Clin Exp Med (2010) 10:185–192
DOI 10.1007/s10238-009-0084-7

ORIGINAL ARTICLE

Antioxidant potential, paraoxonase 1, ceruloplasmin activity and C-reactive protein concentration in diabetic retinopathy

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Received: 2 March 2009 / Accepted: 23 November 2009 / Published online: 11 December 2009

Abstract The aim of this study was to evaluate the ferric-reducing ability of serum (FRAS), paraoxonase 1 (PON1), ceruloplasmin serum oxidase activity and hsCRP level in patients with type1 diabetes mellitus without and with diabetic retinopathy. The study was performed in 76 patients with type 1 diabetes mellitus, 35 without diabetic retinopathy (group 1) and 41 with preproliferative and proliferative retinopathy (group 2). Control group consisted of 35 non-diabetic, age-, gender-, body mass-matched healthy volunteers who came to the outpatient clinic for a routine health check-up. We evaluated FRAS using the method described by Benzie and Strain; PON1 by kinetic spectrophotometric assay with paraoxon as substrate and ceruloplasmin using its oxidative activity with 3-phenylenodiamine as substrate. CRP was measured with a high sensitive enzyme immunoassay. PON1 activity was significantly decreased in patients with diabetic retinopathy (227.66 ± 123.57 U/l) when compared with control (312.04 ± 129.77 U/l). FRAS was significantly decreased in group 2 (439.33 ± 79.87 μmol/l) when compared with group 1 (522.79 ± 167.56 μmol/l) and control (529.80 ± 81.99 μmol/l). Ceruloplasmin activity was significantly elevated in group 1 (58.36 ± 22.56 U/g protein) when compared with control (45.22 ± 14.96 U/g protein). We have found significant increase in hsCRP level in group 2 (3.71 ± 2.47 mg/l) when compared with group 1 (1.75 ± 1.01 mg/l) and control (0.57 ± 0.46 mg/l). The PON1/CRP ratio in control group was significantly increased when compared with diabetic patients and was significantly decreased in group 2 compared with group 1. We have not found gender-dependent difference in studied parameters in both control and in study groups. We have found tendency to decrease the serum activity of FRAS and hsCRP in elder patients but the difference was significant only in group 2. FRAS and PON 1 activity is decreased in patients with type 1 diabetes mellitus with presence of diabetic retinopathy which confirms that oxidative stress could play a role in pathogenesis of diabetic retinopathy. Significantly elevated levels of hsCRP in diabetic patients with the presence of diabetic retinopathy compared with patients without diabetic retinopathy providing a link between inflammation and the development of microvascular complication of diabetes. Because of the significant difference in PON1/CRP ratio

The parts of the results of this study were presented at the 40th Annual Meeting of the Society for Clinical Investigation. Prague, Czech Republic, March 15–18, 2006. The project was carried out with the permission of The Bioethical Board of the Medical University of Silesia (NN-013-165/02).

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between patients without and with the presence of diabetic retinopathy, it seems that PON1:CRP ratio may be used as a biochemical marker for progression of retinopathy. The link between the antioxidant concentration, inflammation and the development of diabetes complications needs further longitudinal studies in order to confirm our findings.

Keywords Diabetes mellitus · Diabetic retinopathy · Antioxidant status · Paraoxonase · Ceruloplasmin · C-reactive protein

Introduction

Type 1 diabetes mellitus accounts for only 5–10 percent of all cases of diabetes but represents an important health problem, since this disorder begins early in life and leads to long-term complications. Diabetic retinopathy is a microvascular complication of diabetes closely correlated with chronic long-standing hyperglycemia [1]. Diabetic retinal neovascularisation is considered to be a consequence of retinal ischemia caused by capillary occlusion. Capillary occlusion is the result of microvascular thrombi in which erythrocytes, platelets and leukocytes each may play a role. Ischemic retina acts as a source of upregulated growth factor production, particularly vascular endothelial growth factor, fibroblast growth factor and hepatocyte growth factor, thereby inducing new vessel formation in the surrounding tissue [2, 3]. Macrovascular complications are the most common causes of early death, and microvascular complications (retinopathy) are the predominant causes of visual loss in type 1 diabetes mellitus [4]. Diabetes mellitus leads to endothelium dysfunction and an accelerated progression of atherosclerosis. Apart from chronic long-standing hyperglycemia [1], several mechanisms are involved in pathogenesis of diabetic retinopathy: autoimmunological mechanisms, polyol pathway, advanced glycation endproducts, protein kinase C, hexosamine pathway as well as blood lipid disturbances [5–8]. Some authors reported that enhanced generation of reactive oxygen species (ROS) may take part in the pathogenesis of diabetic microvascular complications [9, 10].

Paraoxonase 1 (PON1) is a polymorphic protein able to prevent low-density lipoprotein oxidation. In vitro, PON1 hydrolyzes a large variety of endogenous or exogenous substrates, some of which are clearly involved in the progression of arteriosclerosis. A close relationship between PON1 deficiency and accelerated progression of arteriosclerosis has been found in animal models. Moreover, PON1 activity is reduced in high oxidative stress diseases such as coronary heart disease, dyslipidemias (low-HDL syndrome, hypertriglyceridemia, etc.), inflammatory processes, diabetes and certain neuropathies [11].

Ceruloplasmin act as an antioxidant in serum by oxidizing ferrous ion, which could otherwise act as a catalyst in free radical reactions (i.e. Fenton reaction), but in special condition, i.e. at low pH in tissue compartments with inflammation, ceruloplasmin could act as pro-oxidant by donation of free copper ions that induce free radical generation and oxidative tissue damage or LDL oxidation [12, 13]. Ceruloplasmin is also positive acute phase protein, and increase in their serum concentration may inform about inflammation processes in organism [14].

Diabetes mellitus accelerates the atherosclerotic processes. It is known that inflammation plays a key role in atherosclerosis. The prototypic marker of inflammation, C-reactive protein (CRP), is one of the strongest independent predictors of cardiovascular disease [15].

The aim of this study was to evaluate the ferric-reducing ability of serum (FRAS), PON1 and ceruloplasmin serum oxidative activity and C-reactive protein (hsCRP) concentration in patients with diabetes mellitus type1 without and with the presence of retinopathy.

We hypothesized that low PON1 activity and high CRP concentration associated with decreased antioxidant potential found in diabetic patients may be important markers of retinopathy progression and the PON1/CRP ratio may be used as one of the biochemical markers for progression of retinopathy.

Methods

The study included 76 patients with type 1 diabetes mellitus (30 men, 46 women) aged from 20 to 65 (mean ± SD 40.70 ± 11.58) who had been treated for more than 10 years for diabetes. Body mass index (BMI) for 80% of these participants was in normal grade and 20% being in the overweight grade (mean ± SD 21.5 ± 4.7 kg/m^2).

After ophthalmological examination, patients were divided into the following two groups: group 1 consisted of 35 patients aged from 20 to 57 (mean ± SD 38.9 ± 12.1) with a mean duration of diabetes of 17.7 ± 12.2 years, a hemoglobin A1C 6.4 ± 1.3%, BMI 22.8 ± 3.2 kg/m^2 and without diabetic retinopathy; group 2 consisted of 41 patients aged from 26 to 65 (mean ± SD 42.2 ± 11.3) with a mean duration of diabetes 21.4 ± 8.1 years, a hemoglobin A1C 6.9 ± 0.9%, BMI 20.9 ± 2.1 kg/m^2 and with preproliferative (pre PDR) and proliferative (PDR) retinopathy.

There was no significant difference in systolic and diastolic blood pressure and frequency of antihypertensive drug use between study groups.

The eye examination was performed by ophthalmologist and included visual acuity, biomicroscopy and ophthalmoscopy using either a 90-diopter lens or a direct

ophthalmoscopy after pupil dilatation, photography or fluorescein angiography of the retina.

Exclusion criteria were diabetes duration less than 10 years, poor-controlled diabetes (HbA1c >8%), acute, metabolic complication of diabetes (i.e. diabetic ketoacidosis or nonketotic, hyperosmolal diabetic coma), other than diabetes endocrine disease, hepatitis, rheumatologic or neoplastic diseases.

Control group consisted of 35 nondiabetic, age-, gender-, body mass-matched healthy volunteers (coal mine workers and hospital staff) without any known disease (aged from 30 to 60, mean ± SD 40.3 ± 9.9 years) who came to the outpatient clinic for a routine health check-up. Body mass index for 70% of these participants was in normal grade and 30% being in the overweight grade (mean ± SD 22.8 ± 3.2 kg/m^2).

To analyse the effect of age on studied parameters, the control subjects and patients from group 1 and 2 were classified into 2 subgroups, young and middle-aged (30 to 50 years) and older (over 50 years).

Blood was collected after an overnight fast, and serum was obtained by centrifugation and than was stored at −70°C.

In the serum, we evaluated ferric-reducing ability of serum (FRAS) according to the method described for ferric ability of plasma (FRAP) by Benzie and Strain [16]. The principle of the method is areduction in Fe^{3+} ions by antioxidants presented in serum samples to Fe^{2+} ions, which then react with specific ferrous ions chemical—2,4,6-tripyridyl-S-triazine (TPTZ). Fe(II)-TPTZ has an intensive blue color and can be measured at 593 nm. We use a synthetic vitamin E analog (Trolox) as standard and express results as Trolox equivalent in μmol/l. Inter-assay and intra-assay coefficients of variations were, respectively, 3.1 and 5.9%.

Serum paraoxonase 1 (PON1) activity was determined by kinetic spectrophotometry according to Eckerson et al. [17] with paraoxon (*o,o*-diethyl-*o*-(*p*-nitrophenyl)-phosphate) as substrate in glycine/NaOH buffer (pH 10) containing calcium chloride as PON1 activator. Ten minutes before PON1 assay, we inactivate cholinesterase by adding eserine (physostigmine) salicylate to 5 μmol/l final concentration in serum sample. Inter-assay and intra-assay coefficients of variations were, respectively, 2.3 and 7.1%.

Ceruloplasmin (Cp) oxidative activity was measured using 3-phenylenodiamine as substrate according to Richterich [18] and expressed in arbitrary units per gram of total serum protein (U/g TP). One unit of Cp is the equivalent of 1 mg human Cp. Inter-assay and intra-assay coefficients of variations were, respectively, 1.3 and 4.0%. Total serum protein was measured by standard biuret method with inprecision below 2.5%.

All biochemical analysis were performed with use Technicon RA-XT^{TM} (Technicon Instruments Corporation, USA) biochemical analyzer in 37°C.

CRP concentration was measured with a high sensitive ELISA method with use rabbit antibodies against human CRP (DakoCytomation, Denmark) and human CRP standards form Randox (Great Britain). ELISA flat bottom plates (Maxisorp, Nunc, Denmark) were coated with anti-CRP antibody (10 mg per liter of carbonate buffer; pH 9.6; 100 μl/well; 16 h at 4°C), washed 3 times with phosphate-buffered saline with 0.05% Tween 20 (PBST) and blocked with 2% bovine serum albumin (BSA) solution in PBST (200 μl/well; 2 h). Serum samples were diluted 200-fold in 1% BSA in PBST and incubated for 60 min at plates (100 μl/well). After washing step, anti-CRP-HRP conjugate was dispensed on plate for next 60 min. After extensive washing, Sigma Slow Kinetic TMB substrate was dispensed for 10–15 min, and reaction was stopped with 1 M sulphuric acid solution. Absorbances were determined in PowerWave XS plate reader (BioTek, USA) at 450 nm, and results were calculated using KCJunior computer program (BioTek, USA). Assay range of hsCRP assay was between 0 and 20 mg/l, sensitivity—0.02 mg/l and inter-assay and intra-assay coefficients of variations were, respectively, 6.1 and 8.1%.

The statistical analysis was performed by the STATISTICA 8.0 data analysis software system StatSoft, Inc. (2007) using the Mann–Whitney's *U*-test when we compared two study groups and when we compared three different groups using one-way analysis of variance (ANOVA) with post hoc Tukey's HSD test, assuming the levels $P < 0.05$ as statistically significant.

All subjects gave a formal consent before participating in the study, and research followed the tenets of the Declaration of Helsinki.

The project was carried out with the permission of The Bioethical Board of the Medical University of Silesia.

Results

The results of the study are summarized in Tables 1 and 2.

The serum activity of FRAS was significantly decreased in diabetic patients when compared with control subjects (483.55 ± 142.93 vs. 529.80 ± 81.99 μmol/l, $P < 0.05$). There was significant difference in FRAS activity between patients without (group 1) and with diabetic retinopathy (group 2) (522.79 ± 167.56 vs. 439.33 ± 79.87 μmol/l, $P < 0.05$) as well as between patients with diabetic retinopathy and control group (439.33 ± 79.87 vs. 529.80 ± 81.99 μmol/l, $P < 0.05$) (Table 2).

PON1 activity in diabetic 1 patients was not significantly decreased when compared with control group (Table 1), but we have found a significant difference in PON1 activity between group 2 and control patients

Table 1 Demographic data and serum concentration and activity of studied parameters (mean and standard deviation) in diabetes type 1 patients and control group

	Diabetes type 1 ($n = 76$)	Control ($n = 35$)	P value[a]
Gender (M:F)	30:46	15:20	
Age (years)	40.7 ± 11.58	40.3 ± 9.9	NS
Body mass index—BMI (kg/m^2)	21.5 ± 4.7	22.8 ± 3.2	NS
Duration of diabetes (years)	18.9 ± 10.1	0.0	
Glycosylated hemoglobin—HbA1C (%)	6.7 ± 1.0	4.7 ± 0.9	$P < 0.05$
FRAS (μmol/l)	483.55 ± 142.93	529.80 ± 81.99	$P < 0.05$
Ceruloplasmin (U/g protein)	53.88 ± 19.81	45.22 ± 14.96	$P < 0.005$
PON1 (U/l)	266.83 ± 164.94	312.04 ± 129.77	NS
hsCRP (mg/l)	3.41 ± 2.52	0.57 ± 0.46	$P < 0.005$
PON1/hsCRP (U/mg)	192.16 ± 180.71	1157.95 ± 764.87	$P < 0.005$

FRAS ferric-reducing ability of serum, *PON1* paraoxonase1, *hsCRP* high sensitive C-reactive protein

[a] Mann–Whitney *U*-test (NS not significant)

Table 2 Demographic data and serum concentration and activity of studied parameters (mean and standard deviation) in diabetes type 1 patients without and with diabetic retinopathy

	Group 1 ($n = 35$)	Group 2 ($n = 41$)	Control ($n = 35$)	ANOVA P	Post hoc Tukey's HSD test
Gender (M:F)	13:22	17:24	15:20		
Age (years)	38.9 ± 12.1	42.2 ± 11.3	40.3 ± 9.9	0.165893	NS
Body mass index—BMI (kg/m^2)	23.9 ± 2.7	20.9 ± 2.1	22.8 ± 3.2	0.083245	NS
Duration of diabetes (years)	17.7 ± 12.2	21.4 ± 8.1	0.0	NS	
Glycosylated hemoglobin—HbA1C (%)	6.4 ± 1.3	6.9 ± 0.9	4.7 ± 0.9	0.023675	$P < 0.05$
					$P^* < 0.05$
FRAS (μmol/l)	522.79 ± 167.56	439.33 ± 79.87	529.80 ± 81.99	0.018994	$P^* < 0.05$
					$P^{**} < 0.05$
Ceruloplasmin (U/g protein)	58.36 ± 22.56	50.45 ± 14.26	45.22 ± 14.96	0.050046	$P < 0.05$
PON1 (U/l)	289.87 ± 157.07	227.66 ± 123.57	312.04 ± 129.77	0.015949	$P^* < 0.05$
hsCRP (mg/l)	1.75 ± 1.02	3.71 ± 2.47	0.57 ± 0.46	0.000262	$P < 0.05$
					$P^* < 0.05$
					$P^{**} < 0.05$
PON1/hsCRP (U/mg)	208.58 ± 108.12	164.61 ± 74.73	1157.95 ± 764.87	0.000003	$P < 0.05$
					$P^* < 0.05$
					$P^{**} < 0.05$

One-way analysis of variance (ANOVA) with post hoc Tukey's HSD test

Group 1: patients without diabetic retinopathy

Group 2: patients with diabetic retinopathy

FRAS ferric-reducing ability of serum, *PON1* paraoxonase1, *hsCRP* high sensitive C-reactive protein

P control group vs. group 1

P^* control group vs. group 2

P^{**} group 1 vs. group 2

(227.66 ± 123.57 vs. 312.04 ± 129.77 U/l, $P < 0.05$) (Table 2).

Ceruloplasmin activity was significantly elevated in type 1 diabetic patients when compared with control (53.88 ± 19.81 vs. 45.22 ± 14.96 U/g protein $P < 0.005$) (Table 1) and was significantly elevated in group 1 compared with control group (Table 2). We have not found significant difference in ceruloplasmin activity between diabetic patients without and with diabetic retinopathy (58.36 ± 22.56 vs. 50.45 ± 14.26 U/g protein, $P = 0.26$).

Concentration of hsCRP in diabetic patients was significantly elevated when compared with control (3.41 ± 2.52 vs. 0.57 ± 0.46 mg/l, $P < 0.005$) (Table 1), and sCRP level was also significantly elevated in group 2 patients when compared with group 1 (3.71 ± 2.47 vs. 1.75 ± 1.02 mg/l, $P < 0.05$) (Table 2).

The PON1/hsCRP ratio in control group was significantly increased when compared with diabetic patients (1157.95 ± 764.87 mg/U vs. 192.16 ± 180.71 mg/U $P < 0.005$). The PON1/hsCRP ratio was also significantly decreased in group 2 compared with group 1 (164.61 ± 74.73 mg/U vs. 208.58 ± 108.12 mg/U, $P < 0.05$).

In our study, we have not found gender-dependent difference in hsCRP concentration both in control or in study group. We have also not found gender difference in other studied parameters (FRAS, ceruloplasmin and PON1) in control as well as in diabetic patients.

We have not found age-dependent difference in PON1 activity in control as well as in diabetic type 1 patients.

We have found tendency to decrease the serum activity of FRAS in older control patients, but the difference was not significant (509.85 ± 52.71 vs. 531.21 ± 43.84 μmol/l, $P = 0.12$). We obtained similar and not significant results in group 1, but in group 2 we have found significant difference in FRAS activity between younger and middle-aged (461.88 ± 39.42 μmol/l) and older patients (411.43 ± 71.75 μmol/l, $P < 0.05$). The mean value of hsCRP increased significantly with age in group 2 (younger and middle-aged: 3.07 ± 1.27 vs. elder: 4.89 ± 1.12 mg/l, $P < 0.005$). Decrease in FRAS activity and increase in hsCRP concentration was positively associated with duration of diabetes.

Discussion

It is still discussed whether oxidative stress and inflammation are involved in pathogenesis of microvascular diabetic complications. The ambiguous results of the investigations presented by numerous researchers are probably caused by some factors. First, the populations studied in the various investigations were different, with completely different nutritional habits. Moreover, most of the authors determine the level of the oxido-reduction status as a high, medium or low, based on the laboratory norms for their own control groups.

The limitations of our study are rather small sample size and cross-sectional design of the study. The other restrictions in establishing the role of antioxidant factors in the development of the diabetic retinopathy is a lack of direct possibility for the intravital measurement of the antioxidant concentration in the retina. In interpreting the results, it must be assumed that the antioxidative vitamin concentration in the blood serum and the activity of enzymes in the RBCs correlate with their concentrations and activity in the eye.

In our study, the FRAS parameter was chosen for examination since it reflects the concentration of low-molecular nonenzymatic antioxidants and it does not depend (in contrast to total status antioxidant) on the concentration of proteins in blood serum. Defining FRAS enables a more objective evaluation of the nonprotein antioxidant activity [19–21]. Ceruloplasmin (Cp) act as an antioxidant in serum, but ceruloplasmin is also a positive acute phase protein, and increase in their serum concentration may inform also about inflammation processes in organism [12–14]. Results of our study support the data presented by Jones et al. [22] who have reported increased activity of ceruloplasmin in diabetic patients.

In agreement with previous findings [22, 23], we have not found statistical difference in Cp activity between diabetic patients with and without the presence of diabetic retinopathy. Memişoğullari et al. [24] consistent to the results of our study have found increased level of Cp as well as CRP in diabetic patients, when compared with control, and also studied parameters were significantly higher in group with diabetic complications than compared with group without complications. In opposition to results of our study, Abou-Seif and Youssef have found decreased serum activity of Cp in two types of DM in comparison with the corresponding activities of the control subjects [25]. Previous studies have shown that in type 1 diabetes, endothelial dysfunction persists even when glycemia is normalized [26]. Moreover, oxidative stress has recently been considered as a mediator of hyperglycemia-induced endothelial dysfunction [27]. Most of the studies show that oxidative stress is possibly implicated in the pathogenesis of diabetes-related complications, and treatment with antioxidants seemed to be a promising therapeutic supporting option [28, 29]. Because oxidative stress is only one factor contributing to diabetic complications, antioxidant treatment would most likely be more effective if it was coupled with other treatments for diabetic complications [29].

Our results support the results presented by Firoozrai et al. [30] who studied malondialdehyde (MDA) concentration as an index of erythrocyte susceptibility to oxidative stress and antioxidant defense system (reduced glutathione-GSH, glutathione peroxidase-GPx in erythrocytes and FRAP as the total plasma antioxidant capacity) in patients with type 1 diabetes. They found significantly increased concentration of MDA in diabetic patients. FRAP activity was decreased in diabetic 1 patients compared with controls, which is consistent with our results. In our study, we have found significant difference in activity of FRAS between diabetic patients without and with the presence of diabetic retinopathy. In contrast to the previous study and our results, VanderJagt et al. [31] have reported neither plasma markers nor intracellular markers of oxidative stress are different in type 1 diabetic patients with long-term diabetic complications compared to patients without complications.

Aging is associated with changes in physical characteristics and decline of many physiological functions. It has been accepted that the oxidative stress or damage induced by free radicals is related to aging [32, 33].

Paraoxonase is a serum enzyme, which prevents oxidation of low-density lipoprotein by hydrolyzing lipid peroxides [34]. Lipid oxidation may play an important role in the development of micro- and macro-vascular disease. There is evidence that paraoxonase activity is reduced in patients with diabetes. Results of our study support the results presented by other authors. Ikeda et al. [35] have found that patients with diabetic retinopathy had lower PON activity than those without the complication. Authors concluded that serum PON activity was one of the significant factors for retinopathy and that decreased PON activity is involved in diabetic microvascular complications.

Wang et al. [36] have also reported markedly decreased activity of PON1 and significant increased serum concentration of ox-LDL in patients with type 2 diabetes. This results are in opposition to results presented by Sampson et al. who does not support an in vivo action of paraoxonase on LDL oxidation [37]. In contrast to our and others authors results, Kordonouri et al. [38] have found that PON1 activity was significantly higher in patients with retinopathy compared with those without retinopathy. Mackness et al. [39] have found significantly higher levels of CRP and significantly low levels of PON1 activity in both type 1 and 2 diabetic patients, which is consistent with results of our study. The similar results were presented by other authors who have found that plasma concentrations of CRP were higher in type 1 diabetic patients than in control subjects [15, 40, 41]. Authors concluded that correlation of CRP with markers of endothelial dysfunction suggests a relation between activation of the endothelium and chronic inflammation in diabetes [40, 42]. Targher et al. [43] and van Hecke et al. [44] also have found that in diabetic patients with the presence of microvascular complication, plasma biomarkers of inflammation (CRP) and endothelial dysfunction (sICAM-1) were significantly increased in those with more advanced disease compared with those with early complications or without complications, which is consistent with our present and earlier studies [45]. PON1/hsCRP ratio can be a useful indicator for disturbances between intensity of inflammation processes and anti-inflammatory and antioxidant effects of HDL fraction, whose functions are closely related to PON1 activity. Thus, both increase in CRP concentration and decrease in PON1 activity in serum can disturb subtle equilibrium between those two parameters.

Gender differences in CRP concentrations is currently a controversial topic. In some studies, authors have reported similar concentration of CRP in men and women [46, 47], which is similar with our results, whereas others have shown higher concentrations in men [48] or in women [49].

Conclusion

FRAS and PON 1 activity is decreased in patients with type 1 diabetes mellitus with the presence of diabetic retinopathy, which confirms that oxidative stress could play a role in pathogenesis of diabetic retinopathy.

Significantly elevated levels of hsCRP in diabetic patients with the presence of diabetic retinopathy compared with patients without diabetic retinopathy provide a link between inflammation and the development of microvascular complication of diabetes.

Because of the significant difference in PON1/CRP ratio between patients without and with the presence of diabetic retinopathy, it seems that it may be used as a biochemical marker for progression of retinopathy but it requires further evaluation.

The link between the antioxidant concentration, inflammation and the development of diabetes complications need further longitudinal studies in order to confirm our findings.

Conflict of interest statement The authors declare that they have no commercial or financial interest in the publication of this article.

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