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Levels of neuroinflammation markers in type 2 diabetes mellitus patients with diabetic retinopathy and genetically determined hyperhomocysteinemia

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Background: *Hyperhomocysteinemia determined by polymorphisms of genes encoding folate cycle enzymes is closely correlated with markers of systemic inflammation. Prolonged excessive cytokine production in the neural tissue in patients with type 2 diabetes mellitus (T2DM) causes gliosis, posing a threat of neuroinflammation, a potential pathogenetic component of diabetic retinopathy (DR).*
Purpose: *To assess plasma levels of neuroinflammation markers (IL-1β and IL-10) and gliosis marker, non-neuronal enolase (NNE), in T2DM patients with DR and genetically determined hyperhomocysteinemia.*
Methods: *One hundred and six T2DM patients with DR were involved in the study. DR severity was classified according to ETDRS DR severity system (DRSS) as non-proliferatve (NPDR; DRSS level 47–53), proliferative (NPDR; DRSS level 47–53) and advanced (APDR; DRSS level 81-85). The control group was composed of 64 individuals. Cases and controls were comparable for age, sex and way of life. MTHFR C677T (rs1801133), MTHFR A1298C (rs1801131), and MTR A2756G (rs1805087) genotypes were determined using the TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystem). Enzyme-linked immunosorbent assay (ELISA) kits were used to assess plasma levels of L-homocystein, cytokines and NNE.*
Results: *Plasma IL-1β levels were 1.7 times higher in patients with diabetes duration shorter than 15 years compared to those with longer diabetes duration. In addition, plasma NNE levels were higher in the former patients, but the difference was not significant. There was correlation of plasma L-homocystein levels with plasma IL-10 ($R = 0.357$, $p < 0.01$), IL-1b ($R = 0.320$, $p < 0.01$) and NNE ($R = 0.286$, $p < 0.01$) levels. Plasma NNE levels correlated with plasma IL-10 ($R = 0.279$, $p < 0.01$) and IL-1beta ($R = 0.368$, $p < 0.01$).*
Conclusion: *Elevated plasma levels of pro-inflammatory cytokines in patients indicate an important role of neuroinflammation in the pathogenesis of DR. The rs1801131 CC, rs1805087 GG and rs1801131 CC genotypes may be considered risk factors for the development of DR in patients with T2DM.*

Keywords:
cytokines, non-neuronal enolase, diabetic retinopathy

Introduction

Type 2 diabetes mellitus (T2DM) is a global endemic with rapidly increasing prevalence in both developing and developed nations. Diabetic retinopathy (DR) is a dangerous diabetic complication that, if untreated, may cause severe vision loss and even blindness. Research on the pathogenesis of DR is still ongoing and important since there are numerous factors involved [1]. Now, the paradigm is shifting towards a more comprehensive view of diabetic retinal disease as a tissue-specific neurovascular complication. The term "neurovascular unit" (NVU) was first applied to the blood-brain barrier, then to the retina; the retinal NVU is composed of neurons, glial cells and the retinal intravascular network. Recent evidence suggests that DR is the result of a global dysfunction of the NVU: the activation of glial cells (astrocytes, Müller cells, and microglia) and degeneration of neural elements (ganglion, bipolar, horizontal and amacrine cells). These components are considered as target for potential pharmacological therapy in patients with DR [2, 3].

Therefore, it is believed that prolonged hyperglycemia causes not only microvascular damage but also intraretinal inflammation and neuronal degeneration. Neuroinflammation and neurodegeneration are common

phenomena occurring in any stage of DR and entangled with the effects of vascular exudation and retinal ischemia [4-7]. Research on the development of neurodegenerative processes in the presence of genetically determined impaired folate metabolism is underway [8-10]. Every third individual is a carrier of a polymorphism coding for folate cycle enzyme synthesis. Hyperhomocysteinemia is an established risk factor for atherosclerotic and cardiovascular diseases and develops in carriers of a combination of unfavorable polymorphisms [11]. Homocyteine-induced toxicity toward vascular endothelial cells might participate in the formation of retinal vascular diseases [12]. Hyperhomocysteinemia, and impaired folate cycle and vitamin B metabolism have been demonstrated to be involved in the pathogenesis of DR in type 2 diabetes mellitus (T2DM) [13, 14]. The patterns identified pave the way to personalized management of patients with DR and recommendations for the prevention of T2DM complications.

Most studies on the effect of hyperhomocysteinemia on the development of autism, dementia, parkinsonism, and Alzheimer's disease provide reasonable grounds to consider neuroinflammation as an important component of neurodegeneration [15]. There is, however, paucity of data elucidating (a) which brain circuits are especially susceptible to hyperhomocysteinemia-induced damage and (b) the role of hyperhomocysteinemia-induced inflammation in DR.

The structural and functional properties of two brain enolases have been described. Immunocytochemical techniques have established that one brain enolase is restricted to neuronal cells (neuronal-specific enolase or NCE) while the other is localized in glial cells (non-neuronal enolase or NNE). The brain enolases, therefore, represent the first example of functional markers for neuronal and glial cells in the brain. Glial cells are known to be the first responders to systemic inflammation and cytokines [16].

The purpose of the study was assess the levels of neuroinflammation markers (IL-1β, IL-10) and gliosis marker (NNE) in patients with DR associated with T2DM in the presence of genetically determined hyperhomocysteinemia.

Methods

This was a prospective, observational clinical case-control study.

The procedures followed were in accordance with the ethical standards of the Helsinki declaration (1964, amended most recently in 2008) of the World Medical Association. The study was conducted in compliance with the requirements of the Council of Europe Convention on Human Rights and Biomedicine, and relevant laws of Ukraine. Informed consent was obtained from all participants of the study.

The case group included 106 patients with T2DM whose median age (interquartile range or IQR) was 65.68 (48–80) years. Of the 106 patients, 71 (67%) were women and 35 (33%) were men. Diabetes duration ranged from 7 to 14 years in 63 patients, and from 15 to 34 years in 43 patients.

Patients underwent eye examination as per the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol including visual acuity assessment, refractometry, static Humphrey perimetry, tonometry, and biomicroscopy, ophthalmoscopy with the Volk Super Field lens and Goldmann three-mirror lens (Volk Optical, Mentor, OH) and fundus photography (the ETDRS seven standard fields). In addition, they underwent spectral domain optical coherence tomography (SD-OCT; Copernicus REVO, Optopol Technology Sp, zo.o, Zawiercie, Poland). Gonioscopy was performed, if required. DR severity was classified according to ETDRS DR severity system (DRSS) as non-proliferatve (NPDR; DRSS level 47–53), proliferative (NPDR; DRSS level 47–53) and advanced (APDR; DRSS level 81-85) [17]. Group 1 (NPDR) included 58 patients, group 2 (moderate PDR), 25 patients, and group 3 (advanced PDR), 23 patients.

The control group included 64 individuals without diagnosed metabolic abnormalities who were referred to the Clinical and Diagnostic Laboratory (University Clinic, Bogomolets National Medical University) for a medical check-up routine. Of these 64 individuals, 38 (60%) were women. The median age (IQR) for controls was 58.4 (42–79) years.

A venous blood sample was collected and transferred into a 4-ml K3 ethylenediaminetetraacetic acid (EDTA) blood collection tube in the laboratory procedure room. After centrifugation, the plasma was transferred to an Eppendorf tube and frozen at -20ºC until enzyme-linked immunosorbent assay (ELISA) analysis, and the precipitated cell layer was stored at -20ºC until genetic analysis. The cell sample was used in real-time PCR to identify single nucleotide polymorphisms (SNPs). ELISA was used to assess plasma lecytokine, NNE and homocystein levels. Plasma lecytokine, NNE and homocystein levels were determined using HiPo MPP-96 microplate reader (Biosan, Riga, Latvia), Intelliwasher 3D-IW8 plate washer (Biosan), PST-60HL-4 microplate thermoshaker (Biosan), Elabscience Human IL-1β (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China; sensitivity, 4.69 pg/ml; detection range, 7.81-500 pg/ml) ELISA Kit. Elabscience Human IL-10 (E-EL-H6154; sensitivity, 0.94 pg/ml; detection range, 1.56-100 pg/ml) ELISA Kit, and Elabscience Human NNE (E-EL-H1260; sensitivity, 0.19 ng/ml; detection range, 0.31-20 ng/ml) ELISA Kit. Plasma L-homocystein levels were assessed using Axis® Homocysteine Enzyme Immunoassay (EIA) kit (AXIS-SHIELD Diagnostics Ltd, Dundee, Scotland, UK, Cat No. FHCY100). Quantassay 0.8.2.6 software was used for data processing. Methylenetetrahydrofolate reductase (MTHFR) C677T (rs1801133), MTHFR A1298C (rs1801131), and methionine synthase (MTR) A2756G (rs1805087) polymorphisms of folate cycle enzymes were determined. All studies were performed

according to the manufacturers' instructions and standard protocols. Genomic DNA was extracted using PureLink® Genomic DNA Kits for purification of genomic DNA (Invitrogen Corp., Carlsbad, CA, USA). The TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystem) were used to analyze polymorphic DNA loci. The above studies were conducted at the laboratory (Research Institute of Experimental and Clinical Medicine, Bogomolets National Medical University) in accordance with the cooperation agreement.

Data were analyzed using INM SPSS version 23 and MedStat software. Normality of quantitative data was established using the Shapiro-Wilk test. Pearson and Spearman correlation analyses were conducted to examine significant associations between variables. If data samples were non-parametric, the data was presented as median and standard error (SE) of the median. Bar charts are provided with 95% confidence intervals (CI).

Results

First, we conducted the analyses of gene polymorphisms occurring in patients of the case group (n = 106) and controls (Fig. 1). rs1801133 heterozygous C/T genotype was most common (53% of cases and 59% of controls), followed by CC genotype (39% of cases and 22% of controls) and TT genotype (8% of cases and 19% of controls).

Analysis of rs1805087 genotypes found that, among the controls, the major AA genotype was more common than heterozygous AG genotype (64% versus 36%, respectively). We, however, found no GG carriers among the controls. Among patients with DR, rs1805087 AA genotype was most common (51%), followed by AG genotype (42%) and 7%.

Analysis of rs1801131 genotypes found that the heterozygous AC genotype was most common (51% of cases and 54% of controls), followed by AA genotype (32% of cases and 40% of controls) and CC genotype (17% of cases and 6% of controls).

Further analysis of the distribution of respective alleles and odds ratios for the examined parameters found no association of MTHFR C677T, MTHFR A1298C, and MTR A2756G genotypes with the risk of developing DR in the presence of T2DM. We, however, used the above data for further identification of associations of the most common genotypic variants and polymorphisms with phenotypic characteristics of hyperhomocysteinemia, systemic inflammation and neural tissue damage.

Plasma homocysteinemia levels were statistically significantly higher in patients than in controls (Fig. 2). Plasma L-homocystein levels in patients with DR were 1.58 times higher in patients compared to controls (22.21 ± 6.3 µmol/l versus 14.00 ± 5.6 µmol/l; p ≤ 0.05). There was, however, almost no difference in plasma L-homocystein among the groups of patients with DR (group 1, 21.7 ± 7.08 µmol/l, group 2, 22.33 ± 4.9 µmol/l, and group 3, 22.36 ± 5.28 µmol/l).

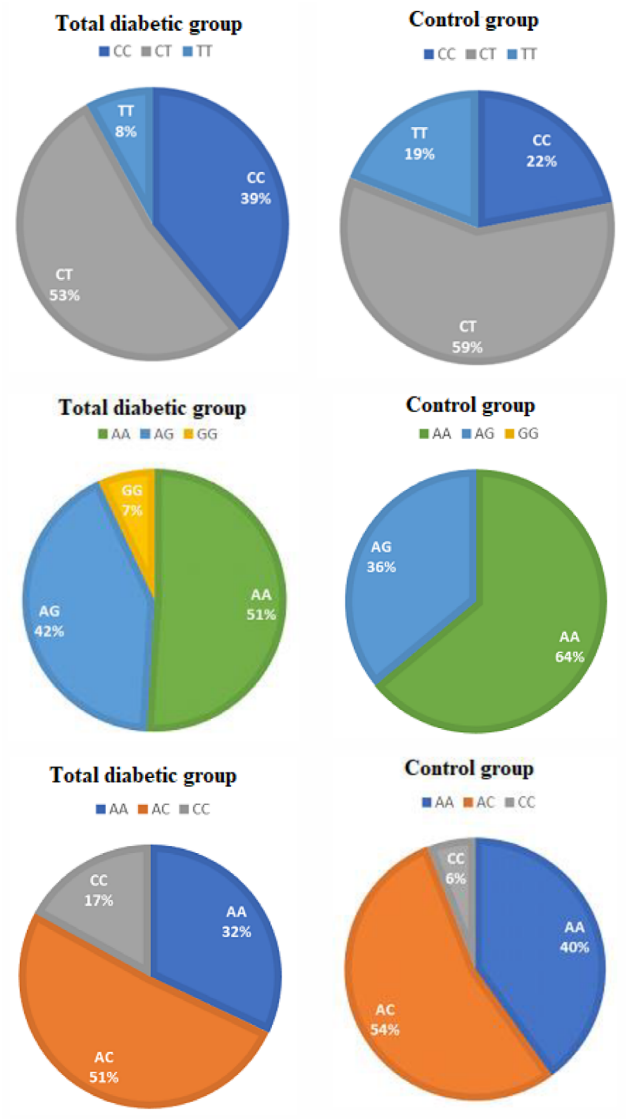

Fig. 1. Pie charts for the frequency distributions of alleles and genotypes of rs1801133 (CC, CT, TT), rs1805087 (AA, AG, GG), rs1801131 (AA, AC, CC) in total patients with DR and controls

Plasma IL-1β levels in total patients with DR were 9 times higher compared to controls (8.7 ± 6.4 pg/ml versus 0.96 ± 0.8 pg/ml; p ≤ 0.05). Patients with NPDR showed the highest plasma IL-1β levels (9.01 ± 6.3 pg/ml), followed by patients with advanced PDR (6.06 ± 3.2 pg/ml) and moderate PDR (8.7 ± 2.6 pg/ml). Therefore, a significant difference in plasma IL-1β level between total T2DM patients with DR and controls indicates the presence of inflammation in these patients.

Plasma IL-10 levels in total patients with DR were twice higher compared to controls, and this difference was also significant (3.37 ± 2.0 pg/ml versus 1.73 ± 1.2 pg/ml; p ≤ 0.05). There was practically no difference in plasma IL-10 level between groups of patients with NPDR (3.37 ± 1.87 pg/ml), moderate PDR (3.24 ± 1.6 pg/ml), and advanced PDR (3.27 ± 1.4 pg/ml). This was almost

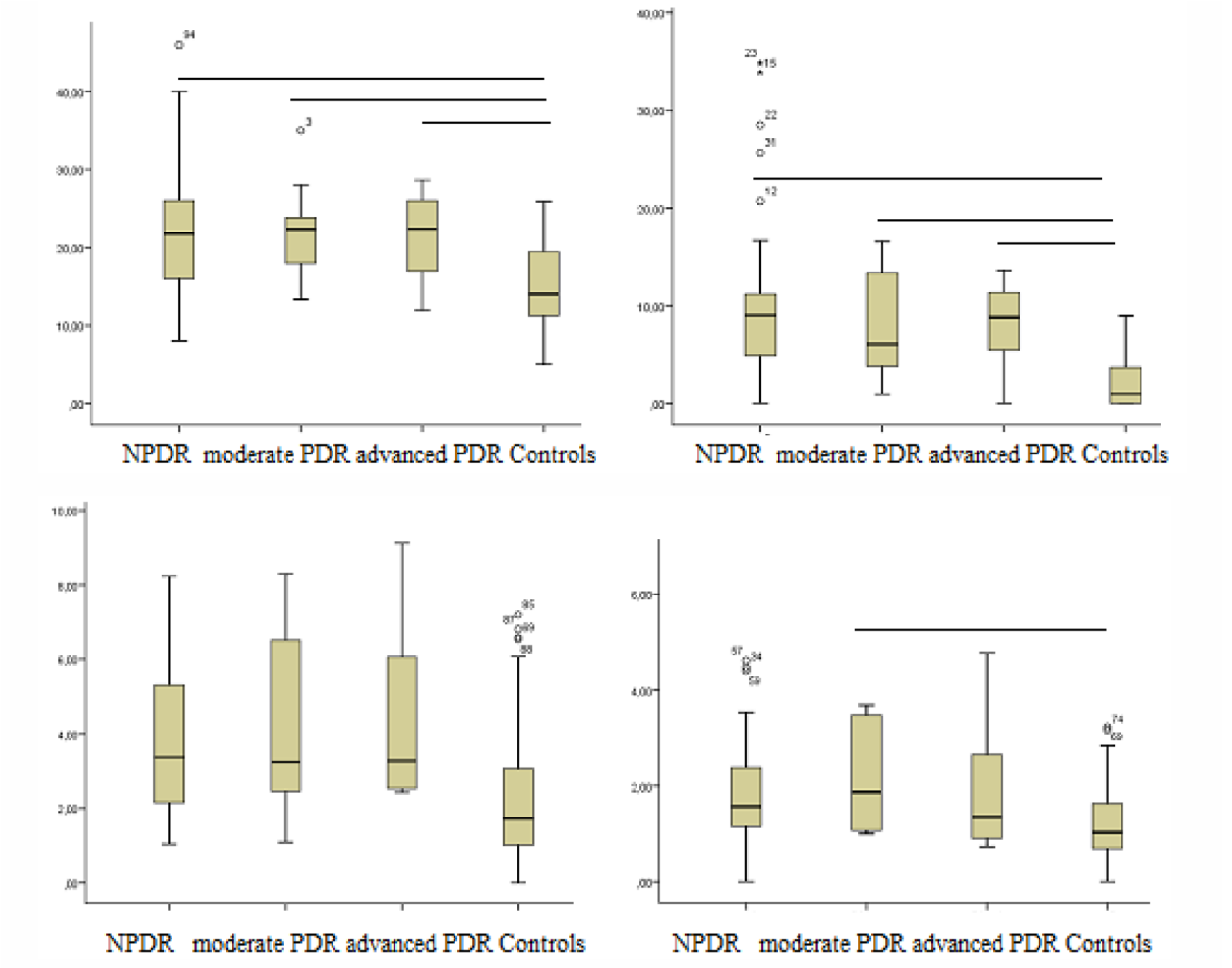

Fig. 2. Box plots depicting plasma L-homocystein (A), IL-1β (B), IL-10 (C), and non-neuronal enolase (NNE) (D) levels in patients with non-proliferative diabetic retinopathy (NPDR), moderate proliferative diabetic retinopathy (PDR) and advanced PDR compared to controls. Notches indicate the 95% confidence interval of the median bolded midline indicates median value, and box indicates the first and third quartiles. Note: *, significant difference ($p < 0.05$) from controls

twice higher than in controls, but there was no significant difference due to high variance.

Plasma NNE levels in total patients with DR were higher compared to controls, but this difference was not significant (1.51 ± 0.33 ng/ml versus 1.07 ± 0.06 ng/ml; $p > 0.05$). There was, however, a significant difference in plasma NNE levels between patients with moderate PDR and controls (1.87 ± 1.3 ng/ml versus 1.07 ± 0.06 ng/ml; $p \leq 0.05$). In addition, plasma NNE levels in patients with NPDR and advanced PDT were 1.56 ± 0.9 ng/ml and 1.35 ± 0.15 ng/ml, respectively.

Therefore, in the current study, indices of systemic inflammation and neural tissue damage were higher in patients compared to controls, indicating a significant role of neuroinflammation in the pathogenesis of DR.

Table 1 compares patients with diabetes duration of 7 to 14 years and those with diabetes duration of 15 to 34 years with regard to mean plasma IL-1β, IL-10 and NNE levels.

Plasma IL-1β levels were 1.7 times higher in the former patients ($p < 0.05$). Plasma NNE levels were higher in patients with a diabetes duration shorter than 15 years, but the difference was not significant. There was practically no difference in plasma IL-10 levels between patients with diabetes duration of 7 to 14 years and those with diabetes duration of 15 to 34 years. It is likely that the synthesis of pro-inflammatory cytokines is more profound in early DR, with these cytokines affecting neural glial cells, causing gliosis and contributing to the development of neuropathy. At later stages of DR, the body becomes adapted to inflammation, and the level of inflammation decreases.

There was bilateral correlation of plasma L-homocystein levels with plasma IL-10 ($R = 0.357$, $p < 0.01$), IL-1b ($R = 0.320$, $p < 0.01$) and NNE ($R = 0.286$, $p < 0.01$) levels (Table 2). In addition, plasma NNE levels correlated with plasma IL-10 ($R = 0.279$, $p < 0.01$) and IL-1beta ($R = 0.368$, $p < 0.01$). This suggests a pathogenetic

Table 1. Plasma levels of inflammation markers in DR patients with T2DM duration ≤ 15 years versus those with T2DM duration > 15 years

Marker	DR patients with T2DM duration ≤ 15 years	DR patients with T2DM duration > 15 years	p
IL-1β, pg/ml	9.4 ± 3.6	5.6 ± 3.4	p = 0.02
IL-10, pg/ml	3.24 ± 1.3	3.38 ± 2.1	p > 0.05
NNE, ng/ml	2.02 ± 1.5	1.34 ± 0.6	p = 0.068

Note: DR, diabetic retinopathy; IL, interleukin; p, significance of difference; T2DM, type 2 diabetes mellitus

Table 2. Correlations between plasma levels of interleukins, NNE, and L-homocystein in T2DM patients with DR

Index	L-homocystein	IL-1b	IL-10	NNE
L-homocystein	1	0.320** p=0.002	0.357** p=0,001	0.286** p=0.007
IL-1b		1	0.355** p=0.001	0,368** p=0.000
IL-10			1	0.279** p=0.009
NNE				1

Note: P-values refer to the significance of the Spearman rank correlation coefficient; **, bilateral significant correlation; DR, diabetic retinopathy; IL, interleukin; NNE, non-neuronal enolase; T2DM, type 2 diabetes mellitus

relationship of hyperhomocysteinemia with markers of systemic and neuroinflammation in T2DM patients with DR.

We assessed plasma interleukin and NNE in patients with different genotypes of folate cycle enzyme genes (Figs. 3-5).

Discussion

Therefore, the rs1801133 CC genotype may be considered as a risk factor for systemic inflammation and gliosis, because significantly increased IL-1 beta levels were found in all carriers of this genotype (especially those with NPDR and moderate PDR). However, the rs1801133 TT genotype may be considered as a protective factor for the development of systemic inflammation and gliosis because the smallest difference in plasma IL-1 beta and NNE levels was noted between patients carrying this genotype and controls. In addition, patients carrying this genotype exhibited the highest plasma levels of IL-10, a potent anti-inflammatory cytokine playing a central role in limiting host immune response to pathogens.

The rs1805087 GG genotype may be considered as a risk factor for the development of DR in patients with T2DM, because, in the current study, carriers of this genotype were found only among patients with DR, and plasma IL-1β levels in these patients (especially those with NPDR) were almost 15 times higher compared to controls. In addition, NPDR patients with the rs1805087 GG genotype showed the greatest difference in plasma NNE levels compared to controls (in these patients, plasma NNE levels were twice as high as in controls). The rs1805087 AA genotype appeared as a protective genotype against DR due to insubstantial differences in plasma NNE levels compared to controls and highest plasma levels of the anti-inflammatory cytokine IL-10.

We believe that the rs1801131 CC genotype is a candidate risk factor for the development of DR in patients with T2DM, because plasma IL-1beta levels were 10 times higher in NPDR patients carrying this genotype compared to controls, and patients with severe PDR carrying this genotype showed increased plasma NNE levels. In addition, the smallest difference in plasma IL-10 levels was observed between patients carrying this genotype and controls.

Our findings are in agreement with a previous report whose authors concluded that the CC genotype of the rs1801133 is a likely risk factor for DR in patients with T2DM because DR patients carrying the genotype exhibited 14 times higher endothelin-1 levels compared to non-carriers [13]. Those authors believe that T2DM patients having the minor homozygous GG genotype of MTR 2756A/G (rs 1805087) are at some risk for DR. Microvascular complications in these patients can develop through several mechanisms which involve elevation of homocysteine, ET-1, and arginase-1, and folate deficiency [13].

Therefore, study findings support the theory that inflammatory processes are involved in retinal neurodegeneration and pathogenesis of DR. It is likely that neuronal damage can occur not only through the formation of advanced glycation end-products and/or excessive reactive oxygen species production, but also through the development of neuroinflammation and gliosis, which definitely represents a threat to retinal neurons.

The relationship between diabetic neuropathy and vasculopathy is still debated: neurogeneration could be due to subclinical changes in intraretinal microvasculature [4]. Retinal neurodegeneration can only be considered as a different pathologic feature of diabetic retinal disease, at least partly independent from microvascular alterations. This new view of DR underpins the need for better phenotyping and stratification of diabetic retinal disease in order to identify a subset of patients with chronic neurodysfunction and validate biomarkers to measure therapy outcomes, considering the emergent development of neuroprotective drugs.

Our finding of correlation of hyperhomocysteinemia with plasma levels of the inflammatory cytokine, IL-1β, and the gliosis marker, NNE, indicates a close

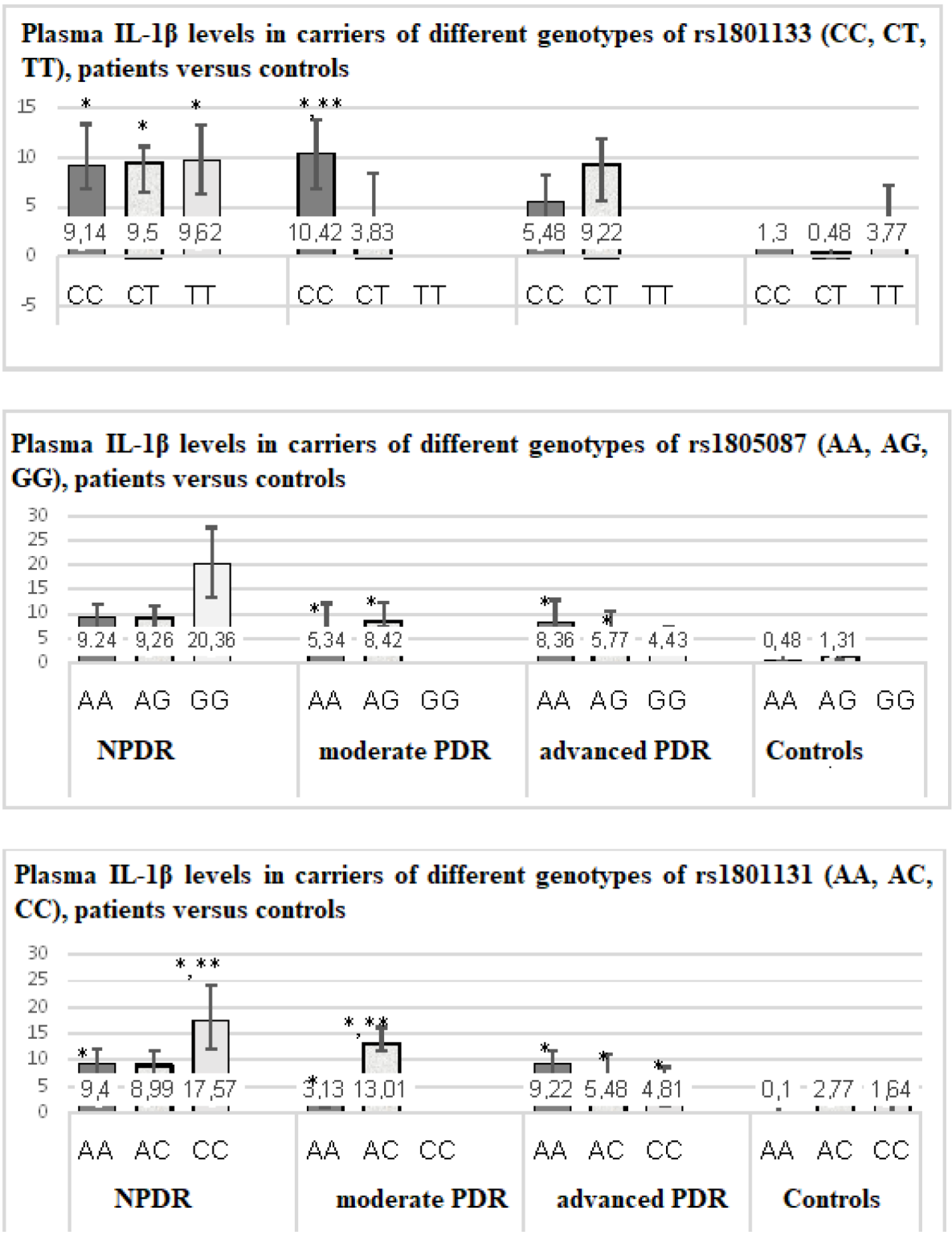

Fig. 3. Box plots depicting plasma IL-1β levels (pg/ml) for carriers of different genotypes of rs1801133 (CC, CT, TT), rs1805087 (AA, AG, GG), rs1801131 (AA, AC, CC), patients with non-proliferative diabetic retinopathy (NPDR), moderate proliferative diabetic retinopathy (PDR) and advanced PDR compared to controls. Notches indicate the 95% confidence interval of the median bolded midline indicates median value, and box indicates the first and third quartiles. Note: *, significant difference ($p < 0.05$) from controls.

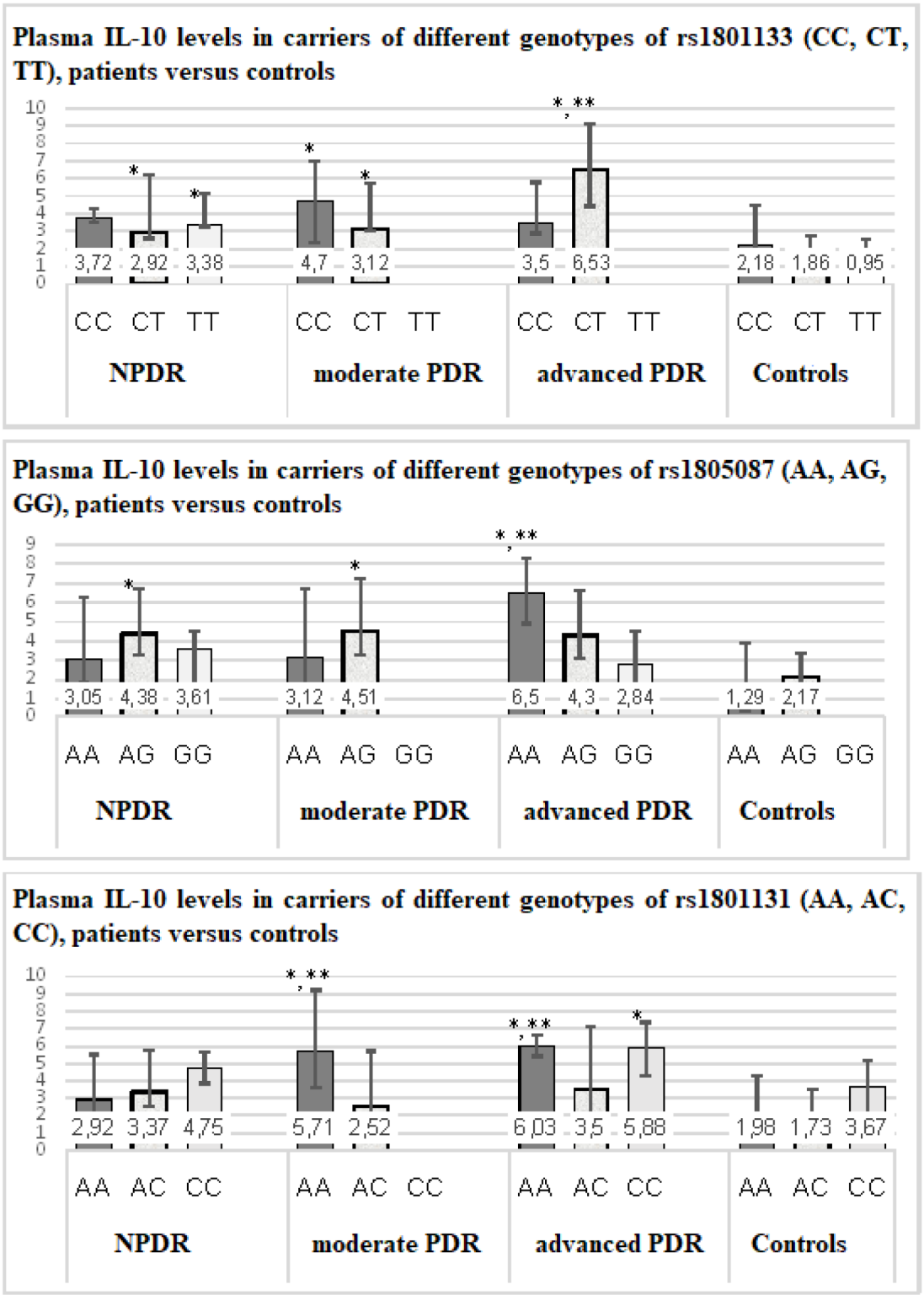

Fig. 4. Box plots depicting plasma IL-10 levels (pg/ml) for carriers of different genotypes of rs1801133 (CC, CT, TT), rs1805087 (AA, AG, GG), rs1801131 (AA, AC, CC), patients with non-proliferative diabetic retinopathy (NPDR), moderate proliferative diabetic retinopathy (PDR) and advanced PDR compared to controls. Notches indicate the 95% confidence interval of the median bolded midline indicates median value, and box indicates the first and third quartiles. Note: *, significant difference ($p < 0.05$) from controls.

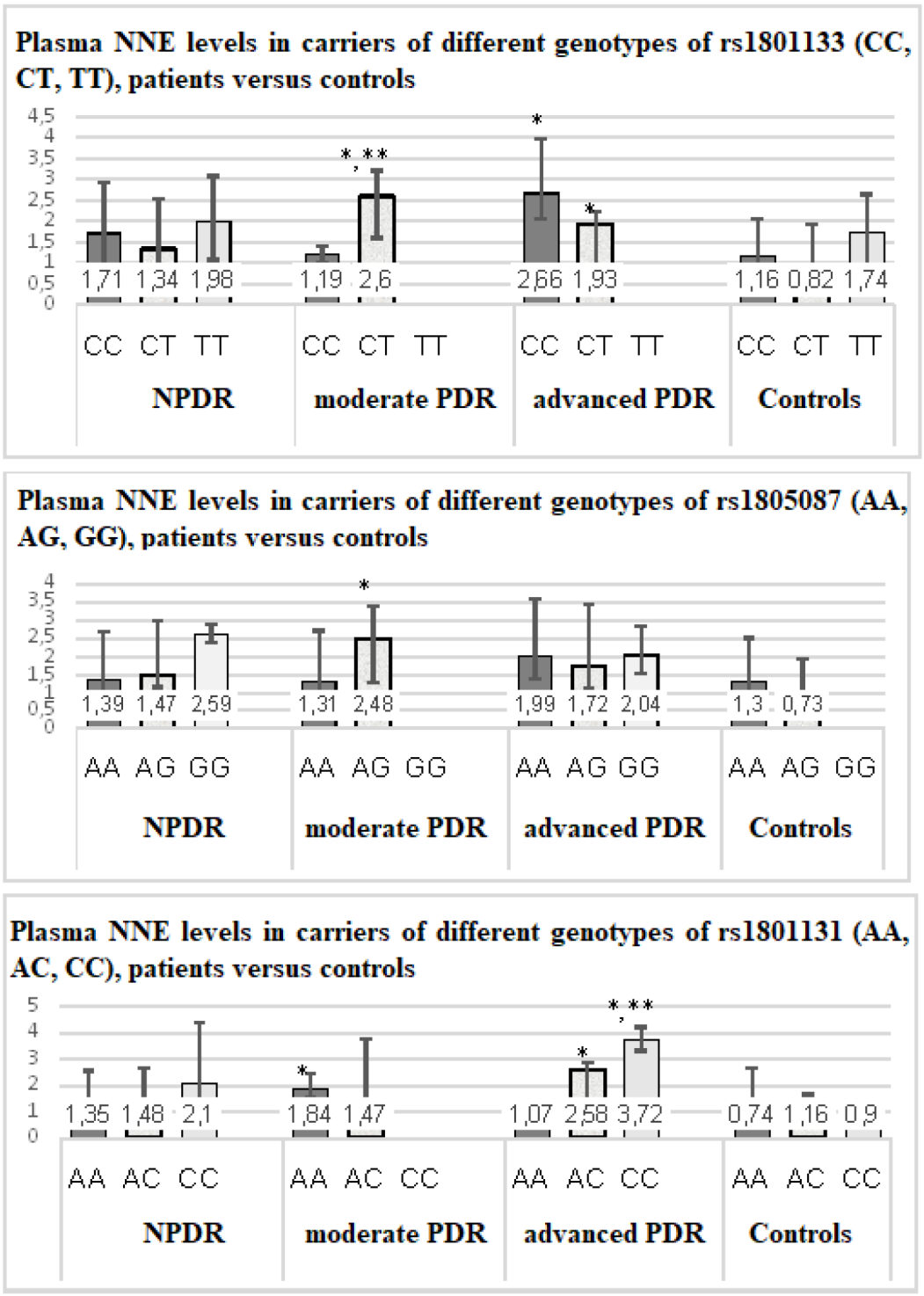

Fig. 5. Box plots depicting plasma non-neuronal enolase (NNE) levels (ng/ml) for carriers of different genotypes of rs1801133 (CC, CT, TT), rs1805087 (AA, AG, GG), rs1801131 (AA, AC, CC), patients with non-proliferative diabetic retinopathy (NPDR), moderate proliferative diabetic retinopathy (PDR) and advanced PDR compared to controls. Notches indicate the 95% confidence interval of the median, bolded midline indicates median value, and box indicates the first and third quartiles. Note: *, significant difference ($p < 0.05$) from controls.

interrelationship between the mechanisms of damage to the retina. The correlation of the above indices with the plasma level of the anti-inflammatory IL-10 demonstrates a systemic mechanism of immune response, which is also characteristic for a chronic low-grade that develops over years.

The finding of typical combinations of the polymorphisms encoding folate cycle enzymes in patients with T2DM provides ground for a discussion of the role of genetically determined hyperhomocysteinemia in the pathogenesis of DR in T2DM. We found certain patterns of polymorphisms related to the markers of low-grade neuroinflammation in carriers of different genotypes, which may be considered as risk factors for the onset or increase the stage of DR. Although we did not establish the association of gene variants with the development of the disease, we characterized the phenotypic characteristics that may be considered as potential risk factors. We believe that in this way we contributed to the knowledge on the disease course in carriers of different genotypes, which improves the potential success of the tools of personalized medicine.

Therefore, our findings provide evidence of neural tissue damage in T2DM patients with DR, which is indicated by increased proinflammatory cytokine and NNE production. Thus, plasma IL-1β levels were 9 times higher in T2DM patients with DR than in controls ($p < 0.05$), and 1.7 higher in DR patients with diabetes duration ≤ 15 years than in those with longer diabetes duration. There was correlation of plasma L-homocystein levels with plasma IL-10 ($R = 0.357$, $p < 0.01$), IL-1b ($R = 0.320$, $p < 0.01$) and NNE ($R = 0.286$, $p < 0.01$) levels. This and the fact that plasma NNE levels correlated with plasma IL-10 ($R = 0.279$, $p < 0.01$) and IL-1beta ($R = 0.368$, $p < 0.01$) suggests a pathogenetic relationship of hyperhomocysteinemia with markers of systemic and neuroinflammation in T2DM patients with DR. Studies on cytokine and neuroinflammation marker levels in carriers of different gene variants encoding folate pathway enzymes provide reasonable grounds to consider the rs1801131 CC, rs1805087 GG and rs1801131 CC genotypes to be candidate risk factors for the development of DR in patients with T2DM, due to increased pro-inflammatory cytokine levels in patient carriers versus non-carriers of these genotypes.

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Disclosures

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Author's contribution: *All authors meet the criteria for authorship, certify that each author has contributed substantially to the work, including conception, design, analysis, writing, and revision of the article, and each author is responsible for its content All authors have approved the final version of the manuscript.*

Ethical statement: *The study was conducted with human participants. The study was approved by the local bioethics committee. All patients gave informed consent to participate in the study. Measures were taken to ensure respect for human rights, human dignity, and moral and ethical standards in accordance with the principles of the Helsinki Declaration of Human Rights, the Council of Europe Convention on Human Rights and Biomedicine, and the relevant laws of Ukraine.*

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