SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Changes in markers of oxidative stress and endothelial dysfunction as a result of alpha-lipoic acid treatment and exercise therapy in patients with diabetic polyneuropathy

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Introduction

Diabetic neuropathy (DN), the most common microvascular complication of type 2 diabetes mellitus (T2DM), is presented as sensory, motor and autonomic neuropathy. Diabetic sensorimotor polyneuropathy is a symmetrical, distal polyneuropathy attributable to metabolic and microvascular alterations as a result of chronic hyperglycemia and associated with severe metabolic changes including mitochondrial dysfunction and enhanced generation of reactive oxygen species. Cardiac autonomic neuropathy (CAN), perhaps the least understood complication of diabetes, is defined as the impairment of cardiovascular autonomic regulation caused by damage to the autonomic nerve fibers that innervate the heart and blood vessels.

Literary review

Previous research indicates that physical activity may improve the neurological function and impaired nerve conduction in DN. The position statement of American Diabetes Association suggests that structured lifestyle interventions which include at least 150 minutes of physical activity per week and dietary changes, are also recommended to delay the progression of microvascular complications in T2DM. Moreover, all adults with T2DM should perform both aerobic and resistance training for optimal glycemic control.

The development of diabetic neuropathy is a multifactorial process, even though the exact pathogenic mechanism is not fully understood. The key metabolic components are the hyperglycemia-induced activation of alternative metabolic pathways including increased polyol and hexosamine pathway flux, mitochondrial dysfunction, protein kinase C activation, the enhanced formation of reactive oxygen species, and the altered generation of endothelial nitric oxide (NO) leading to endothelial dysfunction. The earliest and most representative findings in diabetic sensorimotor peripheral neuropathy are small-nerve fiber degeneration and endoneurial microangiopathy. During the progression of diabetic neuropathy, hyperglycemia-induced oxidative stress, activation of alternative metabolic pathways and decreased antioxidant protection are thought to contribute to endothelial dysfunction and decreased endoneurial blood flow with consecutive hypoxia, causing further nerve damage. Experimental analysis revealed that these changes in nerve microcirculation may be mediated by alterations in NO metabolism. Overproduction of superoxide anion in diabetic neuropathy leads to binding of this free radical of oxygen to NO forming highly reactive peroxynitrite, which inactivates endothelial NO synthase and triggers endothelial cell apoptosis. Inhibition

of endothelial NO synthase by N-nitro-L-arginine resulted in decreased nerve blood flow and this effect may be reversed by L-arginine.

Based on this approach, inhibition of NO synthesis may promote the progression of diabetic neuropathy via endothelial dysfunction caused by reduced endoneurial blood flow and impaired endothelium-dependent vascular relaxation. Experimental studies suggested that reduction in endoneurial blood flow would precede slowing of motor nerve conduction velocity and the generation of reactive oxygen species could be partially responsible for the development of diabetic neuropathy.

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, is reported to increase in patients with diabetes and is suggested to play a role in the pathogenesis of accelerated atherosclerosis. Previous studies documented that ADMA seems to be a predictor of the cardiovascular outcome in diabetic patients. Other investigators have shown that the changes in the ADMA level may contribute to modified vascular and endothelial functions in diabetic neuropathy.

Alpha-lipoic acid (ALA), as a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activity, is involved in mitochondrial oxidative metabolism and has beneficial effects on diabetic neuropathy. ALA has been reported to improve nerve conduction velocity and protects peripheral nerves from hyperglycemia-induced oxidative stress in experimental diabetic neuropathy. ALA may improve cellular redox potential by increasing de novo synthesis of cellular glutathione, enhancing the NADPH oxidase activity of neuronal NO synthase, inhibiting nuclear factor kappa B activity and improving NO-mediated vasodilation. Previous research has demonstrated that ADMA levels decreased after a short-term treatment with ALA in patients with type 2 diabetes. However, the effects of ALA on these oxidative and endothelial markers and their correlations with the severity of cardiac autonomic and peripheral sensorimotor polyneuropathy are not fully clarified.

Fibroblast growth factor 21 (FGF21) is a liver-secreted hormone with several beneficial effects on obesity-related metabolic disorders. FGF21 enhances glucose uptake and oxidation in an insulin-independent manner by inducing the expression of glucose transporter-1 in adipocytes and skeletal myocytes. Previous experimental and human studies have shown that physical activity may increase the serum levels of FGF21 in T2DM. According to a recent meta-analysis, acute exercise significantly increased the serum concentration of FGF21, regardless of body weight and obesity level. The circulating FGF21 acts through cell surface

receptors comprised of FGF receptors (FGFR) in complex with transmembrane protein β-Klotho. The interaction of FGFR with β-Klotho increases sensitivity to FGF21 and the ability to activate intracellular signaling pathways, which eventually leads to metabolic effects. However, it is still not clarified how FGF21 may improve the process of mitochondrial oxidation.

Irisin is a myokine induced by exercise with insulin-sensitizing properties and derived from the C-terminal cleavage of the fibronectin type III domain containing 5 (FNDC5) transmembrane proteins. This proteolytic process is mediated by the peroxisome proliferator-activated receptor-gamma coactivator-1-alpha (PGC-1α). Irisin/FNDC5 acts on skeletal muscle during exercise, resulting in glucose and fatty acid uptake, as well as increased energy expenditure and induces oxidative metabolism through the induction of metabolic genes involved in the regulation of the mitochondrial bioenergetic process.

In addition to energy storage, adipose tissue produces a variety of adipocytokines, including leptin, adiponectin and others, thus having potential endocrine function. Leptin may directly improve insulin resistance in diabetic mice by increasing the oxidation of free fatty acid (FFA). Previous research has shown that low serum level of adiponectin is correlated with insulin resistance and cardiovascular disease in patients with T2DM. Furthermore, an increased proinflammatory response is observed in leptin resistance during obesity and physical activity may reduce inflammation by improving leptin resistance in T2DM. Tumor necrosis factor alpha (TNF-alpha) is a pro-inflammatory adipokine associated with insulin resistance and β cell failure in T2DM and obesity. High level expression of TNF-alpha induces phosphorylation of the insulin receptor substrate 1 and thus prevents the interaction of insulin with an insulin receptor. The anti-inflammatory properties of adiponectin may play an important role in slowing the progression of atherosclerosis in T2DM and may have a beneficial effect on insulin resistance by inhibiting TNF-alpha-induced activation of NF-κB in endothelial cells. Moreover, TNF-alpha enhances activity of hormone sensitive lipase in adipose tissue and thus increases the release of FFA into circulation.

Study designs

Therefore, the aim of our work was to assess the relationship between markers of endothelial dysfunction and NO synthesis in type 2 diabetic patients with peripheral neuropathy after six-month of ALA treatment. Moreover, we looked for associations between the changes of ADMA level and the severity of CAN and peripheral sensory neuropathy after ALA treatment.

Moreover, we aimed to investigate the changes of FGF21 level and their relationships with other inflammatory markers and adipokines in T2DM patients with distal sensory polyneuropathy after six-week of aerobic exercise training program. We hypothesized significant associations between the changes of FGF21 level and the severity of peripheral sensory neuropathy in T2DM patient after physical activity.

Patients and methods

Study populations

In the first study, in which we examined the effects of alpha-lipoic acid treatment on plasma asymmetric dimethylarginine, 52 type 2 diabetic patients with neuropathy (22 men and 32 women, the mean age: 64.15±8.66 years; duration of diabetes was 12.4 years, and duration of diabetic neuropathy: 3.2±1.4 years) were enrolled. All patients were treated daily by oral route with 600 mg ALA for 6 months. Furthermore, 28 age- and gender-matched diabetic control subjects without neuropathy were also enrolled (duration of diabetes was 12.1 years). In the second research, in which we investigated the change of FGF-21 level and its correlation with the severity of diabetic sensory polyneuropathy after six weeks of physical activity, 30 adult individuals with T2DM and distal sensory polyneuropathy (9 men and 21 women; the mean age: 61.97±8.1 years; duration of diabetes was 10.3±3.7 years, and duration of diabetic neuropathy: 8.7±5.6 years) were included. Besides, 32 age- and gender-matched diabetic control subjects without neuropathy were also enrolled (10 men and 22 women; the mean age was 64.37±6.52 years; mean duration of diabetes was 10.9±4.1 years). All patients were controlled with oral antidiabetic agents (metformin, sulfonylurea and/or DDP4-inhibitors), subjects on insulin therapy were excluded. Patients with a history of diabetic proliferative retinopathy, diabetic nephropathy or type 1 diabetes were also excluded. Besides, we excluded subjects with alcoholism, known liver diseases, endocrine and autoimmune disease, haematological and neurological disorders which can be associated with peripheral neuropathy. Patients with prior cardiovascular disease, established coronary artery disease or myocardial infarction, severe congestive heart failure, smokers, pregnant women, subjects with established malignancy were not enrolled in our study. All the patients were recruited from the Diabetic Neuropathy Center of Debrecen, Department of Internal Medicine, University of Debrecen, Faculty of Medicine, Debrecen, Hungary. All participants provided written informed consent. The study protocol was approved by the local and regional ethical committees.

After final enrolment in the second study, DN patients were instructed to march with trekking poles and the aerobic exercise training program was supervised by a physiotherapist and corrected if needed. Glucose levels were determined immediately after the training and one hour later. If significant drop of serum glucose levels were measured, antidiabetic therapy has been modified according to the needs of exercise. The subjects had to perform the exercises for 6 weeks, 3 days a week, occasionally for 70 minutes. The exercise program that was gradually progressed in duration (from 50% to 80% of maximum heart rate), included 10 minutes stretching movements to warm-up, followed by 50 minutes of aerobic training (treadmill and bicycle ergometers), and finally 10 minutes of relaxation activities to cool down. Before and after the intervention, cardio fitness levels were measured by VO_{2max} (ml/kg/min) applying the Rockport 1600 m walking test. Estimation of VO_{2max} from a timed one-mile track walk with duration, incorporating age, gender, body weight and heart rate at the end of the walking test. The body mass index (BMI) and heart rate of the patients were also measured before and after the exercise training program. After 6 weeks of supervised training, all patients underwent blood tests during outpatient care. All patients with neuropathy underwent blood test and neurophysiological examination for objective evaluation of sensory neuropathies using the Neurometer® current perception threshold testing during the outpatient visit. The control subjects were not exposed to the exercise training program, only were used as a benchmark for the comparison of results.

Blood sampling

Venous blood samples were taken after an overnight fasting and sera were prepared immediately. Routine laboratory analyses (total cholesterol, triglyceride, high-density lipoprotein-cholesterol – HDL-C, low-density lipoprotein -cholesterol – LDL-C, glucose, hemoglobin A1c – HbA1c, creatinine and uric acid) were performed in the Department of Laboratory Medicine, University of Debrecen, Faculty of Medicine, Debrecen, Hungary.

ADMA measurement

Serum ADMA concentrations were measured by commercially available competitive enzyme-linked immunosorbent assay kit (ADMA-ELISA; DLD Diagnostika GmbH, Hamburg, Germany) with intra-assay CVs ranging from 5.7 to 6.4 % and inter-assay CVs ranging from 8.3 to 10.3%, respectively. Measurements of ADMA level in sera were performed according to the manufacturer's instructions. The values were expressed as μmol/l.

TNF-alpha measurement

Serum levels of TNF-alpha were assessed using the TNF-alpha Enzyme-Linked Immunosorbent Assay (ELISA) test (R&D Systems Europe Ltd., Abington, England). Measurements of the TNF-alpha levels in the sera were performed according to the recommendations of the manufacturer. The intra-assay CVs ranging from 1.9 to 2.2 % and inter-assay CVs ranging from 6.2 to 6.7 %, respectively. The values were expressed as pg/ml.

Measurement of oxLDL

Serum concentrations of oxidized LDL (oxLDL) were detected by a commercially sandwich ELISA kit (Mercodia AB, Sweden). It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. The coefficient variations of the intra- and inter-assay for measurement of oxLDL were 5.5-7.3 % and 4-6.2 %, respectively and the sensitivity was < 1 mU/l.

ICAM-1 and VCAM-1 measurement

The ICAM-1 and VCAM-1 levels were measured with use of human soluble ICAM-1 and VCAM-1 sandwich ELISA kits (R&D Systems Europe Ltd., Abington, England). ELISA procedures were carried out according to the manufacturer's instructions. The intra-assay CVs and inter-assay CVs ranging were 3.7-5.2 % and 4.4-6.7 % (ICAM-1), 2.3-3.6 % and 5.5-7.8 % (VCAM-1), respectively. The values were expressed as ng/ml.

Assay for nitrite concentration

The nitrite concentration was measured as an indicator of NO production, according to the Griess reaction. Briefly, 300 µl of deprotonized plasma was incubated with an equal volume of Griess reagent (sulfanilamide and N-(1-naphtyl) ethylendiamine dihydrochloride dissolved in 2.5% H3PO4 as 0.5% and 0.05% solution, respectively, and mixed in the volume ratio 1:1 immediately before use) for 10 min at room temperature, in the dark. The optical density was measured spectrophotometrically at 550 nm. Nitrite concentration was determined using sodium nitrite as standard (10-100 µmol/l). The values were expressed as µmol/l.

FGF21 measurement

Serum FGF21 concentration was measured by commercially available sandwich enzyme immunoassay (Human FGF21 ELISA, Biovendor, Brno, Czech Republic) with intra-assay CVs ranging from 1.6 to 2.4 % and inter-assay CVs ranging from 3.1 to 3.5 %, respectively. Measurements of FGF21 level in sera were performed according to the manufacturer's instructions. The values were expressed as pg/mL.

Measurement of irisin levels

Serum concentrations of irisin were detected by a commercially avaible competitive enzyme-linked immunosorbent assay kit (Human Irisin ELISA, Biovendor, Brno, Czech Republic). The coefficient variations of the intra- and inter-assay for measurement of irisin were 4.8-7.9 % and 8.0-9.7 %, respectively and the lowest level of irisin can be measured by this assay is 1 ng/mL according to the manufacturer's instructions.

Adiponectin and Leptin Measurement

Total adiponectin and leptin concentrations in serum of patients enrolled in our study were measured with commercially available sandwich enzyme immunoassay (Human Total Adiponectin/Acrp30 Quantikine and Human Leptin Quantikine Immunoassays, R&D Systems Europe Ltd., Abington, England). The coefficient variations of the intra- and inter-assay for measurement of total adiponectin were 2.5-4.7 % and 5.8-6.9 %, respectively. Precision of leptin measurement was intra-assay CVs ranging from 3.0% to 3.3%, inter-assay CV-s from 3.5% to 5.4%.

Assessment of autonomic and peripheral nerve function

All participants underwent detailed assessment of peripheral neuropathy (DN4 questionnaire in screening for neuropathic pain syndrome, vibration perception threshold, quantitative sensory testing) and in-vivo corneal confocal microscopy by ophthalmologist for the diagnosis of diabetic sensorimotor polyneuropathy. Peripheral sensory nerve function was assessed through current perception threshold testing (CPT) using a Neurometer® (Neurotron Inc., Baltimore, Maryland, USA, 2002). Previously it has been reported that this neurodiagnostic device is capable of detecting peripheral sensory neuropathy in various diseases including diabetes mellitus. Neurometer® CPT testing delivers sinusoidal alternating current stimuli at three different frequencies: 5 Hz, 250 Hz and 2,000 Hz, assessing small unmyelinated C-fibre, small myelinated Aß-fibre and large myelinated Aß-fibre function,

respectively. This intensity alignment is conducted to approach the sensory threshold with a ±50 Mikroampere (μA) range out of a total range of 0 to 9.99 Milliamperes. The current stimuli were applied to dorsal surfaces of the distal phalanges of the index finger and great toe unilaterally via two small electrodes and the intensity was increased until the participants experienced a painless sensation. Neurometer® CPT testing adjusts the level of stimulation based on the patient's response automatically. The participants were presented with 5 to 7 randomly generated sets of stimuli above and below their level of perception and asked to choose which of the two stimuli felt stronger using an automated forced choice protocol. A CPT value (mA) based on the minimal current perceived was calculated once a sufficient number of correct consecutive responses had been obtained.

Autonomic function was assessed by means of Ewing's five standard cardiovascular reflex tests: changes in heart rate during deep inspiration and expiration, heart rate responses to standing up (30/15 ratio), Valsalva maneuver, systolic blood pressure fluctuation to standing up, and changes in diastolic pressure during a sustained handgrip. A score was created to express the severity of autonomic neuropathy, based on the results of the five tests (normal: 0, borderline: 1, abnormal: 2). The composite autonomic score (CAS) was in the interval of 0–10. A score of 0–1 was taken as normal, 2–3 as mild, 4–6 as moderate, and 7–10 as severe CAN.

Statistical methods

Statistical analyses were performed using the Statistica® 13.5.0.17 software (TIBCO Software Inc. USA). Normality of distribution was tested by the Kolmogorov–Smirnov test. Relationship between two categorical variables is calculated by Chi-squared test. In case of normal distribution, the differences between anthropometry and laboratory parameters in diabetic controls and patients before exercise program were analyzed with unpaired t-test. Data were expressed as means ± SD. In case of non-normal distribution, the previously mentioned differences were analyzed by Mann-Whitney u-test. These data were presented as median (lower-upper quartile). Differences before and after exercise program were determined with paired t-test (normal distribution) or Wilcoxon matched paired test (non-normal distribution). Pearson correlation was used to investigate the relationship between variables. The $p \leq 0.05$ probability values were considered statistically significant.

Results

Effect of alpha-lipoic acid treatment on the level of asymmetric dimethylarginine and endothelial dysfunction in diabetic neuropathy

There was no significant change in BMI, glucose, creatinine, uric acid, HbA1c, VCAM-1, ICAM-1 levels and lipid parameters in the patient group after ALA treatment. ADMA level significantly decreased, while NO level significantly increased in patients after ALA treatment. The initial ADMA level was significantly higher in patients compared to controls (0.62±0.11 vs. 0.56±0.10 μmol/l, $p<0.05$). The level of TNF-alpha significantly decreased after treatment with ALA (1.21±0.42 vs. 1.05±0.50 pg/ml, $p<0.05$). A significant improvement of CPT measured by Neurometer CPT testing and lower CAS were detected in patients with diabetic neuropathy after receiving ALA treatment. Both CPT and CAS were higher in patients before ALA treatment compared to controls ($p<0.01$). VCAM-1 level was significantly higher in patients with diabetic neuropathy both before and after ALA treatment compared to control subjects (885.25±356.75 vs. 739.78±127.83 ng/ml, $p<0.05$).

The improvement of CPT values was correlated positively with the change of ADMA levels ($r=0.58$, $p<0.001$). The change of TNF-alpha levels showed a positive correlation with the change of ADMA levels ($r=0.31$, $p<0.05$). The changes of ICAM-1 concentrations were correlated positively with VCAM-1 and TNF-alpha levels ($r=0.43$, $p<0.01$; $r=0.49$, $p<0.01$, respectively). The improvement of CPT values was correlated significantly with the decrease in CAS ($r= 0.77$, $p<0.001$).

The patient group was divided into two subgroups according to the response to ALA treatment, defined by the clinical symptoms and the improvement in CPT and CAS. We identified 36 responders (9 male/27 female) and 18 non-responders (6 male/12 female). Responders mean significant changes in neuropathic pain symptoms measured by standardized Total Symptom Score and significant improvements in CPT and CAS. Decrease in ADMA level was significantly more in responder patients compared to non-responders identified by both CPT ($p<0.05$) and CAS ($p<0.05$).

Change of fibroblast growth factor 21 level correlates with the severity of diabetic sensory polyneuropathy after six-week physical activity

Significant decreases in BMI (31.6±3.94 vs. 31±3.81 kg/m2, $p<0.001$), HbA1c (7.09±0.81 vs. 6.78±0.87 %, $p<0.01$) and TNF-alpha levels (0.7±0.4 vs. 0.57±0.21 pg/ml, $p<0.05$) were observed after 6-week physical activity in DN patients. Circulating FGF21 levels were significantly increased (140.62 [73.19-373.07] vs. 168.89 [111.4-513.69], $p<0.01$); while CPT values as measured by Neurometer® test were significantly improved ($p<0.05$) after 6-week physical activity in DN patients. There were no differences in serum creatinine, uric acid, irisin, adiponectin, leptin, triglyceride, total cholesterol, HDL-C, nonHDL-C, LDL-C levels and enzyme liver parameters in DN patients before and after physical activity. Although the levels of adipocytokines did not differ across groups after physical activity, the level of leptin was significantly decreased after physical activity compared to control subject (30.72±19.98 vs. 20.93±18.97, $p<0.05$).

Significant negative correlations were observed between the changes in FGF21 levels and BMI ($r=-0.4$, $p=0.03$), between changes in FGF21 and the improvement of CPT values ($r=-0.58$, $p<0.001$) and between the changes in FGF21 and TNF-alpha levels ($r=-0.46$, $p=0.01$) in DN patients after 6-week physical activity. We found significant positive correlation between the changes in the levels of adiponectin and FGF21 ($r=0.39$, $p=0.037$) in DN patients after physical activity. We found a significant positive correlation between changes in BMI and TNF-alpha concentrations ($r=0.39$, $p<0.05$) in DN patients after physical activity. Significant negative correlation was observed between the changes in BMI and adiponectin levels ($r=-0.38$, $p<0.05$). There was no association between changes in the levels of TNF-alpha or FGF21 and changes in HbA1c levels in DN patients. We found a significant positive correlation between changes in CPT values and TNF-alpha concentrations ($r=0.62$, $p<0.001$) and a negative correlation between changes in CPT values and adiponectin levels ($r=-0.4$, $p<0.05$). We did not find any correlation between changes in CPT values and irisin levels.

Discussion

Despite efforts to make an early diagnosis, optimal glycemic control remains the best measure available to prevent or stop the progression of diabetic neuropathy. Chronic inflammation increased oxidative stress and impaired antioxidant response associated to hyperglycemia lead to endothelial cell dysfunction and increased expression of adhesion molecules such as VCAM-1 and ICAM-1. The expression of endothelial adhesion molecules is involved in the adhesion of

leukocytes and, subsequently, the progression of atherosclerosis and the occurrence of coronary heart disease. Chronic inflammatory state in obesity and insulin resistance, decreased NO bioavailability, increased VCAM-1 and oxidized low-density lipoprotein (oxLDL) levels may also be participating factors in the vascular complications of type 2 diabetes. A previous in vitro study on human endothelial cells investigated the suggested role of antioxidant ALA in TNF-alpha induced adhesion molecule expression and NF-kappaB signaling. They found that ALA dose-dependently inhibited TNF-alpha-induced IkappaB kinase activation, subsequent degradation of IkappaB, the cytoplasmic NF-kappaB inhibitor, and nuclear translocation of NF-kappaB. We also measured significantly higher VCAM-1 levels compared to controls, however, there was no significant changes in ICAM-1 and VCAM-1 levels after ALA treatment among diabetic patients. To date, there are no data on the effect of ALA on serum levels of VCAM-1 and ICAM-1 in diabetic patient populations. In a previous in vitro study, ALA suppressed the homocysteine-stimulated ICAM-1 and VCAM-1 expression of human aortic endothelial cells. Elevated levels of TNF-alpha are also associated with coronary artery disease via inhibition of NO-mediated dilation of coronary arterioles and subsequent production of superoxide in endothelial cells. Although serum NO level was higher in our patients compared to controls, the difference was not significant.

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO synthase, induces expression of adhesion molecules in endothelial cells, oxidation of LDL in vascular endothelium and also promotes the adhesion of the thrombocytes to the endothelium. Various studies documented that ADMA seems to be not only a cause of endothelial dysfunction, but also a predictor of the cardiovascular risk in type 2 diabetes. ADMA has been closely related to insulin resistance and elevated ADMA levels have demonstrated in type 2 diabetic patients with neuropathy, suggesting a role in the development of diabetic neuropathy. Other investigators have found higher ADMA levels in diabetic patients compared to non-diabetic controls, however, the changes in ADMA concentrations have not shown consistent relationship in subjects with or without diabetic neuropathy. In line with these previous data, we also found significantly higher ADMA levels in our patients with neuropathy before ALA treatment. Indeed, since ADMA is mainly metabolized by dimethylarginine dimethylaminohydrolase (DDAH), it is conceivable that the inhibition of ADMA via up-regulation of DDAH may be a novel therapeutic target for the prevention of CVD in patients with diabetes.

Multiple clinical trials have been proven the pathogenetically oriented, disease-modifying effect of ALA treatment. According to these studies, significant improvements in neurological

function, affecting both sensorimotor and autonomic components of the peripheral nervous system and in components of the CAN, were found in patients with diabetic neuropathy. Our results support the beneficial effect of ALA therapy on symptoms of peripheral neuropathy. The mechanism of improvement of neuropathic symptoms and nerve function is thought to be related to improvement in endothelial dysfunction.

In our study we have found significantly decreased ADMA levels and elevated NO concentration as a result of ALA treatment in type 2 diabetic patients. Moreover, we have found significantly lower serum TNF-alpha level after six-month treatment. These data might suggest that ALA modulates the activity of NO synthase involved in increasing endoneurial blood flow and decreasing the expression of endothelial adhesion molecules. This beneficial effect may be due to improved antioxidant status and endothelial function in diabetic neuropathy.

Although several authors have demonstrated decreased ADMA concentration in type 2 diabetic patients with diabetic nephropathy and neuropathy receiving ALA treatment for 6-12 weeks, there was no previous study focusing on the changes of ADMA levels after a long-term therapy. According to these studies, ADMA was found to be an independent risk factor for cardiovascular outcome in diabetic nephropathy and ALA treatment may be associated with cardiovascular risk reduction, in part by decreasing the plasma level of ADMA. ADMA inhibits the endothelial NO synthase, which may explain partly the impaired endothelial function and reduced endoneurial blood flow. Our findings may indicate a close association between chronic inflammation, increased endothelial ADMA concentrations and a decreased antioxidant activity in response to increased production of ROS in the pathogenesis of neuropathy in type 2 diabetes. We have found a significant positive correlation between the changes of TNF-alpha and ADMA levels after six-month ALA treatment. The same correlation was detected between the changes of serum TNF-alpha and ICAM-1 concentrations in our study. Based on these data, we assume that the elevated ADMA levels and oxidative stress may be responsible for endothelial dysfunction in diabetic neuropathy. However, supplementation with ALA may have a protective effect against diabetic nerve damage by restoring endothelial function and decreasing the production of the inflammatory cytokine TNF-alpha in type 2 diabetes. Our results support the initial hypothesis that increased ADMA level may be a marker of endothelial dysfunction and cardiovascular outcome in diabetic neuropathy. The level of ADMA correlated with the severity of peripheral sensory neuropathy and significantly lower ADMA concentration was detected in patients with decreased CAS after ALA treatment. Therefore, changes in serum ADMA levels may predict the clinical response to ALA treatment.

In or second study, we demonstrated significant correlations between the change of FGF21 concentration and change of body mass index, current perception threshold (measured by Neurometer®), as well as change of TNF-alpha and adiponectin levels in T2DM patients with peripheral neuropathy after six weeks of aerobic exercise. The levels of FGF21 were significantly increased during exercise in DN patients.

Previous research has shown that physical activity may increase the serum level of FGF21 in T2DM [5, 20]. A recent retrospecive study also revealed that the levels of serum FGF21 were elevated in patients with higher BMI compared to individuals with normal or low BMI. Moreover, the FGF21 concentrations were found to be higher in patients who exercised regularly compared to those who exercised only intermittently or not at all. our result may be explained by the activating effect of FGF21 on FGFR1 receptor and β-Klotho cofactor inducing the oxidation of fatty acids and the inhibition of lipogenesis. Experimental studies have shown that treatment with FGF21 increases insulin sensitivity, lowers triglyceride levels, and has a beneficial effect on body weight and fat distribution in obese animal models. Both white and brown adipocytes express high levels of β-Klotho and FGFR1c, a member of the FGFR family, consistent with sensitivity to FGF21 in adipose tissue. Plasma level of FGF21 is induced lipolysis in adipose tissue, especially via activation of hormone sensitive lipase and adipose triglyceride lipase. Further research has shown that FGF21 knockout mice exhibited decreased fasting blood glucose level, glucogenesis, liver beta-oxidation and ketogenesis demonstrating that FGF21 mediated the effect of peroxisome proliferator-activated receptor-alpha during the adaptation to fasting and exercise in skeletal muscle. In insulin resistance, mitochondrial respiratory chain deficiency associated with a compensatory response in skeletal muscle cells via increased expression and decreased degradation of FGF21 mRNA results in enhanced mitochondrial function through a PGC-1a dependent pathway. PGC-1α is a major regulator of mitochondrial biogenesis, by upregulating nuclear respiratory factor and mitochondrial transcription factor A, leading to an overall increase in mitochondrial DNA replication and gene transcription. FGF21 knockout mice fail to induce PGC-1a expression in response to a prolonged fast and have impaired gluconeogenesis and ketogenesis.

Growing evidence suggests that FGF21 may reduce atherosclerosis in cardiovascular disease. Recent research has demonstrated that FGF21 may inhibit arterial calcification in experimental models of vascular injury via various mechanisms including suppression of endoplasmic reticulum stress-mediated apoptosis and inhibiting the osteogenic transition of vascular smooth muscle cells. Paradoxically, there is a positive association between FGF21 levels and a number

of cardiovascular or metabolic diseases, such as coronary heart disease, obesity and T2DM. On the other hand, based on another clinical trial, acute myocardial infarction was also associated with a decrease in circulating FGF21 levels. Thus, a number of studies on this topic have been published with contradictory results in recent years, which have often shown inconsistencies and contradictions between studies in terms of metabolic parameters and medication of patients. The results of our study in patients with DN are in line with previous studies showing that exercise may increase the serum levels of FGF21 not only in patients with T2DM, but also in distal sensory polyneuropathy.

To date, there were no data on the effect of aerobic exercise on FGF21 levels among patients with distal sensory polyneuropathy. This is the first report on beneficial effect of physical activity on FGF21 levels in distal sensory polyneuropathy that strengthens the beneficial effects of physical exercise on sensory symptoms and neuropathic deficits in T2DM patients. The change of FGF21 level correlated with the severity of peripheral sensory neuropathy – defined by Neurometer® - after physical activity. Therefore, increased serum FGF21 levels may predict the clinical response to aerobic exercise. Previous studies have demonstrated a significant improvement in neurological function, affecting both sensorimotor and autonomic components of the peripheral nervous system, in patients with DN during physical exercise programs. The mechanism of improvement of neuropathic symptoms and nerve function is thought to be related to improvement in endothelial dysfunction and reduction of inflammation in DN. Our results support the initial hypothesis that increased FGF21 level may be a marker of chronic inflammation in DN. However, further studies are necessary to validate our results.

Although the levels of adipokines did not differ across groups after physical activity, the increasing tendency in adiponectin level was significantly associated with the magnitude of body weight loss and we found a positive correlation between the increase in the adiponectin level and the FGF21 concentration in DN. FGF21 shows functional similarity to adiponectin, which acts as a downstream effector of FGF21, controling glucose and lipid metabolism in adipocytes and skeletal muscle. Meanwhile, adiponectin may enhance the effect of FGF21 on energy balance and insulin sensitivity in this tissue; thus, the FGF21 – adiponectin axis may be implicated in the regulation of glucose and lipid homeostasis. We have found a significant association between the concentrations of adiponectin and improvements of neurological function, affecting sensorimotor component of the peripheral nervous system in patients with DN. Previous research has been reported that decreased adiponectin levels were associated with a significantly increased risk of DN in T2DM patients. Moreover, there was a strong

relationship between decreased nerve conduction velocity and adiponectin concentration in chronic inflammation and progression of diabetic sensorimotor neuropathy.

Our study revealed that six weeks of aerobic physical activity in patients with DN lead to a significant reduction in the levels of TNF-alpha and hsCRP. Recent studies have demonstrated the efficacy of physical exercise on inflammatory markers in DN. TNF-alpha plays a crucial role in initiating inflammatory processes leading to severe impairment of glucose tolerance and insulin sensitivity which may eventually increase the risk of cardiovascular diseases in T2DM. TNF-alpha stimulates lipolysis in adipose tissue, thus increased plasma concentration of FFA may contribute to atherogenesis in T2DM patients. Moreover, TNF-alpha enhances leptin production, which is known to regulate energy homeostasis by reducing pancreatic insulin secretion and promoting insulin resistance. Therefore, TNF-alpha may indirectly contribute to the development of insulin resistance by inhibiting adiponectin and stimulating leptin via glucose metabolic pathways. Our findings regarding the linear association between the changes of body mass index and TNF-alpha levels were generally consistent with prior research in patients with DN after exercise program. However, aerobic training may have a protective effect against diabetic nerve damage by restoring endothelial function and decreasing the production of the inflammatory cytokine TNF-alpha in T2DM.

While some prior studies have demonstrated positive association or contradictory results, we have not found association between the changes in irisin levels after physical activity. Previous research has shown that FNDC5 expression in skeletal muscle are reduced in obese subjects and circulating irisin levels are related with insulin sensitivity in T2DM. Studies examining the relationship between the circulating irisin levels and training-induced changes have yielded mixed results, with some studies suggesting a strong association and others finding no association. It was hypothesized that the level of serum irisin increased immediately after physical activity and seems to correlate with the intensity of exercise training, as well as prior empirical research suggest the contribution of irisin in the neuroprotective process of physical exercise in T2DM. Therefore, further follow-up studies should be performed to determine the effects of various factors directly or indirectly for changes in levels of irisin in T2DM with peripheral neuropathy.

Summary

Our results highlight the significant role of ALA administration in the long-term treatment of peripheral and cardiac autonomic neuropathy. Monitoring of ADMA serum level may predict the efficacy of ALA treatment detected by Neurometer® CPT testing. Further studies are needed to clarify the additional favourable effects of ALA in neuropathy. Data on other endothelial biomarkers may improve our knowledge about the effect of ALA treatment on endothelial dysfunction. However, the positive effect of ALA on endothelial function through the severity of peripheral and cardiac autonomic neuropathy may underline the importance of oxidative stress in the pathomechanism of diabetic neuropathy and enhances the development of novel antioxidant therapeutic agents.

Our research also demonstrated the potential role of regular physical activity in the treatment of diabetic neuropathy. According to our results, physical activity increased the levels of FGF21 in T2DM patients with distal sensory polyneuropathy. Monitoring of FGF21 levels may predict the efficacy of aerobic exercise in diabetic neuropathy. Data on other biomarkers of inflammation and oxidative stress may improve our knowledge about the effect of physical activity in peripheral sensorimotor neuropathy.

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