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Assessment of vitamin B_{12} levels and cardiovascular risk factors in metformin- and non-metformin-treated type 2 diabetic patients

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Abstract: Oxidative stress enhances cardiovascular risk. Metformin decreases intestinal absorption of vitamin B_{12}. Our objective was the evaluation of type 2 diabetics focusing on differences due to their treatment. A prospective study on 224 type 2 diabetics was realized between 2015-2018 in Targu Mures, Romania, divided into 2 subgroups (metformin vs. other therapy – $2^{nd}/3^{rd}$ generation sulfonylureas, insulin, dietary regimen -, followed for at least one year) and non-diabetic controls (n=25) for oxidative stress level comparison. Serum homocysteine (HC), vitamin B_{12} were determined by chemiluminescence (Immulite One). Lipid peroxidation was assessed by serum malondialdehyde (MDA) measurement (HPLC). Biochemical tests, minerals, cystatin C, high-sensitivity C reactive protein (hs-CRP) were measured on Konelab20Xti, glycated hemoglobin on Nycocard Reader. GraphPad InStat-3 was used for statistics. Negative correlation occured between serum vitamin B_{12} and HC, this vitamin's level was significantly lower and serum zinc was significantly higher in patients on metformin. Hyperhomocysteinemia was present in 87% of the subjects, 46% had zinc deficiency and 41% elevated hs-CRP. Serum cystatin C showed positive correlation with creatinine. Serum MDA was significantly higher in diabetics compared to control patients. Elevated hs-CRP and homocysteine represent raised cardiovascular risk. Intense oxidative stress, vitamin, mineral deficiencies are frequent in diabetic subjects.

Keywords: Cardiovascular risk, homocysteine, metformin, type 2 diabetes, vitamin B_{12}

INTRODUCTION

Type 2 diabetes mellitus is a chronic condition, characterized by disturbances in carbohydrate and energy metabolism, being a major public health problem due to its continuously increasing prevalence and its serious, irreversible complications.

Oxidative stress is the most determining factor of the increase in cardiovascular risk in diabetic patients and worsening the outcome (Ighodaro, 2018; Yaribeygi *et al*., 2020).

Major cardiovascular complications are very common in diabetic and obese patients and directly contribute to the increased morbidity and mortality of these patients (Yang *et al*., 2019; Cercato and Fonseca, 2019).

Intensified lipid peroxidation, elevated homocysteine (HC) and high-sensitivity C reactive protein (hs-CRP) levels represent risk factors for cardiovascular complications in diabetes. HC also contributes to insulin resistance and endothelial dysfunction (Hu *et al*., 2019). The association between the increased level of HC and the prevalence of microvascular complications of diabetes (nephropathy and retinopathy) has been demonstrated by numerous clinical trials (Ye *et al*., 2021).

Metformin, the first-line oral antihyperglycemic agent, blocks the absorption of vitamin B_{12} through a mechanism not yet fully elucidated. Vitamin B_{12} levels may also be lower in metformin therapy due to intensified cellular uptake. The deficiency of this vitamin, decreasing the rate

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of HC remethylation producing methionine, can lead to elevated HC levels (De Jager *et al*., 2010). The vitamin B_{12}-intrinsic factor complex uptake at the level of the terminal ileum requires the availability of calcium. Some studies reported that this side effect of metformin therapy can be reversed with supplemental calcium (Bauman *et al*., 2000; Shaikh and Goldman-Levine, 2014).

According to recent studies hs-CRP is not only a marker of inflammation that predicts major cardiovascular events among healthy individuals, but is also an independent predictor of kidney function decrease in adults with renal function known to be normal (Guessous *et al*., 2014).

Cystatin C, an extracellular cysteine-protease inhibitor, which due to its low molecular weight may serve as a reliable glomerular filtration rate (GFR) marker. It was already proved the correlation of serum cystatin C level with the risk of complications in diabetes mellitus (diabetic nephropathy and neuropathy) and at the same time, it seems to be a good predictor of cardiovascular morbidity and mortality in patients with coronary- and peripheral artery disease. Cystatin C is used for the early detection of nephropathy, being a modern parameter, better compared to creatinine, because is less affected by external factors such as muscle mass, age, gender or diet (Kim *et al*., 2015; Ma *et al*., 2019).

Several studies provided evidence about the altered metabolism of minerals in diabetes and these elements could have pathogenic role in the onset and progression of the disease (Hasanato, 2020; Omidian *et al*., 2021; Bjørklund *et al*., 2020). Abnormal serum level of some trace-elements (Fe, Cu, Cr, Se, Zn, Mn and Ni), as well theirs metabolism alteration, have a demonstrated involvement in the pathogenesis and progression of type-2 diabetes mellitus. There are some controversies in the scientific literature regarding the beneficial effect of trace element supplementation in diabetic patients and results can vary depending on the studied population. Hereditary Copper (Cu) deficiency is associated with diabetes mellitus, Cu being a constituent of the superoxide dismutase, which reduces oxidative stress, promoter of the development of type 2 diabetes, while the high levels of Cu in diabetic patients disrupt the antioxidant functions and enhance the low-density lipoprotein peroxidation, causing vascular complications of the diabetes. Based on a relatively similar mechanism, iron (Fe) excess contributes to diabetic nephropathy. Selenium (Se), with essential role in DNA synthesis, reproduction, thyroid hormone metabolism, protection against infections and oxidative damage, improves glucose homeostasis and insulin resistance. The insufficient concentration of chromium, involved in both carbohydrate and lipid metabolism, represents an increased risk factors for diabetes mellitus and cardiovascular diseases. The low level of nickel (Ni) reduces plasma glucose, depresses reproductive performance and growth. Not least, adequate levels of manganese (Mn) are also required for normal insulin synthesis and secretion. Zinc is essential for the function of over 300 enzymes and 1000 transcription factors of the human organism, being indispensable among others and for the synthesis, storage and secretion of insulin. Zinc deficiency in diabetics is associated with increased oxidation of lipids, damaging the vascular system (Bjørklund *et al*., 2020; Kazi *et al*., 2008; Pattan *et al*., 2021).

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The aim of the study was to evaluate metabolic balance, deficiency of minerals, oxidative stress, cardiovascular risk factors (serum hs-CRP, HC, LDL-cholesterol) and nephropathy (based on creatinine and cystatin C levels) in type 2 diabetic subjects and assess carbohydrate metabolic balance, serum vitamin B_{12} and homocysteine concentration in patients on metformin treatment compared to other diabetic subjects (on diet, insulin or other antidiabetic drugs). Another objective was to compare oxidative stress level of diabetic patients to that of a non-diabetic control group.

MATERIALS AND METHODS

A prospective, cross-sectional clinical study was conducted between 2015-2018 at the Procardia Laboratory in Târgu Mureș, Romania. 224 type 2 diabetic adults were enrolled in two groups depending on their treatment: metformin (n=172) vs. other therapy (2nd and 3rd generation sulfonylureas (gliclazide, glimepiride), insulin, dietary regimen, n=52), the treatment being followed for at least one year by each patient.

The study was approved by the leading board of the medical institution hosting the research and by the Ethical Committee of the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș (No. 61/13.05.2015). Inclusion criteria: HbA1c <10%, documented diagnosis of type 2 diabetes mellitus, willingness to participate (all the enrolled subjects signed the informed consent document). Exclusion criteria: mental diseases, other forms of diabetes (type 1, MODY - maturity-onset diabetes of the young, secondary diabetes), chronic infections (tuberculosis, hepatitis, etc.), pregnancy, malignancy in the last 5 years, autoimmune disease, end stage organ failure, familial hyperlipidemia.

Besides routine laboratory tests-complete blood count (Abacus Junior 30 hematology analyzer, Diatron, Hungary), basic metabolic panel (glycaemia, urea, creatinine), lipid panel (serum triglycerides, total

cholesterol, HDL- and LDL-cholesterol levels) (Konelab^{TM}20XTi, Thermo Fisher Scientific Inc, Waltham, MA, USA; LDL-cholesterol values were determined by the Friedewald formula), urine dipstick analysis (HandUReader, 77 Elektronika Kft, Budapest, Hungary), HbA1c (reflectometry (NycoCard^{TM} Reader II, Axis-Shield Diagnostics Ltd, Scotland)-a series of other parameters were determined. Serum samples were stored at -70^{o}C before being processed for serum vitamin B_{12} and HC assessment by a chemiluminescent method (Immulite® One, Siemens Healthcare GmbH, Erlangen, Germany) and other special parameters.

Oxidative stress was measured by serum malondialdehyde (MDA) concentration, a marker of lipid peroxidation, by TBARS (thiobarbituric acid reactive substances) reaction. The photometric procedure described by Kei Satoh (Kei, 1978) was adapted to a chromatographic procedure by our research team, which was used for processing the patients' samples. 25 non-diabetic patients provided normal values for MDA concentration by this novel HPLC (high pressure liquid chromatography) method. Acetonitrile (VWR International S.A.S., Fontenay-sous-Bois, France) was used for deproteinization of the serum in 3:1 ratio. After 10 minutes of centrifugation at 5000 rpm 1.5ml of TBA (Sigma Aldrich, Chemie GmbH, Munich, Germany) and 2.5 ml H_2SO_4 (Chemical Company, Iași, Romania) were added to 1ml of serum supernatant and boiled for 30 minutes. The HPLC-UV-VIS Dionex equipment (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used, Supelcosil^{TM} LC-18 (Sigma Aldrich, Chemie GmbH, Munich, Germany), 33x4.6mm, 3µm columns, mobile phase: 20mM phosphate buffer (Merck KgaA, Darmstadt, Germany), pH=6, wavelength 530nm, retention time of TBA: 0.58 minutes. Values of minerals (calcium, magnesium, zinc, sodium, potassium, chloride), hs-CRP and cystatin C were measured (Konelab^{TM}20XTi, Thermo Fisher Scientific Inc, Waltham, MA, USA) using reagents from Diagnosticum LTD, Hungary for minerals (except zinc - Sentinel Diagnostics, Milano, Italy) and from Thermo Fisher Scientific Inc, Waltham, MA, USA for hs-CRP and cystatin C. Renal function was assessed by serum creatinine and cystatin C values.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad InStat version 3.0 software. We performed two-tailed t test and assessed linear Pearson correlation, p values under 0.05 being considered statistically significant. The normal distribution of data was checked by Kolmogorov-Smirnov test.

RESULTS

The mean age of the enrolled diabetic patients was 72.76±10.42 (SD), 67% of the subjects were females.

Serum HC was elevated in 87% of the diabetic patients and negative correlation (r=-0.2413, p=0.022) was observed between serum vitamin B_{12} and HC levels (fig. 1).

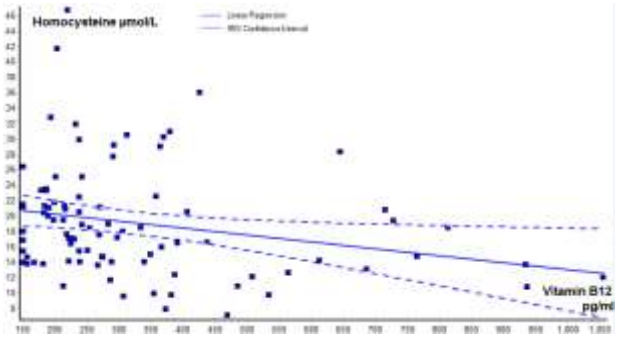

Fig. 1: Inverse correlation between serum homocysteine and B_{12} vitamin levels in type 2 diabetic patients

Three quarters of the patients presented mild hyperhomocysteinemia, which corresponds to values between 12-30 µmol/L (fig. 2).

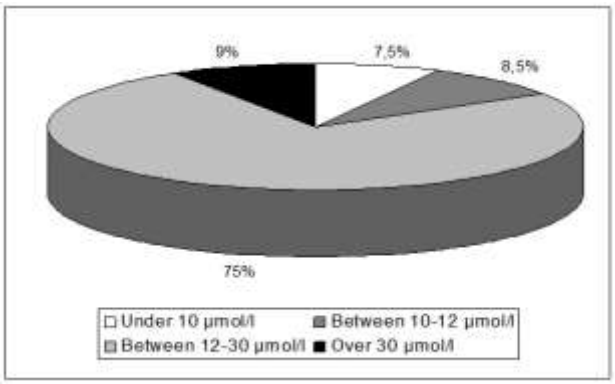

Fig. 2: Distribution of serum homocysteine levels in type 2 diabetic patients

46% of the diabetic patients presented zinc deficiency, the average value was 10.85 µmol/L +/- 2.77 (SD), close to the lower limit of the normal range (reference values: 10.4-16.4 µmol/L). No correlation could be observed between serum zinc and HC levels (p>0.05).

The average magnesium level was 0.86 mmol/L +/- 0.12 (SD), 6% of the patients presented magnesium deficiency. The average calcium concentration was 2.27 mmol/L +/- 0.15 (SD), 22% of the patients presented calcium deficiency, most of them showed mild decrease in this parameter. The average value of potassium concentration was 4.56 mmol/L +/- 0.44 (SD), 3% of the patients presented hyperkalemia. The mean serum chloride level was 101.63 mmol/L +/- 3.59 (SD), 6% of the patients presenting increased values. The average serum sodium concentration was 141.23 mmol/l +/- 3.84 (SD), 6% of the patients had pathological sodium levels, half of them higher, half of them lower values.

Table 1: Comparison of laboratory test results in type 2 diabetic patients on different therapies

Parameters	Units	Metformin-treated diabetic patients	Non-Metformin-treated diabetic patients	Statistical significance
Leukocytes	10^9/L	8.81 ± 2.50	7.81 ± 2.01	p=0.4182
Erythrocytes	10^{12}/L	4.61 ± 0.40	4.20 ± 0.47	p=0.0562
Hemoglobin	g/dl	12.79 ± 2.36	12.82 ± 1.45	p=0.9797
Hematocrit	%	39.40 ± 3.50	38.02 ± 4.35	p=0.4247
MCV	fl	85.67 ± 5.90	89.00 ± 4.73	p=0.2171
Glycemia	mmol/L	6.89 ± 2.26	6.16 ± 2.92	p=0.1446
Cholesterol	mg/dL	188.57 ± 39.64	176 ± 74.80	p=0.6004
Triglycerides	mg/dL	142.73 ± 77.41	109.73 ± 82.17	p=0.2156
HDL	mg/dL	48.71 ± 12.08	46.26 ± 11.87	p=0.5690
LDL	mg/dL	108.88 ± 31.43	112.13 ± 63.59	p=0.8926
Urea	mg/dL	40.40 ± 14.79	44.94 ± 22.24	p=0.5522
Creatinine	mg/dL	1.22 ± 0.36	1.38 ± 0.52	p=0.3603
Cystatin C	mg/L	1.06 ± 0.33	1.21 ± 0.45	p=0.2275
Calcium	mmol/L	2.26 ± 0.15	2.29 ± 0.17	p=0.6826
Magnesium	mmol/L	0.84 ± 0.12	0.89 ± 0.12	p=0.2274
Sodium	mmol/L	141.48 ± 4.71	141.13 ± 2.64	p=0.8379
Potassium	mmol/L	4.64 ± 0.49	4.36 ± 0.35	p=0.1274
Chloride	mmol/L	100.31 ± 14.79	102.41 ± 2.67	p=0.4283
Zinc	µmol/L	11.38 ± 2.61	9.18 ± 2.64	p=0.0005*
MDA	ng/mL	10.62 ± 6.87	9.58 ± 8.06	p=0.5697
Hs-CRP	mg/L	3.32 ± 2.78	4.12 ± 3.20	p=0.3220
Vitamin B_{12}	mg/day	297.35 ± 159.21	386.75 ± 257.46	p=0.0488*
Homocysteine	mg/day	18.99 ± 7.82	19.14 ± 9.55	p=0.9343
HbA1c	%	6.96 ± 1.09	6.48 ± 1.25	p=0.0701

Most of our subjects were patients with well-controlled diabetes (HbA1c <7%), the average HbA1c value was 6.84% +/- 1.14 (SD). Glycosuria was present on the day of sampling in 14% of the patients.

41% of the patients had slightly elevated hs-CRP levels (between 3-10 mg/L, corresponding to increased cardiovascular risk), values higher than 10 mg/L were excluded, considered to be caused by acute infections, inflammations, thus mean hs-CRP value was 3.51 mg/L +/- 2.88 (SD). No significant correlation was found between serum hs-CRP and HC levels (p>0.05).

Results of the main laboratory parameters obtained in the two subgroups of diabetic patients are included in Table 1. Significant differences were obtained only in case of serum zinc and vitamin B_{12} results (marked with asterisk).

We found serum creatinine concentrations slightly exceeding the highest value of the normal range in 40% of the subjects. The average creatinine value of the studied diabetic patients was 1.25 mg/dL +/- 0.35 (SD), the normal range is slightly higher in male patients (0.84-1.2 mg/dL) compared to females (0.66-1.00 mg/dL). The mean cystatin C value of these patients was 1.09 +/- 0.36 (SD), the normal range being 0.47-1.03 mg/L. Positive correlation was found between serum creatinine and cystatin C values in the studied diabetic patients (r=0.5734, p<0.0001) (fig. 3).

MDA level was significantly higher in patients with type 2 diabetes - average: 10.38 ng/mL +/- 7.12 (SD) - compared to non-diabetic non-smoker subjects of similar age - mean value 5.51 ng/mL +/- 1.33 (SD) -, p<0.0001 using unpaired t test with Welch correction.

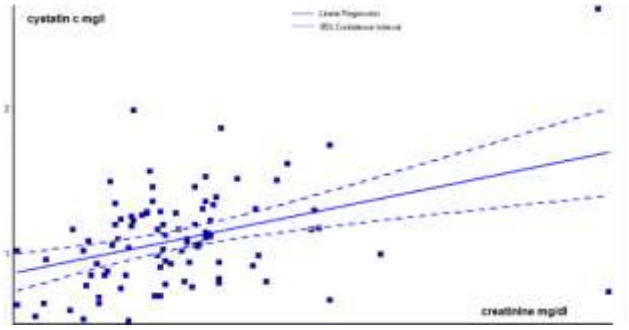

Fig. 3: Correlation between serum creatinine and cystatin C values in type 2 diabetic patients

The average serum LDL-cholesterol level of the studied diabetic subjects was 109.45 mg/dL +/- 37.04 (SD), 11% of the patients presented values exceeding 160 mg/dL.

DISCUSSION

Increased homocysteine level has been identified as a strong predictor of cardiovascular disease (CVD), independent from other classical thrombotic risk factors. According to previous studies, an increase of 3µmol/L in the plasma level of HC can increase the incidence of stroke by almost 20% and the incidence of ischemic cardiac disease by more than 10%. It is controversial whether mild/moderate hyperhomocysteinemia is a causal risk factor or just a predictor of CVD, a biomarker,

treatment of which does not improve the clinical outcome (Smulders and Blom, 2011). In our study group of diabetic patients, mild hyperhomocysteinemia had the highest prevalence, a longer follow-up of these patients could reveal the difference between the incidence of cardiovascular complications in these subjects and those having moderate/severe HC increase (values between 30-100, >100 µmol/L, respectively) compared to those presenting normal or acceptable values (under 10 µmol/L, between 10-12 µmol/L, respectively). In the study group no correlation could be found between serum HC and hs-CRP concentration, which are considered to be independent cardiovascular risk factors.

The level of hs-CRP is influenced by gender, treatment, body mass index and diseases, which makes the interpretation of the results difficult in case of our subjects. Diabetic patients usually present slightly increased hs-CRP levels; some of the subjects had very high values indicating an infectious background. Statin treatment decreases hs-CRP levels and women are more likely to have increased hs-CRP values in comparison with men (Shimoda *et al*., 2016), two third of our patients being female subjects. No correlation was found between serum hs-CRP and triglyceride concentrations, or between serum hs-CRP and HbA1c levels, differently from the data available in the scientific literature (Mazidi *et al*., 2018; Mahajan *et al*., 2009).

Our results revealed that diabetic patients on metformin treatment (patients receiving this medication for at least one year were selected) had significantly lower serum vitamin B_{12} concentrations compared to those on other therapies and in majority of the cases deficiency of this vitamin could be correlated to increased HC levels. These finding are similar to those in the literature (Wang *et al*., 2021; Crăciun *et al*., 2021). Vitamin B_{12} deficiency favors the acceleration and aggravation of atherosclerosis through various molecular mechanisms including cytoskeletal organization, cell differentiation and migration, chronic inflammatory process and oxidative stress. According to recent experimental studies high fat diet contributes to worsening depletion of vitamin B_{12} and exacerbation of hyperhomocysteinemia (Duthie *et al*., 2015).

In our study group most of the patients presented normal serum LDL-cholesterol level probably due to their diet and therapy, only a small percentage had higher values of this parameter, leading to increased cardiovascular risk. All the enrolled diabetic patients follow a restrictive low-calorie diet, rich in fibers, helping them to lose weight and lower their atherogenic cholesterol level.

Zinc deficiency was the most frequent mineral disorder revealed in our study. Zinc plays a key role in sustaining normal biological functions. It exhibits insulino-mimetic, antioxidant and anti-diabetic effects (Vardatsikos *et al*., 2013; Cruz *et al*., 2015). The antioxidant effect of zinc is due to its incorporation in the antioxidant enzyme superoxide dismutase, found in the cell cytoplasm, it also has antiviral effect. Zinc deficiency, which is common in diabetic patients, has been confirmed also by our study (the non-metformin treated group had significantly lower results compared to those on metformin therapy), it has a determining role in the occurrence of microvascular complications, especially the retinopathy (Cruz *et al*., 2015). Several studies proved the zinc supplementation benefits on carbohydrate and lipid metabolism and it can also decrease cardiovascular risk and prevent progression of retinopathy (Ugarte and Osborne, 2014; Feng *et al*., 2021; Tamura, 2021). Zinc associated to metformin therapy proved to be benefic on lipid profile parameters (decreased serum triglyceride, total and LDL-cholesterol, increased HDL-cholesterol) and also reduced HbA1c and oxidative stress levels (Younis *et al*, 2021).

In several studies carried out on type 2 diabetic patients cystatin C values were found elevated compared to healthy controls, but others reported heterogenous results (Ma *et al*., 2019). Our study showed positive correlation between this modern marker and the routinely used creatinine in this group of diabetic patients, so both proved to be reliable parameters for revealing nephropathy.

Significantly higher MDA values were found in the diabetic subjects compared to the non-diabetic ones, which represents an increased level of oxidative stress. One of the worse effects of oxidative stress is the lipid peroxidation, known to induce cell necrosis through damage to the unsaturated fatty acids in the cell membrane inducing atherosclerosis and its complications (Gianazza *et al.,* 2021). Dietary supplements containing efficient antioxidants could be beneficial in prevention of cardiovascular complications (Forman and Zhang, 2021).

Limitations of the study

Direct measurement of LDL-cholesterol was not performed due to its higher costs, although its calculation by the Friedewald formula is not completely accurate in patients presenting increased triglyceride levels (Keti and Muhtaroglu, 2021), a common type of dyslipidemia in diabetes mellitus. Nowadays calculation of non-HDL cholesterol is an important parameter for assessment of cardiovascular risk, therefore direct measurement of LDL-cholesterol does not seem to be so critical. The measurement of vitamins and minerals was quite limited, a more complex evaluation would have been more appropriate, including serum selenium (often deficient in diabetic patients) and vitamin D measurement (statin therapy can interfere with the metabolism of this vitamin). The originality of the study is derived from using an innovative, cost-effective HPLC method validated by our research team for assessment of lipid peroxidation level (Fogarasi *et al*., 2016), which has a precision similar to

other HPLC methods developed for MDA measurement. A strength of our research is the relatively high number of enrolled diabetic subjects and the complexity of laboratory tests, including several parameters (serum MDA, hs-CRP, cystatin C, vitamin B_{12}, homocysteine) which are not regularly performed in case of these patients in Romania. According to our knowledge this is the first study of this kind in our geographical area. The outcome of this study has an important practical value. It can be used by clinicians to select diabetic patients with high cardiovascular risk, so they can benefit of special attention regarding secondary prevention, cardiovascular complications, especially thrombo-embolic events (Varga *et al*., 2017), which are very common in obese and diabetic patients (Tilinca *et al*., 2018b; Bálint *et al*., 2017; Tilinca *et al*., 2018a), causing serious fatality worldwide (Tilea *et al*., 2021; Nicola *et al*., 2018). Furthermore, revealing low mineral and vitamin levels helps the affected patients to receive dietary supplements to correct the deficiency.

This research opens the perspective for larger studies on this field, the assessment of diabetic patients based on different techniques of medical imaging, focusing especially on evaluation of microvascular dysfunction in addition to laboratory investigations.

CONCLUSION

Based on the obtained results in most of the studied diabetic patients an intense oxidative stress (lipid peroxidation) was present. Hyperhomocysteinemia and increased hs-CRP levels constitute major cardiovascular risk factors in this vulnerable group of patients. Vitamin B_{12} and mineral deficiencies (especially zinc and calcium) were common in the studied diabetic subjects. The diagnosis of these deficitary states should be in the focus of the laboratory investigation of diabetic patients.

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