no correlation could be found (r=-0.0637, p=0.7204) and between serum zinc and GSH, with no correlation (r=0.0993, p=0.5705), either. No correlation could be found between serum zinc and MDA values (r=0.2466, p=0.1201). Pearson's test was used for the evaluation of all these possible correlations.

Mean GSH values were significantly higher (p=0.0395) in males, 2.13 ± 0.92 µg/ml, compared to female subjects (1.75 ± 0.60 µg/ml) in the study group. The data followed gaussian distribution based on the Kolmogorov-Smirnov normality test.

The relationship between the age of the patients in the study group and their serum MDA value was also tested, but without finding any correlation (r=0.0631, p=0.5350). No correlation could be observed between the age of the patients and their serum GSH concentrations (r=-0.0063, p=0.9492) or GSSG values (r=0.07525, p=0.6315), using Pearson's test.

Discussion

Several clinical studies revealed significant dif-

ferences in lipid parameters between obese, hypertensive and diabetic patients compared to control subjects. Dyslipidemia including increased serum LDL-cholesterol, elevated triglycerides and decreased serum HDL-cholesterol are frequently associated with diabetes (21). Serum triglyceride concentration was significantly higher and HDL-cholesterol was significantly lower in obese patients compared to control subjects (22). Changes in metabolic parameters (glycated hemoglobin, serum uric acid, lipid profile) can be correlated with the risk of developing chronic kidney disease in type 2 diabetic patients (23), thus these can be predictive factors for certain complications. Lipid profile and some lipid ratios have been proposed as markers of predicting carbohydrate metabolic balance in type 2 diabetics (21). The obtained differences between the diabetic and non-diabetic subgroups regarding serum glucose and triglyceride concentration were expected, and also the correlation between serum glucose and glycated hemoglobin, two thirds of the diabetic subjects enrolled in the study having good carbohydrate metabolic balance according to the latest guidelines.

Measured and derived lipid parameters can have predictive role in certain cardiovascular complications, some of them might have different values depending on the patients' gender. Serum triglyceride/HDL-cholesterol ratio showed positive correlation with the occurrence of silent brain infarcts, especially in males, while total cholesterol/triglyceride ratio was negatively correlated with this pathology (24). Our study also showed certain differences between genders regarding protective biomarkers against cardiovascular diseases and inverse correlation between the protective HDL-cholesterol and serum triglyceride concentration. Changes of these lipid parameters can be in relationship with the background disease (such as hypertriglyceridemia is common in diabetes mellitus) and with the patients'current medication.

Changes in the structure of lipids and proteins, caused by oxidation, glycosylation of proteins and other chemical reactions can contribute to the development of atherosclerosis in hypertensive and diabetic patients (25).

Recent scientific data support the influence of lifestyle-related factors, such as unhealthy diet and lack of exercise, on the development of metabolic syndrome by causing epigenetic changes, influencing gene expression, having hyperglycemia, hypertriglyceridemia and low values of HDL-cholesterol as a consequence (26).

A recent Romanian study revealed reduced levels of protective antioxidants and increased levels of oxidative stress and inflammatory markers in obese subjects compared to a non-obese control group (27).

Our results regarding significantly lower GSH values obtained in the hypertensive patients with or without diabetes mellitus compared to the control group were similar to those reported by such studies (28).

Gender-related dimorphism of glutathione metabolism was suspected in several other human and animal studies, some of them finding higher levels of GSH in males than females, and others finding an opposite relation depending on the location of studied tissues (serum, brain, liver, heart, endothelial cells, erythrocytes). The causes of these differences are not yet totally clarified (29).

Serum zinc measurement is not a routine analysis in Romania. Zinc is a trace element important for human metabolism, being essential for the activity of certain enzymes, including antioxidant enzymes (such as the cytoplasmic Cu/Zn superoxide dismutase), its deficiency being involved in the ethiopathogenesis of several chronic diseases, such as age-related cardiovascular pathology. Recent studies revealed the role of zinc deficiency in the ethiopathogenesis of hypertension, partially by promoting sodium reabsorption (30). Zinc deficiency is involved

in chronic inflammation and enhances oxidative stress, being an important risk factor for atherosclerosis, lowering HDL-cholesterol levels and facilitating LDL oxidation. Zinc protects thiol group containing molecules from oxidation and enhances glutathione biosynthesis, plays a key role in the development of diabetes mellitus and its complications, being involved in insulin homeostasis. Zinc supplementation was found benefic in hypertensive and diabetic patients (31). Our study revealed a high incidence of zinc deficiency in the studied patients, which increases the risk of cardiovascular complications. Based on these results, we can recommend a more complex measurement of microelements in patients suffering from these pathologies, to reveal deficiency states which require temporary supplementation of certain minerals, avoiding excesses.

In a recent study, intensified lipid peroxidation and lower activity of antioxidant enzymes could be observed in hypertensive patients compared to healthy control patients of the same age. Oxidative stress was more obvious in case of female hypertensive patients (32).

Due to the many reactions that take place between oxygen derived free radicals (sometimes interacting also with nitrogen derived free radicals) and considering that only hydroxyl free radical is able to damage cell membranes and to produce serum MDA increase, a correlation between serum MDA and GSH (a substance able to neutralize only some forms of oxygen derived reactive species) has a relatively low probability to be detected by these types of biomarker measurements. Furthermore, GSH and HDL-cholesterol values are not expected to be linked due to the different mechanisms they act or are produced.

The implication of oxidative stress in the development of cardiovascular complications and insulin resistance in type 2 diabetic subjects, and in the endothelium dysfunction common in hypertension has been proven by several studies. Scientifically valid antioxidant strategies should be included in the therapeutic scheme of diabetic and hypertensive patients, especially in case of those suffering from obesity, besides treatment of dyslipidemia (33-35).

A limitation of the study is due to the assessment of only a few oxidative stress biomarkers, some of them lacking normal ranges for the studied age group since they are novel, recently developed methods. Some of the patients included in the study group and the control group were smokers, which can influence oxidative stress parameters, but no significant difference could be observed in the smokers' distribution between the two groups. Another limitation was the LDL-cholesterol calculation by the Friedewald formula, which is not completely accurate in patients having increased triglyceride levels (36), a common type of dyslipidemia in diabetes mellitus. Nowadays, calculation of non- HDL cholesterol is considered to be an important parameter for assessment of cardiovascular risk, so direct measurement of LDL-cholesterol seems not to be so critical.

The importance and novelty of the study is derived from the assessment of several laboratory parameters which are not routinely used, including methods developed and validated by our research group, in patients being at high risk for cardiovascular complications. Data provided by our study can contribute to choosing the most appropriate parameters for cardiovascular risk evaluation, and might have implications in the therapeutic strategies used in case of these patient groups.

Conclusions

Protective biochemical factors against cardiovascular affections show gender differences. Intense oxidative stress is present in patients suffering from hypertension with/without diabetes mellitus compared to control subjects based on the differences in glutathione levels. Zinc deficiency is very frequent in the study group and

hsCRP is increased in several subjects. Statistical correlation could be found between some lipid parameters and two kidney function tests. Oxidative imbalance can contribute to the development of cardiovascular complications, thus proven antioxidant strategies associated to conventional treatment might be benefic in secondary prevention.

Abbreviations

BMI – body mass index
DBP – dyastolic blood pressure
GSH – reduced glutathione
GSSG – oxidized glutathione
HbA1c – glycated hemoglobin
HDL – high density lipoprotein
HPLC – high pressure liquid chromatography
hsCRP – high sensitive C reactive protein
LDL – low density lipoproteins
MDA – malondialdehyde
SBP – systolic blood pressure
SGL – serum glucose level
STC – serum total cholesterol
STG – serum triglycerides
TBA – thiobarbituric acid

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Authors' contributions

ENN – Contributed by conceptualization, investigation and drafting the article
EF – Contributed mainly to the methodology, validation, resources and submitting the article
MDC – Had contribution to the methodology, validation and critical review of the article
ZP – Contributed to the investigation and formal statistical analysis of the collected data
SP – Had contribution to drafting the article
KINy – Contributed to investigation and resources for the research
TP - Contributed to investigation and resources for the research
RGT – Had contribution to critical review of the article
MCT – Had contribution to critical review of the article
MGS – Contributed to conceptualization, investigation and critical review of the article

Conflict-of-Interest Statement

The authors declare no conflict of interest.

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