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

Associations between the serum level of progranulin and the markers of carbohydrate- and lipid metabolism and inflammatory processes in patients with familial hypercholesterolemia and diabetic polyneuropathy

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Introduction

Cardiovascular diseases (CVD) have been considered the leading cause of death in our country and in developed countries of the world for decades. Although the atherosclerosis as an underlying cause of CVD affects almost the entire population with advancing age, certain pathologies, such as familial hypercholesterolemia (FH) and type 2 diabetes mellitus (T2DM), appear in a significantly higher proportion and at an earlier age and lead to atherosclerotic vascular complications. Because of the increased cardiovascular risk, laboratory markers that can indicate the degree of atherosclerosis, help us identifying very high-risk patient groups, or help us predicting the degree of response to drug therapy are of increased importance in these pathologies. In addition, the discovery of these proteins and their role may help to map the therapeutic possibilities of a new drug in the future. Although intensive research has been conducted in recent decades to identify such molecules (mainly proteins), the number and applicability of biomarkers that can be used in everyday medical practice is limited. However, as a result of extensive research, it has become clear that during the development of atherosclerosis, disturbances in lipid and carbohydrate metabolism, chronic inflammatory and immune processes, oxidative stress, and the resulting endothelial dysfunction, vessel wall inflammation, plaque formation, plaque instability and the process of thrombus formation is very closely related to each other, and the regulatory processes often take place in a complex manner, mutually affecting the aggravation of individual components. One of the newly recognized actors of this complex regulatory process is progranulin (PGRN), which was initially identified as a growth factor and studied mainly in connection with the development of neurodegenerative diseases. However, it has now become clear that PGRN has a wide-ranging role in many areas of the body's functioning, including the regeneration of endothelial cells and neurons. However, the role of PGRN in FH and diabetic polyneuropathy as a complication of T2DM has not been fully elucidated.

Review of literature

Progranulin

PGRN, also known as acrogranin, proepithelin, GP88, granulin–epithelin precursor (GEP) or PC cell-derived growth factor (PCDGF), is highly expressed in mammalian cells. The coded PGRN is a 593 amino acid protein with a molecular weight of 68.5 kDa, rich in cysteine, which is secreted in a highly glycosylated form. PGRN can be expressed by many cell types, including epithelial cells, macrophages, T cells, neutrophil granulocytes, neurons, adipocytes, fibroblasts,

and chondrocytes. It is also produced by neurons, astrocytomas, microglia and endothelial cells in the central nervous system in response to various stressor effects, such as hypoxia, reduced glucose concentration, acidosis, or oxidative stress. In addition to the entire PGRN protein, 6 KDa peptides, so-called granulins (GRN A-G) also play an active role, mainly involved in the regulation of inflammatory and neuroprotective processes. Meanwhile, the direct binding of the secretory leukocyte protease inhibitor (SLPI) to the PGRN protein prevents enzymatic cleavage. The main apolipoprotein of HDL, apolipoprotein A1 (ApoA1), forms a complex with the PGRN molecule and also inhibits its conversion to granulin. The multifunctional nature of the protein soon became clear. Its regulatory role has been suggested in many biological and pathological processes that are crucial for the body, including cell growth and cell division, cell migration, cell death and regeneration, as well as angiogenesis, regulation of lysosomal homeostasis, phagocytic stimulation, immune cell activation, cytokine production, and in connection with the regulation of insulin resistance. As a result of all this, its role in the process of tumorigenesis, embryogenesis, wound healing and nerve cell regeneration emerged. The existence of a specific receptor for PGRN has not been proven at present. However, PGRN binds to several cell surface receptors, among which tumor necrosis factor receptors 1 and 2 (TNFR1 and TNFR2) are responsible for the anti-inflammatory role of the protein, while sortilin may be responsible for the neuronal effects. The affinity of PGRN to the TNF2 receptor is greater than that of tumor necrosis factor-α (TNF α), and it binds to TNFR1 and TNFR2 with equal affinity.

The role of PGRN in the development of atherosclerosis

Nowadays more and more data are available regarding the regulatory role of PGRN in atherosclerosis. Deletion of PGRN in ApoE knockout mice increased the development of atherosclerosis. PGRN was expressed in human carotid artery samples and exerted an anti-inflammatory effect. The serum level of PGRN proved to be an independent predictor of carotid artery atherosclerosis and was identified as a predictor of total mortality and functional outcome in patients with acute ischemic stroke. It has been described as a new biomarker of cardiovascular risk in patients with polycystic ovary syndrome. Increased production of PGRN in endothelial foam cells and intimal vascular smooth muscle cells has been described in tissue studies of human atherosclerotic plaques. In addition, PGRN -/- ApoE -/- knockout mice showed more severe atherosclerosis compared to PGRN +/+ Apo-E-/- mice. PGRN reduced monocyte chemoattractant protein-1-induced monocyte chemotaxis and vascular smooth

muscle cell interkeukin-8 production. However, the lack of PGRN induced the development of atherosclerosis in mice through the enhancement of the production of inflammatory cytokines and adhesion molecules, as well as the reduction of endothelial nitric oxide synthase (eNOS) activity. In addition, the lack of PGRN increased cholesterol accumulation in macrophages.

Familial hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal, dominantly inherited disease characterized by significantly elevated total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels observed since childhood, which leads to early generalized atherosclerosis and the serious complications, including ischemic heart disease, ischemic cerebral infarction and peripheral arterial diseases. The occurrence of heterozygous forms is common, previously it was considered to have a frequency of 1:500, but more recently the prevalence in the European population is currently approx. at 1:300, but the frequency can be as high as 1:200. In a previous study using data mining methods, the prevalence of heterozygous FH (HeFH) was 1:340 in the population of North-Eastern Hungary. Homozygotes occur with a probability of 1:1,000,000 in the average population. An important characteristic of the disease is the early onset of atherosclerosis, which in progressive cases causes the manifestation of cardiovascular diseases already in childhood in the homozygous form, and in young adulthood in the heterozygous form. The last decade has brought revolutionary changes in the treatment of FH. In the case of both heterozygous and homozygous FH, drugs with new mechanisms of action have been put on the clinical market. In the case of heterozygous FH, high-intensity statin (rosuvastatin 20-40 mg or atorvastatin 40-80 mg daily) and ezetimibe 10 mg daily treatment with PCSK9-inhibiting monoclonal antibodies (evolocumab and alirocumab) and small interfering RNA (inclisiran) de-emphasized significantly previously widely used form of extracorporeal treatment, the selective LDL apheresis treatment.

Diabetic polyneuropathy

Among the microvascular complications of type 2 diabetes mellitus, neuropathic complications are very common and can affect any part of the body. The four main types are peripheral neuropathy, proximal neuropathy, focal neuropathy, and autonomic neuropathy, of which the peripheral form is the most common. However, the development of autonomic neuropathy, including cardiac autonomic neuropathy, also significantly affects the quality of life and life prospects of patients.

The development of neuropathy in type 2 diabetes patients is a complex process, in which osmotic damage caused by high blood sugar levels, accelerated glycolysis, alternative metabolic pathways (polyol pathway, protein kinase-C (PKC) pathway and increased glycated end product (AGE) pathway and the hexosamine pathway), the reduced antioxidant capacity, the oxidative stress resulting from the production of reactive oxygen free radicals (such as superoxide (•O2-) anion), with increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and damage to mitochondrial oxidation associated with (ADP-ribose) polymerase (PARP) activation, endothelial dysfunction, and the development of local inflammatory processes triggered by activation of the nuclear factor-kappa B (NFκB) pathway and cyclooxygenase-2 (COX-2) play a key role. Various growth factors and genetic factors contribute to this, which ultimately lead to the development of nerve fiber damage. Diabetic neuropathy can be detected in about 15% of patients with type 2 diabetes mellitus at the moment of diagnosis, and during the 10-year duration of the disease, approx. causes complaints in 50%. The incidence of diabetic neuropathy increases significantly with age. The most common symptoms are the gradually developing increasing numbness. pinprick-like sensation, burning pain along the course of the affected nerve. The screening test with a calibrated tuning fork provides information on the sensation of vibration (deep sensation), which characterizes the functioning of the thick myelinated fibers. The monofilament test is used to examine superficial sensation and thin nerve fibers. The Neurometer® instrument is suitable for examining sensory function, which allows the examination of thick and thin myelinated as well as unmyelinated thin sensory fibers. The method gives a quantitative result, so its application allows monitoring the effectiveness of the treatment.

Peripheral neuropathy is often associated, even in an asymptomatic form, with cardiac autonomic neuropathy, for which Ewing's cardiac autonomic reflex tests are still considered a standard method in clinical practice. Within the five reflex tests, two large groups can be distinguished. The function of the parasympathetic innervation is examined by the deep breathing test, the Valsalva maneuver and measuring the heart rate change during standing up suddenly from lying down. The change in blood pressure values during standing up from lying down and the handgrip test primarily provides us with information about the functioning of the sympathetic nervous system.

The most important elements of diabetic neuropathy therapy are improving glycemic control, lifestyle management and reducing neuropathic pain. Diet, regular physical activity, proper control of lipid levels and blood pressure are particularly important elements in lifestyle treatment. Disease modifying drugs target the pathomechanism of the disease. These include

alpha-lipoic acid (ALA) and benfotiamine, as well as omega-3 fatty acids and aldose reductase inhibitors. Serotonin and norepinephrine reuptake inhibitors (duloxetine, velaflaxin), tricyclic antidepressants (amitriptyline, nortriptyline, desipramine, imipramine), anticonvulsants (pregabalin, gabapentin) and, in case of their ineffectiveness, opioid analgesics can be used to reduce pain. ALA has several clinically significant effects. It participates in the regulation of glucose and lipid metabolism and gene transcription. By increasing the activity of the GLUT4 transporter, it improves glucose uptake into muscle and fat cells. ALA has an antioxidant effect, as it is able to restore the intrinsic antioxidant system, including increasing the synthesis of glutathione. In addition, by forming a chelate, it is able to effectively remove heavy metals (iron, copper and zinc) from the circulation, which otherwise increase oxidative stress. Moreover, unlike other antioxidants, ALA can react with both fat-soluble and water-soluble components. ALA and its reduced form (dihydrolipoic acid – DHLA) are capable of inactivating reactive oxygen radicals. Through the activation of the NFκB signaling pathway, it increases the activity of nitric oxide synthetase (NOS), and at the same time inhibits the production of proinflammatory cytokines and adhesion molecules.

Aims

In our research, we aimed in untreated HeFH patients and in a matched control population:

- determining the serum PGRN level,
- examination of the correlation of the PGRN level with the serum lipid parameters,
- within this, the ratio of HDL and LDL subfractions,
- as well as inflammatory and endothelial function parameters.

We aimed to evaluate peripheral polyneuropathy T2DM patients before treatment and after 3 months of treatment with 600 mg of alpha-lipoic acid per day, as well as in a matched control population:

- determining the serum PGRN level,
- examination of the correlation of the PGRN level with the serum lipid parameters,
- with changes in the threshold value of the current sensation and the value of the composite autonomic score (CAS),
- as well as inflammatory and endothelial function parameters.

Materials and methods

Study Population

HeFH patients and control subjects

We enrolled 81 patients (females and males) with HeFH and 33 age- and gendermatched healthy control subjects. Patients were recruited from the Lipid Outpatient Clinic of the Department of Internal Medicine, University of Debrecen. All HeFH patients were either heterozygous with a confirmed LDL receptor gene mutation or fulfilled the Dutch Lipid Clinic Network diagnostic criteria for FH. The patients were referred to our Lipid Outpatient Clinic by GPs and other specialists, such as cardiologists and neurologists, to verify the diagnosis of HeFH and initiate optimal therapy. These were scheduled medical appointments from 08:00–10:00 AM and we asked the patients to arrive after 12 h of fasting. All patients were newly diagnosed without ongoing lipid-lowering medical treatment. The sample of patients referred to our General Outpatient Clinic for routine wellbeing physicals was used as the control. Only patients that were taking no medications and had no previous chronic diseases or acute illnesses in the past 3 months were selected as controls. Furthermore, from the physical examination of the controls, their electrocardiograms and laboratory tests, including lipid parameters, were free of any abnormalities. Patients with a previous history of type 1 or type 2 diabetes were excluded from the study, as well as subjects with alcoholism, known liver diseases, autoimmune or endocrine diseases and neurological or hematological disorders, which can be associated with peripheral polyneuropathy. Pregnant women or subjects with established malignancy were also excluded. All participants provided written informed consent. The study protocol was approved by the local and regional ethical committees (DE RKEB/IKEB 4775-2017, date obtained: 3 April 2020 and ETT TUKEB 34952-1/2017/EKU, date obtained: 30 June 2017) and the study was carried out in accordance with the Declaration of Helsinki.

Patients with type 2 diabetes with neuropathy and control subjects

This prospective study was performed in patients with type 2 diabetes with neuropathy as well as in age- and sex-matched control subjects who had diabetes without neuropathy. All subjects underwent a detailed neurological assessment to identify patients with diabetic neuropathy. Distal sensorimotor polyneuropathy was diagnosed by the presence of neuropathic symptoms and by vibration perception thresholds on the hallux of both feet using a 128-Hz tuning fork. Detection of neuropathic pain was performed according to the DN4 (Douleur Neuropathique en 4 Questions) questionnaire assessing numbness, pain, hypoesthesia, and tingling sensations. The Semmes–Weinstein monofilament test was used to identify sensory changes in protective

sensations of the feet.16 All patients were treated for 6 months with 600 mg ALA (WORWAG € Pharma GmBH, Boblingen, Germany) € administered daily by the oral route. In addition, all patients were adequately controlled with oral antidiabetic drugs (metformin, sulfonylurea, and/or dipeptidyl peptidase-4 inhibitors); subjects on insulin therapy were excluded. Patients with a previous history of diabetic proliferative retinopathy, diabetic nephropathy (estimated glomerular filtration rate < 60 mL/minute/ 1.73 m2 and/or persistent albuminuria), or type 1 diabetes were not included in the study. We excluded subjects with alcoholism, known liver diseases, autoimmune and endocrine diseases, or neurological and hematological disorders that may be associated with peripheral polyneuropathy. Subjects with prior cardiovascular disease, established coronary artery disease or myocardial infarction, or severe congestive heart failure (New York Heart Association class III–IV) were not included in the study, and smokers, pregnant women, and subjects with established malignancies were also excluded. All patients were recruited from the Diabetic Neuropathy Center of Debrecen, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary. All participants provided written informed consent. The study protocol was approved by the Regional and Institutional Ethics Committee, University of Debrecen, Clinical Center (UDCC REC/ IEC; 4775-2017) and by the Medical Research Council of Hungary, National Scientific and Ethical Committee (5287-2/ 2019/EUIG). We followed the STROBE € (Strengthening the Reporting of Observational Studies in Epidemiology) Statement guidelines for reporting observational studies. The study was performed in accordance with the Declaration of Helsinki.

Blood sampling

Venous blood samples were drawn after overnight fasting and sera were prepared immediately. Routine laboratory investigations (triglycerides, total cholesterol, low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], creatinine, uric acid, glucose, and hemoglobin A1c [HbA1c]) were performed with fresh sera using the Cobas c501 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany) in the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary. NonHDL-C was calculated as the total cholesterol minus HDL-C. The sera for enzyme activity measurements and for enzymelinked immunosorbent assay (ELISA) determinations were stored at -70C until analysis. The reagents were purchased from the same vendor and the tests were performed according to the recommendations of the manufacturer.

Measurement of Progranulin and Asymmetric Dimethyl Arginine (ADMA)

Serum PGRN and ADMA concentrations were measured using the commercially available competitive enzyme-linked immunosorbent assay (ELISA) (BioVendor, Brno, Czech Republic and DLD Diagnostika GmBH, Hamburg, Germany), with intra-assay CVs ranging from 3.38 to 4.35% and inter-assay CVs ranging from 6.36 to 7.99% in the case of PGRN and intra-assay CVs of 4.9–5.4% and inter-assay CVs of 4.3–9.6% in the case of ADMA. The values were expressed as ng/mL and μmol/L, respectively.

Measurement of TNFα

Serum TNFα was assessed using the TNFα ELISA (R&D Systems Europe Ltd., Abington, UK). The measurement of serum TNFα was performed according to the recommendations of the manufacturer. The intra-assay CVs ranged from 1.9 to 2.2% and inter-assay CVs from 6.2 to 6.7%. The values were expressed as pg/mL.

Measurement of oxLDL

The serum concentrations of oxidized LDL (oxLDL) were detected busing the commercial sandwich ELISA (Mercodia AB, Uppsala, Sweden), which is based on a direct sandwich technique where two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. The sensitivity of the oxLDL measurements was < 1 mU/L and the intra- and inter-assay coefficients of variation were 5.5–7.3% and 4–6.2%, respectively.

Measurement of sICAM-1, sVCAM-1 and sCD40L

The serum sICAM-1, sVCAM-1 and sCD40L were measured using the sandwich ELISA (R&D Systems Europe Ltd., Abington, UK). The ELISA procedures were carried out according to the manufacturer's instructions. The intra-assay and inter-assay CVs ranged between 3.7–5.2% and 4.4–6.7% (ICAM-1), 2.3–3.6% and 5.5–7.8% (VCAM-1) and 4.5–5.4% and 6.0–6.4% (sCD40L). The values were expressed as ng/mL

Measurement of Serum Myeloperoxidase

The serum concentrations of MPO were measured using the commercially available sandwich ELISA (R&D Systems Europe Ltd., Abington, UK). The intra- and inter-assay coefficients of variation were 6.5–9.4%. The ELISA assay was performed according to the manufacturer's instructions.

Determination of PON1 Enzyme Activity

The serum PON1 arylesterase activity was measured using phenylacetate substrate (Sigma Aldrich, Budapest, Hungary) and the hydrolysis of the phenylacetate was monitored at 270 nm at room temperature, as formerly described in. The serum PON1 paraoxonase activity was assayed on a microtiter plate via a kinetic semiautomated method using paraoxon (O,Odiethyl-O-p-nitrophenyl phosphate, Sigma-Aldrich, Budapest, Hungary) as a substrate. The hydrolysis of the paraoxon was followed at 405 nm at room temperature.

Lipoprotein Subfraction Analyses

The LDL and HDL lipoprotein subfractions were distributed using electrophoresis on polyacrylamide gel with the Lipoprint System (Quantimetrix Corporation, Redondo Beach, CA, USA), as previously described in [15]. Briefly, 25 μL of each patient's serum sample was transferred to polyacrylamide gel tubes containing 200 and 300 μL of loading gel and lipophilic dye (Sudan Black, Sigma Aldrich, Budapest, Hungary). After 30 min of photopolymerization, the tubes were electrophorized using a 3 mA electric current. Each electrophoresis chamber involved a quality control provided by the manufacturer (Liposure Serum Lipoprotein Control, Quantimetrix Corporation, Redondo Beach, CA, USA). The subfraction bands were scanned with an ArtixScan M1 digital scanner (Microtek International Inc., Hsinchu, Taiwan) and analyzed with Lipoware software (Quantimetrix Corporation, Redondo Beach, CA, USA). During the LDL subfraction analysis, up to seven LDL subfractions were distributed. The proportion of large LDL (large LDL %) was defined as the summed percentages of LDL1 and LDL2, whereas the proportion of small LDL (small dense LDL %) was defined as the sum of LDL3–LDL7. The cholesterol concentrations of LDL subfractions were determined by multiplying the relative area under the curve (AUC) of subfractions by the total cholesterol concentration. We calculated the total LDL-C as the sum of cholesterol in the IDL (MidA–C) and LDL subfractions (LDL1–LDL7), which correlated with the directly determined LDL–C. In the HDL subfraction tests, large (from HDL1 to HDL3), intermediate (from HDL4 to HDL7) and small (HDL8 to HDL10) subfractions were distributed between LDL and albumin peaks. The cholesterol contents of the HDL subfractions were calculated using the Lipoware software (Quantimetrix Corp., Redondo Beach, CA, USA), based on the relative AUC of the subfraction bands.

Assessment of autonomic and peripheral nerve function

All participants underwent detailed assessments of peripheral neuropathy (DN4 questionnaire to screen for neuropathic pain syndrome, vibration perception threshold, and quantitative

sensory testing) and in vivo corneal confocal microscopy by an ophthalmologist for the diagnosis of diabetic sensorimotor polyneuropathy. Peripheral sensory nerve function was assessed by current perception threshold testing (CPT) using a NeurometerVR (Neurotron Inc., Baltimore, MD, USA). It has been previously reported that this neurodiagnostic device is capable of detecting peripheral sensory neuropathy in various diseases, including diabetes mellitus.17 NeurometerVR CPT testing involved the delivery of sinusoidal alternating current stimuli at three different frequencies: 5 Hz, 250 Hz, and 2000 Hz, to assess the function of small unmyelinated C-fibers, small myelinated Aß fibers, and large myelinated Aß fibers, respectively. An intensity alignment was conducted to approach the sensory threshold with a – 50 mA range, out of a total range of 0 to 9.99 mA.18,19 Current stimuli were applied to the dorsal surfaces of the distal phalanges of the index finger and hallux unilaterally via two small electrodes. The intensity was increased until the participants experienced a painless sensation. NeurometerVR CPT testing automatically adjusts the level of stimulation based on the patient's response. Participants were presented with five to seven randomly generated sets of stimuli above and below their level of perception and were 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 using 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), the Valsalva maneuver, systolic blood pressure fluctuation to standing up, and changes in diastolic pressure during a sustained handgrip.20 A score was created to express the severity of autonomic neuropathy based on the results of the five tests (for each test, normal: 0, borderline: 1, abnormal: 2). The composite autonomic score (CAS) ranged from 0 to 10. A CAS of 0 to 1 was taken as normal, 2 to 3 as mild autonomic dysfunction, 4 to 6 as moderate autonomic dysfunction, and 7 to 10 as severe autonomic dysfunction.

Statistical Methods

The statistical analyses were performed using the Statistica 13.5.0.17 software (TIBCO Software Inc. Palo Alto, CA, USA) and GraphPad Prism 6.01 (GraphPad Prism Software Inc., San Diego, CA, USA). We performed the statistical power analysis using the calculator of SPH Analytics (SPH Analytics LTD., Alpharetta, GA, USA) to validate the difference in serum PGRN concentrations between FH (group 1) and control individuals (group 2). The statistical power was above 0.8 (0.98). The relationship between the two categorical variables was

calculated with a chi-squared test. The normality of distribution was tested by the Kolmogorov–Smirnov test. Comparisons between the groups were performed using Student's unpaired t-test in the case of normally distributed variables and the Mann–Whitney U test in the case of variables with non-normal distribution. The data were expressed as mean ± SD or median (upper–lower quartiles). Pearson correlation was used to investigate the relationship between the selected variables. One-way ANOVA was performed to study the effects of VLDL and HDL-C on serum PGRN in HeFH patients and controls. A multiple regression analysis was performed to determine the best predicted PGRN concentrations of the variables. $p \leq 0.05$ probability values were considered statistically significant.

Results

The anthropometric and laboratory parameters of the study individuals

Significantly higher serum total cholesterol, LDL-C, triglyceride, apoB100 and Lp(a) were found in HeFH patients compared to the control subjects, while serum HDL-C and ApoA1 did not differ significantly. Although PON1 arylesterase activity was significantly higher in HeFH patients compared to the controls, we could not find any significant differences in PON1 paraoxonase or salt stimulated paraoxonase activities. Serum myeloperoxidase, oxLDL, sICAM-1 and TNFα were significantly higher in HeFH patients compared to the controls, while there were no significant differences in serum hsCRP, sVCAM-1, sCD40L or ADMA between the two study groups. Serum PGRN did not differ significantly between HeFH patients and the controls.

The concentrations of lipoprotein subfractions in the study participants

The proportion and concentration of VLDL and the concentration of IDL subfractions were significantly higher in HeFH patients compared to the controls. Significantly higher small dense LDL ratios and concentrations, as well as lower mean LDL sizes, were also found in HeFH patients compared to the controls.

Serum progranulin in HeFH patients with and without previous acute myocardial infarction (, with and without previous vascular complications, with and without previous stroke and with and without carotid artery atherosclerotic disease

Slightly, but not significantly, higher PGRN concentrations were found in patients with a positive history of acute myocardial infarction (AMI) (n = 5, mean age at first AMI: 53.8 ± 5.9 years) compared to patients without AMI (n = 75) (43.07 vs. 37.46 ng/mL, p = 0.06) (Figure 4). There were no differences in PGRN between patients with and without vascular complications, stroke and CAAD in their history.

The correlations between serum triglyceride, lipoproteins, lipid subfractions, oxLDL, TNFα. sVCAM-1 and PGRN in heterozygous familial hypercholesterolomic patients

We found significant positive correlations between age (r = 0.21; p = 0.03), the ratio of VLDL subfractions (r = 0.24; p = 0.02) and serum PRGN, while the correlation between PGRN and VLDL was on the border of statistical significance (r = 0.19; p = 0.057). Significant negative correlations were found between the ratio of IDL subfractions (r = −0.24; p = 0.01), mean LDL

size ($r = -0.32$; $p < 0.001$) and PGRN in the whole study population. Serum triglyceride correlated positively with PGRN both in HeFH patients and the whole study population, but not in the controls. A significant negative correlation was found between HDL-C and PRGN in the whole study population, but not in the separate groups. We found significant negative correlations between the ratios of large HDL subfractions and serum PGRN in all patient groups, while there were significant positive correlations between the ratios of small HDL and serum PGRN in all populations. Serum TNFα correlated positively with PGRN in HeFH patients, controls and in the whole study population. There were significant positive correlations between serum sVCAM-1 and PGRN in HeFH patients and in the whole study population, but not in the controls. We did not find significant correlations between serum oxLDL and PGRN in HeFH patients ($r = 0.19$; $p = 0.11$) or in the whole study population ($r = 0.13$; $p = 0.20$); however, there was a significant correlation in the control subjects ($r = 0.45$; $p < 0.05$).

Clinical and laboratory data from patients with diabetes with and without neuropathy

Fifty-four patients with type 2 diabetes with neuropathy (22 men and 32 women, mean age: 64.1± 8.7 years; mean known type 2 diabetes duration before the initiation of our study: 12.4 years [interquartile range: 4.1– 14.7 years], and duration of diabetic neuropathy: 3.2 ± 1.4 years) were enrolled in the study. In addition, 24 age- and sex-matched control subjects, who had diabetes without neuropathy, were also enrolled in the study (mean known type 2 diabetes duration before the initiation of our study: 12.1 years [interquartile range: 4.0–14.6 years]. There were no significant differences in age, body mass index, waist circumference, or levels of glucose, creatinine, uric acid, HbA1c, ICAM-1, PGRN, or oxLDL and other lipid parameters between patients with and without neuropathy. TNFa levels were significantly higher in patients with neuropathy compared with controls.

Effects of ALA treatment in patients with diabetes with neuropathy

Patients with diabetes with neuropathy were treated with oral ALA daily for 6 months. TNFa levels were significantly lower after ALA treatment ($p=0.003$). In addition, significant improvements in CPT and CAS were observed after ALA treatment ($p < 0.05$ and $p < 0.01$, respectively). The levels of PGRN were significantly higher after ALA treatment (before ALA: 34.89 ± 7.13 ng/mL vs. after ALA: 36.23 ±7.93 ng/mL; $p < 0.05$). There was a significant negative correlation between PGRN and HDL-C levels ($r = -0.38$, $p<0.005$) and a significant positive correlation between PGRN and nonHDL levels ($r= 0.29$, $p < 0.05$) before ALA treatment. Significant positive correlations were demonstrated between PGRN levels and

ICAM-1 (r=0.45, p=0.001), oxLDL (r=0.36; p=0.009), and TNFα (r=0.37, p=0.007) levels in patients with diabetic neuropathy before ALA treatment. There were also significant positive correlations between PGRN levels and ICAM-1 (r= 0.49; p < 0.001), VCAM-1 (r =0.27; p=0.05), and TNFα (r=0.29; p=0.038) levels after ALA treatment. Moreover, a significant negative correlation was revealed between the changes in CPT and PGRN levels (r=0.31; p=0.037).

To test whether the associations detected in the univariate analyses were independent of other inflammatory parameters, we performed a multiple regression analysis using PGRN levels as the dependent variable. The model included ICAM-1, VCAM1, oxLDL, and TNFa levels. Using the backward stepwise analysis, PGRN levels were predicted by serum ICAM-1 levels both before (beta= 0.439; p < 0.001) and after (beta =0.488; p < 0.001) ALA treatment.

Summary of new findings

During the examination of untreated HeFH patients:

1. The patients' serum PGRN level did not differ significantly compared to the control group. We found markedly, but not significantly, higher serum PGRN levels in HeFH patients with a positive history of AMI compared to those without a positive history of AMI.
2. A significant positive correlation was found between the VLDL subfraction ratio and the serum PGRN level, while a significant negative correlation was found between the IDL ratio, the mean LDL particle size and the PGRN levels in the entire studied population.
3. Serum triglyceride levels were positively correlated with PGRN levels in HeFH patients and the entire study population. A significant negative correlation was found between HDL-C and PGRN serum levels in the entire study population. A significant negative correlation was observed between the ratio of the large HDL subfraction and the serum PGRN level, while a significant positive correlation was found between the ratio of small HDL and the serum level of PGRN in all studied populations.
4. A significant positive correlation between serum TNFα and PGRN levels was described in the whole study population. Serum sVCAM-1 was significantly and positively correlated with PGRN levels in HeFH patients and the whole study population.
5. The best predictor of the serum PGRN level in the backward stepwise multiple regression analysis was the ratio of sVCAM-1, lgTNFα and the small HDL subfraction.

During the examination of patients with diabetic neuropathy:

6. As a result of the 6-month ALA treatment, the serum PGRN level increased significantly.
7. The pre-treatment serum PGRN level showed a positive correlation with serum sICAM-1, oxLDL and TNF-α levels.
8. After 6 months of ALA treatment, serum PGRN level showed a positive correlation with serum sICAM-1, sVCAM-1 and TNF-α levels.
9. Changes in CPT was negatively correlated with the serum PGRN level of diabetic neuropathy patients before ALA treatment.
10. Based on the multiple regression analysis, the independent predictor of serum PRGN in patients with diabetic neuropathy was the serum sICAM-1 level both before treatment and after 6 months of ALA treatment.

Discussion

This is the first report to demonstrate the serum concentration of PGRN in patients with untreated familial hypercholesterolemia and its correlations with lipid parameters and the oxidative and inflammatory markers of atherosclerosis. PGRN has received attention as an important modulator of the inflammatory process as it binds directly to TNF receptors and disrupts TNFα signaling. PGRN has been shown to inhibit the TNFα-induced phosphorylation of p38 and c-Jun N-terminal kinase (JNK) and impair the nuclear translocation of nuclear factor-κB (NF-κB). Inflammation plays a pivotal role in the pathomechanism of atherosclerosis and contributes to all of its stages, from plaque initiation to maturation and rupture. In human endothelial cells, PGRN inhibits the atherosclerotic process induced by lipopolysaccharide through the activation of the Akt–eNOS pathway and the attenuation of the NF-κB pathway, resulting in the decreased expression of sVCAM-1, sICAM-1 and monocyte chemoattracant protein-1 (MCP1). Moreover, the deletion of PGRN exacerbated atherosclerosis in ApoE knockout mice through the promotion of inflammation, accumulation of excessive cholesterol in macrophages and altered activity and amount of HDL-associated protein demonstrates the anti-atherogenic effect of PGRN. We found significant positive correlations between TNFα, sVCAM-1 and PGRN in HeFH patients and in the whole study population, which may indicate that vascular inflammation associated with enhanced atherogenesis in HeFH patients induces the expression of PGRN, which in turn attenuates the inflammatory process mediated by TNFα and sICAM-1. HDL-C levels in FH populations have been extensively studied, but limited data are available on changes in the function, distribution, and concentration of HDL subfractions within this special patient population. Beyond LDL cholesterol, high HDL cholesterol is one of the main markers of longer CVD risk-free survival in HeFH. In contrast, most previous studies could not find differences between the HDL-C concentrations of HeFH patients and non-FH subjects. Moreover, enhanced cholesteryl ester transfer protein activity in HeFH leads to the triglyceride enrichment of HDL particles that are catabolized by an ApoE receptor pathway, resulting in a smaller HDL particle size. The elevated concentrations of small pre-βHDL particles and lower levels of large HDL2 particles are also reported by another study. Our results were in line with these data from the literature. The HDL-C concentration was similar in HeFH patients and the controls, but the concentrations and ratios of large HDL subfractions were significantly lower and the concentrations and ratios of small HDL subfractions were significantly higher in HeFH patients compared to the control population.

HDL functions, including the activities of HDL-associated enzymes, are often impaired in HeFH patients. HDL-associated PON1 hydrolyzes reactive oxygen species, resulting in decreased endogenous oxidative stress and the prevention of atherogenesis [29]. Some previous studies have observed decreased PON1 arylesterase activity in FH patients [30,31]. In contrast, we could not find significant differences between the PON1 paraoxonase, salt stimulated paraoxonase or arylesterase activities of HeFH patients and the controls, although the higher oxLDL and increased MPO activity demonstrated the increased oxidative stress in our HeFH population, which was similar to some other previous observations.

Previously, a significant negative correlation between HDL-C and PGRN was described in patients with myocardial infarction. In our HeFH patients and in the whole study population, PGRN correlated positively with triglyceride and there was a negative correlation between HDL-C and PGRN in the whole study group, but not in HeFH patients. However, there were significant negative correlations between the ratio of large HDL and PGRN and significant positive correlations between the ratio of small HDL and PGRN in all participants, as well as each group separately. These correlations between PGRN and HDL subfractions are novel data. Furthermore, based on the multiple regression analysis, PGRN was best predicted by the small HDL subfraction, which supports the putative role of PGRN in HDL metabolism.

Previous studies using carotid ultrasounds have demonstrated atherosclerotic plaque in 90% of asymptomatic patients with FH but without known atherosclerotic diseases. PGRN has been found to be expressed in human atherosclerotic lesions. Moreover, PGRN levels are significantly higher in patients with myocardial infarction compared to control subjects. Therefore, we expected a higher serum concentration of PGRN in HeFH patients compared to normocholesterolemic subjects, but we could not find any significant differences between the two groups. We also compared our HeFH patients with AMI in their history to those who had not previously had AMI. Although serum PGRN was higher in HeFH patients with previous AMI, the difference was not significant, mostly because of the marked inter-individual variability of PGRN in the non-AMI group. Moreover, PGRN may be higher during the acute or subacute phase of AMI compared to the chronic phase. Further studies on a larger AMI population with long-term follow-ups are needed to clarify the kinetic changes of serum PGRN in AMI.

There is a considerable residual risk of clinical events in a large proportion of patients with atherosclerotic diseases, possibly driven in part by inflammatory processes. Lipid-lowering therapy used in HeFH may also modulate the inflammatory effects of atherosclerosis, both

indirectly by attenuating LDL cholesterol mediated inflammation as well as directly by modulating inflammatory signaling.

TNFα plays a major role in the inflammatory response of vascular endothelial cells via the induction of cell adhesion molecules, including VCAM-1 and ICAM-1, which induce neutrophil adhesion to endothelial cells. Therefore, PGRN has a dual mechanism of action: by suppressing neutrophil recruitment, it both inhibits neutrophil chemotaxis by reducing TNFa-induced ICAM-1 expression and ameliorates endothelial inflammation. A previous has study reported that higher PGRN levels are associated with more microvascular complications in patients with type 2 diabetes, including patients with diabetic nephropathy, neuropathy, and retinopathy. To date, however, there are no data regarding the effects of ALA on PGRN levels among patients with type 2 diabetes. This is the first report of the effects of ALA treatment on PGRN levels in diabetic neuropathy. Our findings indicate that ALA treatment has beneficial effects on sensory symptoms and neuropathic deficits in patients with diabetes. The endothelium regulates inflammatory processes in the walls of blood vessels by producing biologically active agents. In diabetic neuropathy, the activation of alternative metabolic pathways is strongly associated with intracellular hyperglycemia-induced oxidative stress, and endothelial cells are unable to compensate for increased oxidative stress with nitric oxide production, which may lead to increased oxLDL. Other investigators have reported that oxLDL-induced activation of NF-κβ attenuates the expression of cell-adhesion molecules (e.g., ICAM-1 and VCAM-1) and provokes inflammation in endothelial cells. These processes in diabetic neuropathy lead to functional changes of the vasa vasorum, thus causing neuronal ischemia and direct axonal damage. It has been reported that PGRN may alleviate neuronal injury induced by ischemia–reperfusion in mice via the inhibition of neutrophil recruitment, resulting in the decreased activation of NF- κβ and matrix metalloproteinase-9. We identified a significant positive correlation between PGRN and TNFa levels in patients with diabetic neuropathy. As has previously been reported, PGRN directly binds to TNFR in vitro, and we hypothesize that a compensatory increase in PGRN levels occurs as a result of TNFa-induced activation of NF-κB, which is associated with neuroinflammation and oxidative stress in diabetic neuropathy. In the present study, we also revealed a positive correlation between PGRN and ICAM-1 levels, as well as between PGRN and oxLDL levels. Correlations among these markers indicate that the action of PGRN may play an important role in inflammatory processes by inhibiting TNFa-induced activation of the NFκB and MAPK, as a competitive antagonist of TNFR. Furthermore, because of its anti-inflammatory effects, PGRN may be a useful marker for assessing the levels of oxidative stress in diabetic neuropathy. We hypothesize that the elevation in PGRN levels

may represent a compensatory response in diabetic neuropathy to reduce the NF-κB-mediated expression of chemokines and intercellular adhesion molecules (e.g., ICAM-1 and VCAM-1) and to protect endothelial cells from atherosclerotic inflammation.

Although the correlation was not significant, the positive tendency between PGRN and LDL-C levels and the significant negative correlation between PGRN and HDL-C concentrations support the aforementioned findings. The levels of non-HDL-C, which includes all cholesterol present in lipoprotein particles that are considered to be atherogenic, including LDL, very-LDL, very-LDL remnants, and lipoprotein(a), correlated positively with PGRN levels. We propose that alterations in PGRN levels may be closely related to endothelial dysfunction and dyslipidemia in diabetic neuropathy. Pr evious research has demonstrated that PGRN is protected from degradation by its binding proteins, including secretory leukocyte protease inhibitor and apolipoprotein A-I, which is the predominant protein in plasma HDL. Other investigators have suggested that the anti-inflammatory effects of HDL on macrophages may be caused by the suppression of PGRN cleavage and granulin production. The significant positive correlation between PGRN and HDL-C levels in the present study thus supports the protective role of HDL in PGRN cleavage.

However, further studies are necessary to validate our conclusions. Some limitations of our study should be noted. The relatively small size of the study population and the low number of men in our sample may reduce the power of the analysis; however, the significant correlations between inflammatory markers, CPT, and PGRN levels highlight the association between PGRN and neuronal repair. Therefore, larger studies with long-term follow-up in this patient population are necessary to reveal the effects of PGRN on neuronal repair mechanisms in diabetes-induced oxidative stress.

Summary

Progranulin (PGRN) is a growth factor that has been shown to be expressed in many tissues, including epithelia, myeloid- and lymphoid-derived cell lines, neurons, and microglia. PGRN has been hypothesized to directly bind to tumor necrosis factor receptor (TNFR), suppress tumor necrosis factor alpha (TNFα)-mediated inflammation and exert anti-atherogenic effects. However, the serum concentrations of PGRN in patients with heterozygous familial hypercholesterolemia (HeFH) have not been studied. Moreover, PGRN may alleviate neuronal injury induced by ischemia–reperfusion in mice via the inhibition of neutrophil recruitment. The antioxidant alpha-lipoic acid (ALA) is used in diabetic neuropathy to improve nerve conduction and relieve neuropathic pain, but its effects on PGRN levels have not yet been elucidated. Therefore, we aimed to measure PGRN in the sera of patients with untreated HeFH. Furthermore, we aimed to assess the relationship between inflammation and endothelial dysfunction markers including TNFα, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular adhesion molecule-1(sVCAM-1) and PGRN levels in in HeFH patients and in type 2 diabetic patients with peripheral neuropathy after 6 months of ALA treatment. Moreover, we examined the association between changes in PGRN levels and the severity of peripheral sensory neuropathy after ALA treatment.

Therefore, 81 untreated patients with HeFH and 32 healthy control subjects were included. We diagnosed HeFH using the Dutch Lipid Clinic Network criteria. Serum PGRN, sICAM-1, sVCAM-1, oxidized LDL (oxLDL) and TNFα concentrations were determined by enzyme-linked immunosorbent assay. Lipoprotein subfractions were detected by Lipoprint gel electrophoresis. Furthermore, in a prospective study, 54 patients with type 2 diabetes and peripheral neuropathy received 600 mg of ALA daily for 6 months. Current perception threshold (CPT) testing was used to assess sensory neuropathy. Twenty-four patients with diabetes without neuropathy were also included in the study.

We could not find a significant difference between the PGRN concentrations of the HeFH patients and controls. We found significant positive correlations between triglyceride, TNFα, sVCAM-1, the ratio of small HDL subfraction and PGRN, while significant negative correlations were found between the ratio of large HDL subfraction and PGRN both in the whole study population and in HeFH patients. PGRN was predicted by sVCAM-1, logTNFα and the ratio of small HDL subfraction. After 6 months of ALA treatment of diabetic patients with peripheral neuropathy, serum PGRN levels were significantly increased, and CPT values

were significantly improved. Furthermore, there were significant positive correlations among TNFα, sICAM-1, and PGRN levels both before and after ALA treatment. A significant negative correlation was observed between the improvements in CPT and the PGRN levels. Furthermore, sICAM-1 levels were an independent predictor of PGRN levels in diabetic patients with peripheral neuropathy.

The strong correlations between HDL subfractions, inflammatory markers and PGRN suggest that PGRN may exert its anti-atherogenic effect in HeFH through the alteration of HDL composition and the amelioration of inflammation rather than through decreasing oxidative stress. Furthermore, changes in serum PGRN levels indicate that ALA treatment may have beneficial effects on endothelial function and neuronal inflammation in diabetic patients with peripheral neuropathy. Further studies are needed to clarify the role of PGRN in regulating the atherosclerotic process in HeFH and the importance of PGRN in neuronal repair among patients with diabetic neuropathy. Understanding the role of PGRN in athero-inflammation and neuronal repair may alter our therapeutic strategy for patients with familial hypercholesterolemia and diabetic patients with peripheral neuropathy.

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List of publications related to the dissertation

1. **Nádró, B.**, Lőrincz, H., Juhász, L., Szentpéteri, A., Sztanek, F., Varga, É., Páll, D., Paragh, G., Harangi, M.: Determination of Serum Progranulin in Patients with Untreated Familial Hypercholesterolemia.
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List of other publications

3. Kovács, B., Németh, Á., Daróczy, B., Karányi, Z., Maroda, L., Diószegi, Á., **Nádró, B.**, Szabó, T., Harangi, M., Páll, D.: Determining the prevalence of childhood hypertension and its concomitant metabolic abnormalities using data mining methods in the Northeastern region of Hungary.
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Vessel Plus. 1, 91-95, 2017.

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