THE ROLE OF ADIPONECTIN AND TOLL-LIKE RECEPTOR 4 GENE POLYMORPHISMS ON NON-PROLIFERATIVE RETINOPATHY IN TYPE 2 DIABETES MELLITUS PATIENTS. A CASE-CONTROL STUDY IN ROMANIAN CAUCASIANS PATIENTS

C.S. Aioanei^{1,*}, R.F. Ilies¹, C. Bala², M.F. Petrisor¹, M.D. Porojan³, R.A. Popp¹, A. Catana¹

“Iuliu Hatieganu” University of Medicine and Pharmacy - ¹Department of Molecular Sciences, ²Department of Diabetes and Nutrition, ³Department of Internal Medicine, Cluj-Napoca, Cluj, Romania

Abstract

Context. Persistent inflammation and impaired neovascularization are important contributors to the development of diabetic retinopathy (DR). Gene polymorphisms of adiponectin (APN) were demonstrated to have an important role on the plasma level and activity of adiponectin. APN has anti-inflammatory, anti-diabetic and anti-atherogenic properties. Toll-Like Receptor 4 (TLR4) is a critical mediator of innate immunity. Polymorphisms in TLR-4 gene were shown to be associated with impaired inflammatory response in diabetes.

Objective. The aim of the study was to analyze the association of +276G>T variant of APN gene and Asp299Gly and Thr399Ile of TLR-4 gene variants in relationship with T2DM and DR in an Eastern European population group.

Design. The distribution of the mutant alleles in 198 T2DM patients with DR and 200 non-T2DM controls was examined. Genomic DNA from T2DM patients and healthy controls genotyped through the use of PCR-RFLP assay.

Results. Genotype and allele frequencies of the Asp299Gly and Thr399Ile polymorphisms differed between T2DM patients and non diabetic subjects ($P<0.001$). Moreover, the presence of the minor alleles of these polymorphisms were significantly identified as protective factors against T2DM, under a dominant model of Fisher's exact test ($\chi^2=4.988$, $\phi=0.745$, $OR=0.767$, 95% $CI=0.602-0.867$, $P<0.001$; respectively $\chi^2=5.254$, $\phi=0.820$, $OR=0.487$, 95% $CI=0.211-0.648$, $P<0.001$). Genotype analysis for the adiponectin 276G>T gene polymorphism yielded no significant association with T2DM, but revealed a borderline significance for the association with DR ($\chi^2=5.632$, $\phi=0.423$, $OR=1.101$, 95% $CI=0.887-1.203$, $P=0.009$).

Conclusions. We found an association between the TLR4 Asp299Gly and Thr399Ile polymorphisms and protection for DR. The APN genetic polymorphism is not associated with T2DM.

Key words: Type 2 diabetes mellitus, diabetic retinopathy, Adiponectin, Toll-like receptor 4, single nucleotide polymorphisms, case-control study.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a major medical problem throughout the world. T2DM causes an array of long-term systemic complications that have considerable impact on the patient's as well. T2DM is a metabolic disease characterized by the association between deficient insulin secretion, peripheral insulin resistance and dysregulated immune response (1-3).

Patients with diabetes often develop ophthalmic complications, such as corneal abnormalities, glaucoma, iris neovascularization, cataracts, and neuropathies. The most common and potentially most blinding of these complications, however, is diabetic retinopathy (DR). DR is the second most frequent microvascular complication of T2DM. DR is defined by damage to the blood vessels of the light-sensitive tissue of retina. Later on, the formation of new friable blood vessels in the retina may produce vision loss if not managed well (4, 5).

Impaired neovascularization and continuous inflammation contribute to the development and evolution of DR. Dysregulated immune response is identified in individuals suffering from T2DM. The hyperglycemic status has been demonstrated by several cellular studies to be responsible for the decreased response of immune cells and persistent inflammation. The functional and structural modifications of the retina in diabetes are persistent with inflammation in time. Anti-inflammatory drugs have been demonstrated to be highly efficient in reducing the development of DR (6, 7).

Adiponectin (APN) is the most abundant cytokine secreted by the adipose tissues (8). APN exerts anti-inflammatory, anti-diabetic and anti-atherogenic properties. Low circulating plasma levels are associated with T2DM (9). Gene polymorphisms

*Correspondence to: Casian Simon Aioanei, “Iuliu Hatieganu” University of Medicine and Pharmacy - Department of Molecular Sciences, 6 Pasteur street, Cluj-Napoca, Cluj, 400012, Romania, E-mail: aioanci.casian@gmail.com

of APN have been demonstrated to play an important role on the plasma APN level and activity (10). The particular +276G>T in the region of APN gene is one of the most common gene variants associated with T2DM, through low circulating plasma levels and insulin resistance, but it remains unknown in terms of its association with DR on a Caucasian population (11).

Toll-like receptor 4 is a transmembrane polypeptide, part of the toll-like receptor family, that belongs to the pattern recognition receptor family. It is mainly responsible for recognizing lipopolysaccharide (LPS), component present in several Gram-negative bacteria. It also recognizes viral polypeptide, polysaccharide and endogenous particles: heat shock protein, low-density lipoprotein (11). Recently, two missense, co-segregating genetic variants (Asp299Gly and Thr399Ile) have been discovered in the coding region of the human TLR4 gene at exon 3, leading to impaired inflammatory signal transduction. Therefore, TLR4 is now believed responsible for DR through inflammation-induced angiogenesis (12, 13). Considering the potential role of TLR4 pathway in diabetes, many studies have been performed to analyze the association between TLR4 polymorphisms and T2DM, but a few analyzed the association with DR and none were carried out in our geographic region.

The aim of the current study was to analyze the possible association of +276G>T variant of APN gene and Asp299Gly and Thr399Ile of TLR-4 gene variants in relationship with T2DM and nonproliferative retinopathy in a Caucasian of origin Eastern European population group.

MATERIAL AND METHODS

Sample Collection

An ethnical heterogeneous group of 398 unrelated Caucasians individuals were included in the case-control study. The distribution of the mutant alleles in 198 T2DM patients and 200 non-T2DM healthy controls was examined, belonging to different ethnicities. We excluded independent risk factors for DR, all individuals were with no history of dyslipidemia or elevated blood pressure. Clinical records for serology status and demographic data were extracted from medical records of the patients (glycemic panel, lipid panel, body-mass index, weight, height). Similarly, we recorded the demographic parameters of the control subjects. Wherever possible, this data was correlated to obtain meaningful information to determine the risk factors of the disease. We also determined the

significance of all the parameters for statistical evaluation. In addition, glycosylated hemoglobin (HbA1c) levels for the study group were determined.

Ophthalmological assessment of the study group was necessary. We also obtained this data from the medical records, where a standard fundus retinography and a binocular indirect ophthalmoscopy were performed for all the diabetic individuals. Only 120 individuals were diagnosed with DR. There was no history of chronic diseases of the eye reported for the control group. The sampling was carried out from the County Hospital of Cluj-Napoca between 2016-2017.

The study protocol respected the ethical and deontological code according to the Declaration of Helsinki. The Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy gave the approval for conducting the study. For the molecular analysis, all patients gave their written informed consent.

Genotyping Adiponectin 276G>T (rs1501299), TLR4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791)

Genomic DNA was isolated from 400 µL of EDTA-treated venous peripheral blood using Wizzard Genomic DNA Purification Kit, Promega (Madison, Wisconsin, USA). The samples were stored frozen at -20°C and later used for the molecular analyses.

The alleles of the 276G>T Adiponectin of TLR4 Asp299Gly and Thr399Ile polymorphisms were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, as previously described, with minor modifications (14, 15). The methodology related to the used primers and cycling conditions is provided in Table 1.

The PCR amplification products were digested at 37°C with 5 U of fast restriction enzyme Mva1269I for Adiponectin 276G>T polymorphism, and fast restriction endonucleases BccI and BslI for TLR4 Asp299Gly and Thr399Ile polymorphisms, respectively under the same conditions. Digestion products were separated by electrophoresis through 3% high resolution agarose gel stained with Ethidium Bromide.

In the case of TLR4 Asp299Gly and Thr399Ile polymorphisms, after digestion the wild-type alleles were 140bp for the Asp299Gly (A allele) and 110bp for the Thr399Ile (T allele). The sizes for polymorphic alleles were 77bp and 63bp for Asp299Gly (G allele), and 89bp and 22bp for Thr399Ile (C allele); whereas heterozygotes present all 3 fragments. In the case of Adiponectin 276G>T polymorphism, the digestion fragments were:

468bp for the wild-type (T allele), 320bp and 148bp (G allele) and all 3 for the heterozygote status.

RESULTS

Statistical analysis

Statistical analysis was carried out using SPSS for Windows software (SPSS, Inc. Chicago, IL). The Chi-squared (χ^2) test was used to measure the Hardy-Weinberg Equilibrium. Data are presented as mean $\pm$ SD for continuous variables and as percentages for categorical variables. Data of clinical and demographic value were compared using the phi coefficient and the Pearson's χ^2 test. In addition, Fisher's exact test performed by comparative analysis according to dominant and recessive models was used. Allele frequencies analysis was performed with Student t test. The effect of the risk alleles was estimated by Odds Ratio (OR) with 95% confidence intervals (CI). The OR measurements were based on the chi-square test with values adjusted for the matching variables. P values (two-tailed) of <0.05 were considered statistically significant (16, 17).

Demography study

Demographic data and clinical characteristics of the cases and controls are shown in Table 2. No differences were revealed by the statistical analysis between the two groups related to the distribution of gender, weight, body mass index, abdominal circumference, blood pressure. Biochemical parameters for the control group were in normal limits. The differences in age mean have no influence on the variant genes investigated. The lipid panel of the control group was in normal limits.

Analyses of single nucleotide polymorphisms (SNP)

The alleles frequencies and genotypes distribution of the APN 276G>T and TLR4 Asp299Gly and Thr399Ile SNP were evaluated and correlated for the carrier status and the susceptibility for developing diabetes and DR. In both groups the frequencies of

Table 1. Summary of primers, PCR conditions, RE digestion and RFLP allele location of TLR4 and Adiponectin genes

SNP ID	Forward primer	Reverse primer	Restriction enzyme used	Product size (bp) and genotype	Cycling conditions
rs4986790 Asp299Gly	CTGCTCTA-GAGGGCCT-GTG	TTCAATAGT-CACACTCAC-CAG	BccI	140 = AA 140, 77, 63 = AG 77, 63 = GG 110 = TT	94°C for 5 min; 35 cycles at 94°C for 40 s; 58°C for 45 s; 72°C for 40 s; followed by 72°C for 10 min.
rs4986791 Thr399Ile	CTAC-CAAGCCTT-GAGTTTCTG	AAGCT-CAGATCTA-AATACT	BsII	110, 89, 22 = TC 89, 22 = CC	94°C for 5 min; 35 cycles at 94°C for 40 s; 58°C for 45 s; 72°C for 40 s; followed by 72°C for 10 min.
rs1501299 276G>T	TCTCTCCAT-GGCTGA-CAGTG	AGATGCAG-CAAAGCCAA	Mva1269I	320, 148=GG 468=TT 320, 148, 468=GT	95°C for 10 min; 35 cycles at 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; followed by 72°C for 7 min.

Abbreviations: OR, odds ratio; CI, confidence interval.

Table 2. Biochemical and demographic parameters of the case and control groups. Data are presented as mean $\pm$ SD or as a number(percent-age)

Parameters	Values		OR	95% CI	P-value
	Cases (n=198)	Controls (n=200)			
Age mean years	68.72 $\pm$ 11.58	58.1 $\pm$ 09	1.122	0.865–1.608	0.001
Male n (%)	105 (53,03)	143 (71,5)	1.101	0.788–1.455	0.345
Female n (%)	93 (46,96)	57 (28,5)	1.098	0.623–1.128	0.331
Body mass index (kg/m ²)	26.7 $\pm$ 7.1	24.8 $\pm$ 4.9	1.129	0.801–1.698	0.198
Weight (kg)	82.4 $\pm$ 16.7	79.9 $\pm$ 18.2	1.258	0.822–1.691	0.901
Abdominal circumference (cm)	91.3 $\pm$ 03.9	N/A	N/A	N/A	N/A
Systolic blood pressure (mmHg)	123.98 $\pm$ 11.65	122.65 $\pm$ 17.25	1.265	0.656–1.820	0.308
Diastolic blood pressure (mmHg)	71.96 $\pm$ 10.12	68.51 $\pm$ 8.98	1.223	0.705–1.911	0.322
Cholesterol (mg/dL)	198.61 $\pm$ 41.71	180.78 $\pm$ 46.01	1.299	0.739–1.810	0.417
HDL (mg/dL)	48.85 $\pm$ 9.88	58.28 $\pm$ 12.01	1.302	0.631–1.908	0.554
LDL (mg/dL)	98.68 $\pm$ 9.78	89.91 $\pm$ 9.96	1.367	0.657–1.798	0.323
Triglyceride (mg/dL)	157.85 $\pm$ 46.28	151.045 $\pm$ 79.08	1.371	0.779–1.850	0.668
HbA1c (%)	6.89 $\pm$ 0.10	N/A	N/A	N/A	N/A
Fasting blood glucose (mg/dL)	123 $\pm$ 30.59	86.2 $\pm$ 13	1.112	0.756–1.789	0.346
Disease's duration (years)	7.19 $\pm$ 4.5	N/A	N/A	N/A	N/A
Retinopathy, n (%)	120 (61.58)	N/A	N/A	N/A	N/A

Notes: Data shown as mean $\pm$ SD unless otherwise specified. Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation; N/A, not applicable; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycosylated hemoglobin.

genotypes and alleles of the APN 276G>T and TLR4 Asp299Gly and Thr399Ile polymorphisms for the control group were comparable with those recorded in the Europeans populations. Hardy-Weinberg Equilibrium was respected for both the genotypes distribution and allelic frequencies of the APN 276G>T and TLR4 Asp299Gly and Thr399Ile SNP. The summarization of alleles and genotype frequencies is presented in Table 3.

The dominant and recessive models of Fisher's exact test for the comparative analysis were used to establish significant risk values for the associations between the carrier status and the predisposition for T2DM and DR and both of them combined in the case-control study.

Fisher's exact test (dominant and recessive models) was initially used for the comparative analysis to evaluate the risk value associated with the carrier status and the predisposition for type 2 diabetes. The dominant model did not reveal any statistical difference for the gene variants carriers of APN 276G>T and TLR4 Asp299Gly and Thr399Ile between the two groups studied ($P=0.015$). Under the recessive model only the CC genotype of variant gene Thr399Ile does identify slightly decreased risk for developing diabetes ($\chi^2 = 7.851$, $\phi=0.569$, $OR=0.975$ 95% $CI=0.746-1.102$, $P=0.02$). The analysis of the association between diabetic retinopathy, glycosylated hemoglobin and the variant genotypes investigated failed to demonstrate any statistically significant differences (APN 276G>T polymorphism: $\chi^2=9.874$, $\phi=0.702$, $OR=2.547$,

95% $CI=2.282-2.767$, $P=0.030$; TLR4 Asp299Gly gene variant: $\chi^2=6.032$, $\phi=0.425$, $OR=0.568$, 95% $CI=0.362-0.8674$, $P=0.036$; Thr399Ile gene variant: $\chi^2=5.271$, $\phi=0.566$, $OR=0.718$, 95% $CI=0.592-0.889$, $P=0.029$).

Comparative analysis under the dominant model (Fisher's exact test, ORs) was performed to assess the diabetic retinopathy risk in the study group compared to that of the control group for the APN 276G>T polymorphism and TLR4 Asp299Gly and Thr399Ile polymorphisms. In the case of APN 276G>T under the dominant model, there is a minimal statistically elevated risk for developing diabetic retinopathy ($\chi^2=5.632$, $\phi=0.423$, $OR = 1.101$, 95% $CI=0.887-1.203$, $P=0.009$). The comparative analysis of TLR4 Asp299Gly and Thr399Ile gene variants revealed a decreased risk for developing diabetic retinopathy ($\chi^2=4.988$, $\phi=0.745$, $OR=0.767$, 95% $CI=0.602-0.867$, $P<0.001$; respectively $\chi^2=5.254$, $\phi=0.820$, $OR=0.487$, 95% $CI=0. 0.211-0.648$, $P<0.001$). TLR4 gene variants act as a protective factor under the dominant model of Fisher's exact test, both separately and combined ($\chi^2=4.852$, $\phi=0.856$, $OR=0.456$, 95% $CI=0.202-0.608$, $P<0.001$). The carriers of variant gene Thr399Ile CC genotype highlighted the most statistically significant protective value against diabetic retinopathy, followed closely by the heterozygote carriers of Asp299Gly SNP.

Comparative analysis using Fisher's exact test under the recessive model was also used to evaluate the risk value for developing diabetic retinopathy

Table 3. Genotype distribution and frequency of alleles in diabetic and control subjects

SNP ID	Variant	Cases n (%)	Controls n (%)	OR (95% CI)	P-value
rs4986790 Asp299Gly	AA	150 (75.75)	143 (71.50)	1.013(0.901-1.118)	<0.001
	AG	39 (19.69)	47 (23.50)	0.919 (0.844-1.125)	0.005
	GG	9 (4.54)	10 (5.00)	0.922 (0.865-1.114)	<0.001
	AG + GG	48 (24.24)	57 (28.50)	0.768 (0.602-0.866)	0.008
	A allele frequency	339 (86.47)	333 (83.25)	1.023 (0.856-1.214)	<0.001
	G allele frequency	53 (13.52)	67 (16.75)	0.757 (0.624-0.897)	<0.001
rs4986791 Thr399Ile	TT	22 (11.11)	13 (6.50)	1.011 (0.932-1.225)	<0.001
	TC	50 (25.25)	35 (17.50)	1.056(0.899-1.212)	<0.001
	CC	126 (63.63)	152 (76.00)	0.560 (0.250-0.821)	<0.001
	CC + TC	146 (73.73)	187 (93.5)	0.489 (0.211-0.649)	<0.001
	C allele frequency	302 (76.26)	339 (84.75)	0.521 (0.325-0.701)	<0.001
	T allele frequency	94 (23.73)	61 (15.25)	1.110 (0.921-1.321)	0.003
rs1501299 276G>T	GG	93 (46.96)	92 (46)	1.050 (0.856-1.154)	0.023
	GT	79 (39.89)	88 (44)	1.023 (0.902-1.255)	0.014
	TT	26 (13.13)	20 (10)	1.042 (0.876-1.188)	<0.001
	GG + GT	172 (86.86)	180 (90)	1.102 (0.887-1.201)	0.009
	G allele frequency	265 (66.91)	272 (68)	1.066 (0.936-1.302)	<0.001
	T allele frequency	131 (33.08)	128 (32)	1.041 (0.879-1.126)	0.006

Abbreviations: OR, odds ratio; CI, confidence interval.

for the gene variants carriers of APN 276G>T and TLR4 Asp299Gly and Thr399Ile between the two groups studied. For the 276G>T polymorphism, the recessive model highlighted no statistically increased risk ($\chi^2=11.52$, $\phi=0.350$, $OR=1.051$, 95% $CI=0.856-1.156$, $P=0.009$). Fisher's exact test-recessive model, that was performed for TLR4 Asp299Gly and Thr399Ile gene variants does identify a similar statistically significant risk value as in the case of the dominant model but modestly increased ($\chi^2=5.001$, $\phi=0.752$, $OR=0.789$, 95% $CI=0.620-0.882$, $P<0.001$; respectively $\chi^2=5.302$, $\phi=0.835$, $OR=0.510$, 95% $CI=0.241-0.674$, $P<0.001$). The CC genotype of variant gene Thr399Ile remains the most significant protective factor for diabetic retinopathy even under the recessive model. Following multivariate analysis, the difference between diabetic and non-diabetic subjects, with regard to TLR4 mutations alone, remained significant ($P=0.002$).

T2DM evaluated in association with metabolic syndrome, and variant genotypes of APN 276G>T and TLR4 Asp299Gly and Thr399Ile investigated did not present any statistical differences under the Chi-square test ($\chi^2=5.302$, $OR=1.654$, 95% $CI=1.451-1.821$, $P=0.017$).

DISCUSSION

Several risk factors have been identified and made responsible for the development and advancement of diabetes and DR, starting from genetic predisposition, in addition to environmental factors and summarizing with biochemical interplay (18, 19). Multiple candidate gene association studies have identified several genes and loci responsible for the outcome of diabetes and DR. Besides the deficient insulin secretion and peripheral insulin resistance characteristic to diabetes, a dysregulated immune response is also implicated.

Hemostatic abnormalities, elevated vascular permeability and tissue inflammation followed by ischemia-induced neovascularization of retina are the defining aspects of DR, occurring in approximately 75% of diabetic patients within 15 years of evolution (20). DR includes abnormalities of the endothelium such as endothelial dysfunction, microvascular rarefaction and decreased collateralization. The alterations in the inflammatory and innate immune response are related to the variation in protein functioning and cytokine level due to the polymorphisms in the implicated genes (21). The genetic susceptibility to DR is multifactorial and the risk factors involved are identified with the alterations in

the immune system response and inflammatory cascade.

Genetic variants of APN and TLR4 were intensively studied in the last 2 decades regarding their implication in diabetes and other metabolic diseases in several populations. There is few data reported regarding the importance and distribution of TLR-4 Asp299Gly and Thr399Ile and APN +276G>T polymorphisms on the predisposition for T2DM and DR in European populations, and no valid data for a Caucasian population group.

Adiponectin is an immunomodulatory, pro-angiogenic, anti-apoptotic adipocytokine protecting against inflammation-induced injury of the endothelial cells. It offers vascular protection in a variety of diseases including diabetes and its vascular complications (22). In diabetic non-proliferative retinopathy, adiponectin behaves as endogenously up-regulated anti-inflammatory cytokine confining retinal inflammation and onset of retinopathy (23, 24).

Genetic variant of APN 276G>T may lead to the loss of its beneficial properties, especially the protective vascular effect in the case of DR, making the variant gene a risk factor. Several meta-analysis and experimental studies carried out on Asian populations, place 276G>T variant of APN gene among the genetic risk factors for both T2DM and DR (25, 26). The present results may not come in complete agreement because of the borderline statistical significance, but a large sample may demonstrate the risk associated (27).

The activation of TLR4 stimulates pro-inflammatory pathways and induces cytokines expression in a variety of cell types including endothelial cells of the retina. In the case of an installed retinopathy, the expression of TLR4 is responsible for the augmentation of inflammation-induced injury of the retina. In contrast, it can promote the onset of retinopathy in an individual with no signs of retinopathy described yet. The TLR4 Asp299Gly and Thr399Ile polymorphisms, when present, offer a protective status by down-regulating the signaling pathway of the inflammatory response being associated with moderate injuries (28). Those 2 genetic variants are proposed by Asian population-based studies as suitable candidate molecular markers for DR study. Our results come in agreement with these studies, placing TLR4 Asp299Gly and Thr399Ile polymorphisms among the protective factors against development and evolution of DR (29, 30).

In addition, there is also demonstrated a relationship between adiponectin and TLR4. Adiponectin can inhibit the inflammation response and reduce autoimmune-induced injuries in endothelial

cells by interfering with TLR4 signalling pathway (31, 32). The expression of adiponectin in the retina can inhibit the expression of TLR4 and its major targets, and it is capable of attenuating the immune cells infiltration (33, 29, 34).

To our knowledge there is no data reported regarding the association between adiponectin 276G>T and TLR4 Asp299Gly and Thr399Ile variant genes and diabetic retinopathy in a Caucasian population group. Adiponectin 276G>T was reported as a risk factor in other populations for obesity, diabetes and other metabolic diseases. Our study concludes that adiponectin 276G>T polymorphism is a potential vascular biomarker in both diabetes and non-proliferative retinopathy, but borderline statistical significance was obtained to support its risk factor effect described in the literature. The limitations of our study regarding the relatively restricted sample size have prevented us from achieving the statistical significance to demonstrate its harmful effect. In the case of gene variants of TLR4 Asp299Gly and Thr399Ile, the results obtained in the current study indicate that both polymorphisms act like a protective factor against diabetic retinopathy in type 2 diabetes mellitus.

In conclusion, from the present study, APN 276G>T polymorphism is not associated with a high risk for developing diabetic retinopathy as literature described, reaching a borderline statistical significance in our study. An increased sample size is required to prove if there may be a risk associated with APN 276G>T gene variant in the Caucasian population. TLR4 gene variants Asp299Gly and Thr399Ile offer a protective status against inflammation-induced injuries of the retina associated with diabetic retinopathy. The protective effect is conferred both individually and combined, for the present polymorphisms investigated. Further molecular and clinical studies are required to clarify the molecular mechanism that participate in the adiponectin anti-inflammatory property by inhibiting TLR4 signalling in diabetic retinopathy.

Conflict of interest

The authors declares that he has no conflict of interest.

Acknowledgements

This work is published within/under the internal grant No.: 4995/22/08.03.2016, financed by “Iuliu Hatieganu” University of Medicine and Pharmacy, Department of Molecular Sciences, Discipline of Medical Genetics, Cluj-Napoca, Romania.

References

1. Yau JW, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, Chen SJ, Dekker JM, Fletcher A, Grauslund J, Haffner S, Hamman RF, Ikram MK, Kayama T, Klein BE, Klein R, Krishnaiah S, Mayurasakorn K, O'Hare JP, Orchard TJ, Porta M, Rema M, Roy MS, Sharma T, Shaw J, Taylor H, Tielsch JM, Varma R, Wang JJ, Wang N, West S, Xu L, Yasuda M, Zhang X, Mitchell P, Wong TY. Meta-Analysis for Eye Disease (META-EYE) Study Group. Prevalence and Major Risk Factors of Diabetic Retinopathy. *Diabetes Care*. 2012;35(3):556–564.
2. Badawi A, Klip A, Haddad P, Cole DE, Bailo BG, El-Sohemy A, Karmali M. Type 2 diabetes mellitus and inflammation: Prospects for biomarkers of risk and nutritional intervention. *Diabetes Metab Syndr Obes*. 2010;3:173–186.
3. Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Res Clin Pract*. 2014;105(2):141–150.
4. Stolar M. Glycemic Control and Complications in Type 2 Diabetes Mellitus. *Am J Med*. 2010;123(3):3–11.
5. Dregan A, Charlton J, Chowienzyk P, Gulliford MC. Chronic inflammatory disorders and risk of type 2 diabetes mellitus, coronary heart disease, and stroke: A population-based cohort study. *Circulation*. 2014;130(10):837–844.
6. Tang J, Kern TS. Inflammation in diabetic retinopathy. *Prog Retin Eye Res*. 2011;30(5):343–358.
7. Ola MS, Nawaz MI, Siddiquei MM, Al-Amro S, Abu El-Asrar AM. Recent advances in understanding the biochemical and molecular mechanism of diabetic retinopathy. *J Diabetes Complications*. 2012;26(1):56–64.
8. Aleidi S, Issa A, Bustanji H, Khalil M, Bustanji Y. Adiponectin serum levels correlate with insulin resistance in type 2 diabetic patients. *Saudi Pharm J*. 2015;23(3):250–256.
9. Misu H, Ishikura K, Kurita S, Takeshita Y, Ota T, Saito Y, Takahashi K, Kaneko S, Takamura T. Inverse correlation between serum levels of selenoprotein p and adiponectin in patients with type 2 diabetes. *PLoS One*. 2012;7(4):1–7.
10. Daniele G, Guardado Mendoza R, Winnier D, Fiorentino TV, Pengou Z, Cornell J, Andreozzi F, Jenkinson C, Cersosimo E, Federici M, Tripathy D, Folli F. The inflammatory status score including IL-6, TNF- α , osteopontin, fractalkine, MCP-1 and adiponectin underlies whole-body insulin resistance and hyperglycemia in type 2 diabetes mellitus. *Acta Diabetol*. 2014;51(1):123–131.
11. Peters KE, Beilby J, Cadby G, Warrington NM, Bruce DG, Davis WA, Davis TM, Wiltshire S, Knuiman M, McQuillan BM, Palmer LJ, Thompson PL HJ. A comprehensive investigation of variants in genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1/R2), and their association with serum adiponectin, type 2 diabetes, insulin resistance and the metabolic syndrome. *BMC Med Genet*. 2013;14:15.
12. Rajamani U, Jialal I. Hyperglycemia Induces Toll-Like Receptor-2 and -4 Expression and Activity in Human Microvascular Retinal Endothelial Cells: Implications for Diabetic Retinopathy. *J Diabetes Res*. 2014;2014:790902.
13. Singh K, Kant S, Singh VK, Agrawal NK, Gupta SK, Singh K. Toll-like receptor 4 polymorphisms and their haplotypes modulate the risk of developing diabetic retinopathy in type 2 diabetes patients. *Mol Vis*. 2014;20:704–713.
14. Zhang N, Shi YH, Hao CF, Gu HF, Li Y, Zhao YR, Wang LC, Chen ZJ. Association of +45G15G(T/G) and +276(G/T) polymorphisms in the ADIPOQ gene with polycystic ovary syndrome among Han Chinese women. *Eur J Endocrinol*. 2008;158(2):255–260.
15. Singh K, Singh VK, Agrawal NK, Gupta SK, Singh K. Association of Toll-Like Receptor 4 Polymorphisms with Diabetic Foot Ulcers and Application of Artificial Neural Network in DFU Risk Assessment in Type 2 Diabetes Patients. *Biomed Res Int*. 2013;2013:1–9.

16. Clarke GM, Anderson CA, Pettersson FH, Cardon LR, Morris AP, Zondervan KT. Basic statistical analysis in genetic case-control studies. *Nat Protoc*. 2011;6(2):121–133.
17. Montana G. Statistical methods in genetics. *Brief Bioinform*. 2006;7(3):297–308.
18. Medicine G, McCarthy MI. Genomics, Type 2 Diabetes, and Obesity. 2013;2339–2350.
19. Alharthi AS, Almutairi MZK, Alswat AHK. Prevalence and Potential Risk Factors of Diabetic Retinopathy among Type 2 Diabetics Patients in Diabetic Center, Taif City, KSA. 2018;70:1455–1463.
20. Dutta S, Manideep BC, Basha SM, Caytiles RD, Iyengar NCSN. Classification of Diabetic Retinopathy Images by Using Deep Learning Models. *Int J Grid Distrib Comput*. 2018;11(1):89–106.
21. Noda K, Nakao S, Zandi S, Sun D, Hayes KC, Hafezi-Moghadam A. Retinopathy in a novel model of metabolic syndrome and type 2 diabetes: new insight on the inflammatory paradigm. *FASEB J*. 2014;28(5):2038–2046.
22. Ghoshal K. Adiponectin: Probe of the molecular paradigm associating diabetes and obesity. *World J Diabetes*. 2015;6(1):151.
23. Tajtáková M, Petrášová D, Petrovicová J, Pytliak M, Semanová Z. Adiponectin as a biomarker of clinical manifestation of metabolic syndrome. *Endocr Regul*. 2006;40(1):15–19.
24. Ouchi N, Kihara S, Arita Y, Okamoto Y. Basic Science Reports Endothelial NF- B Signaling Through a. *Intern Med*. 2000;1296–1301.
25. Ouchi N, Walsh K. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta*. 2007;380(1–2):24–30.
26. Omac T, Nagaoka T, Yoshida A. Relationship Between Retinal Blood Flow and Serum Adiponectin Concentrations in Patients With Type 2 Diabetes Mellitus. *Investig Ophthalmology Vis Sci*. 2015;56(6):4143.
27. Choe EY, Wang HJ, Kwon O, Kim KJ, Kim BS, Lee BW, Ahn CW, Cha BS, Lee HC, Kang ES, Mantzoros CS. Variants of the adiponectin gene and diabetic microvascular complications in patients with type 2 diabetes. *Metabolism*. 2013;62(5):677–685.
28. Nguyen DV, Shaw LC, Grant MB. Inflammation in the pathogenesis of microvascular complications in diabetes. *Front Endocrinol (Lausanne)*. 2012;3:1–7.
29. Rajamani U, Jialal I. Hyperglycemia induces toll-like receptor-2 and -4 expression and activity in human microvascular retinal endothelial cells: Implications for diabetic retinopathy. *J Diabetes Res*. 2014;2014:7–10.
30. Shi H, Kokoeva MV, Inouye K, Tzamelis I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid – induced insulin resistance. *J Clin Invest*. 2006;116(11):3015–3025.
31. Jie Tang, Chieh Allen Lee, Yunpeng Du, Yan Sun, Eric Pearlman, Nader Sheibani, Timothy S. Kern. MyD88-Dependent Pathways in Leukocytes Affect the Retina in Diabetes. *PLoS One*. 2013;8(7):1–10.
32. Jenke A, Wilk S, Poller W, Eriksson U, Valaperti A, Rauch BH, Stroux A, Liu P, Schultheiss HP, Scheibenbogen C, Skurk C. Adiponectin protects against toll-like receptor 4-mediated cardiac inflammation and injury. *Cardiovasc Res*. 2013;99(3):422–431.
33. Omac T, Nagaoka T, Tanano I, Yoshida A. Adiponectin-induced dilation of isolated porcine retinal arterioles via production of nitric oxide from endothelial cells. *Investig Ophthalmol Vis Sci*. 2013;54(7):4586–4594.
34. Wang L, Wang J, Fang J, Zhou H, Liu X, Su SB. High glucose induces and activates Toll-like receptor 4 in endothelial cells of diabetic retinopathy. *Diabetol Metab Syndr*. 2015;7(1):89.