

# Comparison of serum NO, TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus

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LABORATORY STUDY

## Abstract

**Background** Vitreal interleukin (IL)-1beta (IL-1 $\beta$ ), IL-6, IL-8, tumor necrosis factor-alpha (TNF- $\alpha$ ) and nitric oxide (NO) levels have previously been determined in patients with proliferative diabetic retinopathy (PDR). However, at present there is no cohort study linking serum levels of NO and many inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , soluble IL-2 receptor (sIL-2R), IL-6 and IL-8 to the grade of the microvascular complications.

**Purpose** To determine the relation between the stages of DR and the levels of serum NO, TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6 and chemokine IL-8 in patients with diabetes compared with healthy controls.

**Methods** Fifty-three consecutive patients with diabetes (25 men, 28 women) with or without DR and 15 non-diabetic healthy subjects (seven men, eight women) as controls were included in this prospective study. As an indicator for NO, serum total nitrite (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>) levels (end-product of NO) were measured by the Griess reaction. Serum TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6 and IL-8 levels were determined by a spectrophotometric technique using an Immulite chemiluminescent immunometric assay. The patients with diabetes were classified into three groups according to the stage of DR: no DR (NDR;  $n = 16$ ), non-proliferative DR (NPDR;  $n = 18$ ) and PDR ( $n = 19$ ). The data were analysed using a Mann-Whitney *U*-test and the results were expressed as mean  $\pm$  SE (range).

**Results** The levels of IL-1 $\beta$  and IL-6 were below the detection limits of the assay (for each,  $<5.0$  pg/ml) in all patients with

diabetes and controls. Soluble IL-2R levels ranged from 260 to 958 U/ml, with the highest values observed in the patients with PDR. In 47 of the 53 samples (89%) tested for diabetic patients, IL-8 levels were above the detection limits of the assay (5.0 pg/ml). IL-8 levels ranged from  $<5.0$  to 25.0 pg/ml, with the highest mean values observed in PDR patients. TNF- $\alpha$  was detectable in 46 of 53 patients with diabetes (87%), ranging from  $<4.0$  to 26.4 pg/ml, with again the highest values obtained in the patients with PDR. Serum NO levels ranged from 80 to 188  $\mu$ mol/l, with the highest values obtained in patients with PDR. Taken together, the mean serum NO, sIL-2R, IL-8 and TNF- $\alpha$  levels increased with the stage of DR and the highest levels were found in patients with PDR. The PDR patients had significantly (for each,  $P < 0.001$ ) higher serum NO ( $166.8 \pm 3.2$   $\mu$ mol/l), sIL-2R ( $807.9 \pm 33.3$  U/ml), IL-8 ( $17.9 \pm 0.4$  pg/ml) and TNF- $\alpha$  ( $15.0 \pm 0.8$  pg/ml) levels compared with NPDR patients ( $149.5 \pm 2.1$ ,  $659.4 \pm 23.4$ ,  $12.9 \pm 1.1$ ,  $11.5 \pm 0.6$ , respectively), NDR patients ( $115.9 \pm 5.8$ ,  $373.8 \pm 15.0$ ,  $8.3 \pm 1.0$ ,  $6.6 \pm 0.9$ , respectively) and controls ( $116.6 \pm 2.3$ ,  $392.4 \pm 16.6$ ,  $7.2 \pm 0.3$ ,  $7.3 \pm 0.5$ , respectively). Serum levels of these parameters for NPDR patients were also significantly (for each,  $P < 0.01$ ) higher compared with those of NDR patients and controls. On the other hand, serum NO, sIL-2R, IL-8 and TNF- $\alpha$  levels of patients with NDR were comparable with those of controls (for each,  $P > 0.05$ ).

**Conclusion** The results of the present study suggest that NO, sIL-2R, IL-8 and TNF- $\alpha$  may play important roles in the

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Financial/proprietary  
interest: None

Received: 19 April 2001  
Accepted in revised form:  
13 August 2001

**pathophysiology and progression of DR. We think that these potentially inflammatory cytokines and NO with their endothelial implications may act together during the course and progression of DR. These molecules may serve as therapeutic targets for the treatment and/or prevention of diabetes with its systemic and ocular microvascular complications.**

*Eye* (2002) **16**, 163–170. DOI: 10.1038/sj/EYE/6700095

*Keywords:* chemokine; cytokine; diabetes mellitus; Griess reaction; immunoassay; interleukin; nitric oxide

## Introduction

Systemic microvascular disease and diabetic retinopathy (DR) is currently the principal cause of morbidity and mortality in patients with diabetes mellitus, yet the mechanism behind it is not fully understood.<sup>1</sup> It has been demonstrated that immune phenomena and inflammatory reactions might be involved in the pathogenesis and progression, especially in proliferative DR (PDR). Endothelial dysfunction with increased generation of oxygen-derived free radicals (oxidative stress) is a critical factor and endothelium-dependent vasodilatation is impaired in either type of human diabetes.<sup>2</sup> The feature of early stages of the disease is capillary pericyte loss, while progressive disease involves activation and proliferation of retinal vascular endothelial cells.<sup>3</sup> The two fundamental abnormalities in DR are increased retinal vascular permeability and progressive retinal vascular closure, leading to tissue hypoxia and ischaemia with neovascularisation of the retina by angiogenic factors.<sup>4</sup>

Recently, vitreous nitric oxide (NO),<sup>5</sup> tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL) 1- $\beta$  (IL 1 $\beta$ ), IL-6<sup>6</sup> and IL-8<sup>7</sup> levels have been studied in patients with PDR. However, previous studies have measured only a single cytokine in the vitreous sample, ignoring any potential interrelation between the levels of different cytokines, chemokine and NO. In addition, there are no studies in which serum has been analysed for NO, TNF- $\alpha$ , IL-1 $\beta$ , soluble IL-2 receptor (sIL-2R), IL-6 and IL-8 in the same patients as a cohort study linking their levels to the grade of the retinopathy. This is an important point because the breakdown of the blood-retinal barrier (BRB) in DR, especially in advanced cases, facilitates the extracapillary leakage of serum proteins and their passage from the blood stream to the vitreous fluid, thus enabling the serum to have an effect on intravitreal protein levels. Furthermore, high intravitreal levels of a particular protein in diabetic

patients do not necessarily mean its intraocular production only.

Therefore, this study has attempted to overcome these limitations in our understanding by measuring the serum levels of NO, several cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6 and chemokine IL-8, known to be proinflammatory molecules. We wished to test the hypothesis that no one factor alone is predominant in the pathophysiology of the progression of DR, that not only ocular synthesis is responsible in DR but that a regular profile of levels of different molecules might be operating together, and that the profile might differ according to the clinical stage of DR.

## Research design and methods

### Study population

Blood samples were obtained from 53 patients (25 men, 28 women) with non-insulin-dependent diabetes mellitus (NIDDM) (40–72 years old) and 15 healthy hospital staff volunteers (7 men, 8 women) from a similar ethnic background without diabetes (40–70 years old) who served as controls. The nature of the study was explained and all subjects gave their oral consent to participate. The Ethics Review Board of the university approved the study protocol. Patients were excluded if they had a history of intraocular ischaemia due to causes other than DR, such as retinal vein occlusion. All patients were in good diabetic control without microalbuminuria. Exclusion criteria included: (1) age over 75 years; (2) systemic hypertension (defined as systolic blood pressure > 145 mmHg, diastolic blood pressure > 90 mmHg); (3) ischaemic cerebrovascular disorders; (4) renal dysfunction (serum creatinine concentration > 1.5 mg/dl); (5) hepatic disorder (serum ALT > 32 IU/l, serum AST > 40 IU/l); (6) history of malignancy; (7) presence of haematological diseases; (8) hyperlipidaemia; (9) ischaemic cardiovascular disorders. These criteria were applied to all control subjects, whereas only criteria (4), (5), (6), (7) and (9) were applied to the diabetics.

The levels of DR were determined by fundus findings according to the Early Treatment of Diabetic Retinopathy Study (ETDRS).<sup>8</sup> All study patients underwent a complete ocular examination including visual field testing, slit-lamp biomicroscopy and indirect ophthalmoscopy. Patients with diabetes were classified as having no DR (NDR;  $n = 16$ ; mean age 50.5 years, range 45–59 years), non-proliferative DR (NPDR;  $n = 18$ ; mean age 55.0 years, range 40–63 years) or PDR ( $n = 19$ ; mean age 66.6 years, range 57–72 years). Since some medications reduce the transcription of the pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-8, and TNF-

$\alpha$ ),<sup>9</sup> or affect the function of cells that make NO, a detailed history of drug use was obtained in both groups and such patients were not included in the study.

### Blood samples

In both groups, whole blood samples (total 5 ml) were obtained by venipuncture from a peripheral vein, avoiding haemolysis, into plain tubes; patients were in a resting position in the morning hours (0830–0930 hours) after an overnight fast. None of the patients or healthy controls was receiving any topical or systemic medication on admission. Following centrifugation of the blood samples at 800 g for 10 min, serum was separated and kept at  $-20^{\circ}\text{C}$  until the time of cytokine, chemokine and NO metabolite analysis.

### Cytokine and chemokine analysis

Cytokine analysis was performed according to the Immulite (Diagnostic Products, Los Angeles, CA, USA) chemiluminescent immunometric assay. The technique is based on a solid-phase (bead) two-site chemiluminescent enzyme immunometric assay. The solid phase, a polystyrene bead, is coated with either a monoclonal specific antibody (TNF- $\alpha$ , IL-1 $\beta$ ) or an anti-ligand (sIL-2R, IL-6, IL-8). Patient serum and alkaline phosphatase-conjugated monoclonal antibody or ligand-labelled antibody are incubated for 30–60 min at  $37^{\circ}\text{C}$ . Unbound conjugate is then removed by a centrifugal wash ( $\times 3$ ), after which a chemiluminescent substrate (a phosphate ester of adamantyl dioxetane) is added, and the test unit incubated for a further 10 min. The Immulite system automatically handles sample and reagent additions, the incubation and separation step, and measurement of the photon output via the temperature-controlled luminometer. It calculates test results for controls and patient samples from the observed signal, using a stored master curve, and generates a printed report.<sup>10</sup> The values of the inter-assay precision study were similar to those from the intra-assay study, with a coefficient of variation (CV) ranging from 2% to 11.5%. CVs for the measured cytokines were usually around 5%. The linearity is satisfactory, with a regression coefficient higher than 0.99 ( $r^2$ ); slopes close to 1.0 were obtained. There is an excellent practicality of the system, and good stability of the calibration curve (15 days). It is a good reliable method yielding a good precision, along with a satisfactory detection limit.<sup>11</sup> The antibodies used in the Immulite assay for TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6 and IL-8 are highly specific for each cytokine and chemokine,

with no cross-reactivity to other interleukins that may be present in the serum samples.

For each new cytokine calibration, a master curve is constructed by the manufacturer using a material calibrated against the National Institute for Biological Standards and Control (NIBSC) standards. For each new cytokine reagent lot, an adjustment of the calibration slope is made by the user by measuring two serum matrix vials (low and high) designated as 'adjusters'. It should be mentioned that between-run and within-run precision data were similar, which is very important for stat measurement.

### Total nitrite ( $\text{NO}_2^- + \text{NO}_3^-$ ) analysis by the Griess reaction for nitric oxide

This assay provides reagents for use in the determination of nitrite ( $\text{NO}_2^-$ ) as an indicator of NO production in biological samples. NO has a brief half-life and is rapidly converted to the stable end-products  $\text{NO}_2^-$  and nitrate ( $\text{NO}_3^-$ ) in typical oxygenated aqueous solutions. Because an excellent colorimetric reagent (the Griess reagent) exists for the determination of  $\text{NO}_2^-$ , it is common practice to use enzymatic or chemical reduction to convert all  $\text{NO}_3^-$  to  $\text{NO}_2^-$  in a sample and to measure total nitrite as an indicator of NO production as described before.<sup>12–14</sup> This assay provides for enzymatic reduction of  $\text{NO}_3^-$  by nitrate reductase, followed by spectrophotometric analysis of total nitrite using Griess reagent. In addition to providing all necessary components in a microtitre format, it employs affinity-purified *Zea mays* nitrate reductase and NADH, thereby circumventing some of the potential problems reported for  $\text{NO}_2^-$  measurement using nicotinamide adenine dinucleotide phosphate (NADPH)-dependent nitrate reductases.

Spectrophotometric quantitation of  $\text{NO}_2^-$  using Griess reagent is straightforward and sensitive, but does not measure  $\text{NO}_3^-$ , causing a possible underestimation of NO. This method employs the NADH-dependent enzyme nitrate reductase for enzymatic reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  prior to quantitation of  $\text{NO}_2^-$  using Griess reagent. In short, serum (250  $\mu\text{l}$ ) was incubated at room temperature with 250  $\mu\text{l}$  of substrate buffer (imidazole 0.1 mol/l, NADPH 210  $\mu\text{mol/l}$ , flavine adenine dinucleotide 3.8  $\mu\text{mol/l}$ ; pH 7.6) in the presence of nitrate reductase (*Aspergillus niger*, Sigma) for 45 min to convert  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . Excess reduced NADPH, which interferes with the chemical detection of  $\text{NO}_2^-$ , was oxidised by continuation of the incubation of 5  $\mu\text{g}$  (1 ml) of LDH (Sigma), 0.2 mmol/l (120  $\mu\text{l}$ ) pyruvate (Sigma) and 79 ml of water. Total nitrite was then analysed by reacting the samples with Griess reagent (1%

sulphanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5%  $\text{H}_3\text{PO}_4$  spectroquant: Merck, Darmstadt, Germany). Reacted samples were treated with 500  $\mu\text{l}$  of trichloroacetic acid (20%), centrifuged for 15 min at 8000 *g* and the absorbance at 548 nm compared with that of an  $\text{NaNO}_2$  standard (0–100  $\mu\text{mol/l}$ ). This method can be used to accurately measure as little as 1  $\mu\text{mol}$  of  $\text{NO}_2^-$  (final concentration in the assay). Very little sample is required (5–85  $\mu\text{l}$  for most samples).

### Statistical analysis

Results were analysed statistically by using the Mann–Whitney *U*-test, and all values were expressed as mean  $\pm$  standard error (SE) and range.  $P < 0.05$  was considered as a significant difference between the groups.

### Results

All values for IL-1 $\beta$  and IL-6 in diabetic patients and controls were below the detection limits of the assay (for each,  $<5.0$  pg/ml). Serum NO levels in patients with PDR ( $166.8 \pm 3.2$  (135–188)  $\mu\text{mol/l}$ ) were significantly higher compared with NPDR ( $149.5 \pm 2.1$  (125–162)  $\mu\text{mol/l}$ ,  $P < 0.001$ ), NDR ( $115.9 \pm 5.8$  (80–150)  $\mu\text{mol/l}$ ,  $P < 0.001$ ) and controls ( $116.6 \pm 2.3$  (101–135)  $\mu\text{mol/l}$ ,  $P < 0.001$ ). Soluble IL-2R levels ranged from 260 to 958 U/ml, with the highest mean values observed in the patients with PDR. Patients with PDR had significantly higher (for each,  $P < 0.001$ ) serum sIL-2R levels ( $807.9 \pm 33.3$  (480–958) U/ml) compared with those of NPDR ( $659.4 \pm 23.4$  (410–785) U/ml), NDR ( $373.8 \pm 15.0$  (260–493) U/ml) and controls ( $392.4 \pm 16.6$  (260–555) U/ml) (Table 1). Serum NO and sIL-2R levels were correlated with the progression of DR (Figure 1).

The concentrations of IL-8 were detectable in 47 of 53 (89%) patients with diabetes and in 13 of 15 (87%) healthy controls. The mean serum IL-8 concentration was significantly higher in patients with PDR ( $17.9 \pm 0.4$  (15.0–21.0) pg/ml) than in patients with NPDR ( $12.9 \pm 1.1$  ( $<5.0$ –25.0) pg/ml,  $P < 0.001$ ), NDR ( $8.3 \pm 1.0$  ( $<5.0$ –22.4) pg/ml,  $P < 0.001$ ) and controls ( $7.2 \pm 0.3$  ( $<5.0$ –9.0) pg/ml,  $P < 0.001$ ). TNF- $\alpha$  was detectable in 46 of 53 (87%) patients with diabetes. The highest TNF- $\alpha$  concentrations were observed in patients with PDR ( $15.0 \pm 0.8$  (10.0–26.4) pg/ml) and were significantly higher compared with NPDR ( $11.5 \pm 0.6$  (6.4–16.4) pg/ml,  $P < 0.001$ ), NDR ( $6.6 \pm 0.9$  ( $<4.0$ –16.2) pg/ml,  $P < 0.001$ ) and controls ( $7.3 \pm 0.5$  (5.6–13.0) pg/ml,  $P < 0.001$ ). Serum IL-8 and TNF- $\alpha$

levels were also correlated with the progression of DR (Figure 2).

The same findings were observed for patients with NPDR when compared with NDR and healthy controls (for each,  $P < 0.01$ ). On the other hand, the difference between NDR patients and healthy controls was not significant for the mean serum TNF- $\alpha$ , sIL-2R, IL-8 and NO levels (for each,  $P > 0.05$ ).

### Discussion

Progression of DR from NPDR to PDR is a serious complication of diabetes. This progression results in the activation and proliferation of vascular endothelial cells with leukocyte adhesion to the diabetic retinal vasculature. Overall, DR is characterised by a notable increase in antibody-dependent immune response. In addition, degeneration and loss of pericytes are seen as a result of systemic metabolic abnormalities associated with prolonged hyperglycaemia.<sup>15</sup> The early changes in the systemic and retinal vasculature may lead to a compromised blood flow, resulting eventually in abnormally leaky vessels. It has been demonstrated that retinal blood flow is increased and correlated with progressing non-proliferative retinopathy levels with venous dilatation, resulting in the inability of the retinal vessels to autoregulate.<sup>16</sup> Breakdown of the BRB with increased vascular permeability is an important component of the progression of DR from the non-proliferative state through retinal neovascularisation.

Although the increased intraocular levels of NO<sup>5</sup> and some cytokines<sup>6,7</sup> have been demonstrated in DR, the exact source of these molecules is not well known. However, the possibility exists that the increased levels are derived from the blood by the breakdown of the BRB. Therefore, they may contribute to the elevation of vitreous cytokine and NO levels, thus involving in the progression of DR. In addition, this is not surprising since vitreous haemorrhage is frequently associated with the progression of the disease and is not an independent variable. Furthermore, all eyes with PDR had panretinal photocoagulation before vitrectomy. It is therefore possible that laser photocoagulation results in the increased levels. In this respect, our previous study clearly demonstrated that IL-6, IL-8 and NO levels were significantly increased in rabbit vitreous after laser photocoagulation.<sup>17</sup> Therefore, it is likely that the increased vitreous levels of cytokines and NO found in previous studies may not only reflect the intraocular production, but may also arise from the systemic blood by the breakdown of the BRB with increased vascular permeability and vitreous haemorrhage as the disease progresses. On this basis, it seemed relevant to determine the serum levels of these

**Table 1** The serum NO, IL-1 $\beta$ , sIL-2R, IL-6, IL-8 and TNF- $\alpha$  levels in patients with PDR, NPDR, NDR and healthy controls, with statistical analysis

	PDR n = 19 (9 M, 10 F)		NPDR n = 18 (8 M, 10 F)		NDR n = 16 (8 M, 8 F)		Controls n = 15 (7 M, 8 F)	
	Mean $\pm$ SE	Range	Mean $\pm$ SE	Range	Mean $\pm$ SE	Range	Mean $\pm$ SE	Range
Age (years)	66.6 $\pm$ 0.7	57–72	55.0 $\pm$ 1.2	40–63	50.5 $\pm$ 1.0	45–59	54.1 $\pm$ 2.0	46–70
Duration (years)	23.7 $\pm$ 1.3	15–35	9.6 $\pm$ 1.1	6–20	6.0 $\pm$ 0.6	2–12	–	–
Mean BP (mmHg) <sup>d</sup>	90.2 $\pm$ 1.7	105–82	88.4 $\pm$ 1.5	101–80	86.1 $\pm$ 1.4	95.4–81	84.5 $\pm$ 1.4	92–80
NO ( $\mu$ mol/l)	166.8 $\pm$ 3.2 <sup>a</sup>	135–188	149.5 $\pm$ 2.1 <sup>b</sup>	125–162	115.9 $\pm$ 5.8 <sup>c</sup>	80–150	116.6 $\pm$ 2.3	101–135
IL-1 $\beta$ (pg/ml)	<5.0	–	<5.0	–	<5.0	–	<5.0	–
sIL-2R (U/ml)	807.9 $\pm$ 33.3 <sup>a</sup>	480–958	659.4 $\pm$ 23.4 <sup>b</sup>	410–785	373.8 $\pm$ 15.0 <sup>c</sup>	260–493	392.4 $\pm$ 16.6	260–555
IL-6 (pg/ml)	<5.0	–	<5.0	–	<5.0	–	<5.0	–
IL-8 (pg/ml)	17.9 $\pm$ 0.4 <sup>a</sup>	15.0–21.0	12.9 $\pm$ 1.1 <sup>b</sup>	<5.0–25.0	8.3 $\pm$ 1.0 <sup>c</sup>	<5.0–22.4	7.2 $\pm$ 0.3	<5.0–9.0
TNF- $\alpha$ (pg/ml)	15.0 $\pm$ 0.8 <sup>a</sup>	10.0–26.4	11.5 $\pm$ 0.6 <sup>b</sup>	6.4–16.4	6.6 $\pm$ 0.9 <sup>c</sup>	<4.0–16.2	7.3 $\pm$ 0.5	5.6–13.0

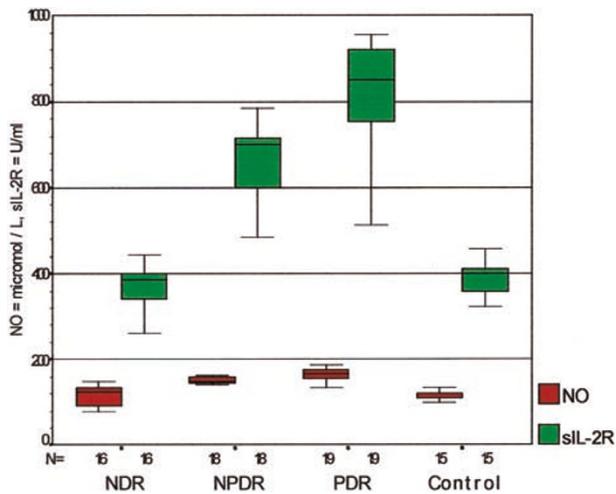
NO, nitric oxide; IL, interleukin; TNF, tumor necrosis factor; PDR, patients with proliferative diabetic retinopathy; NPDR, patients with non-proliferative diabetic retinopathy; NDR, patients with no diabetic retinopathy; M, male; F, female; BP, blood pressure.

<sup>a</sup>Significantly higher than all other groups including healthy controls by Mann-Whitney *U*-test (for each, *P* < 0.001).

<sup>b</sup>Significantly higher than NDR patients and healthy controls by Mann-Whitney *U*-test (for each, *P* < 0.01).

<sup>c</sup>Not different when compared with healthy controls by Mann-Whitney *U*-test (*P* > 0.05).

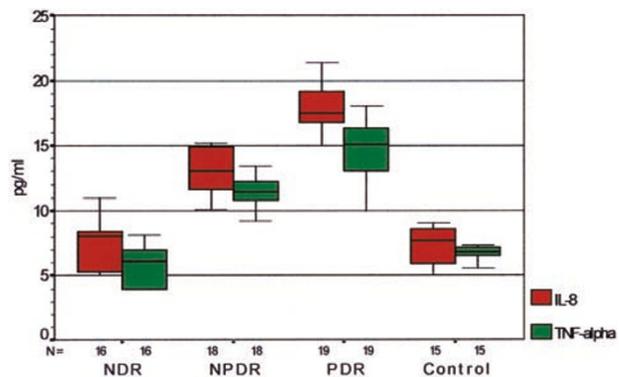
<sup>d</sup>Mean blood pressure = 1/3 (systolic pressure – diastolic pressure) + diastolic pressure<sup>16</sup>



**Figure 1** Mean serum NO and sIL-2R levels according to the stage of diabetic retinopathy (DR): no DR (NDR), non-proliferative DR (NPDR), proliferative DR (PDR) and controls. Levels of both molecules were significantly higher in PDR and NPDR patients than controls.

cytokines and NO according to the grade of DR. Therefore, in this study we investigated the serum of patients with DR for the presence of TNF- $\alpha$ , IL-1 $\beta$ , sIL-2R, IL-6, IL-8 and NO in an attempt to determine whether or not there is a correlation between the levels of these molecules and clinical retinopathy.

NO, known as endothelial derived relaxing factor (EDRF), is a gas that transmits signals in the organism and is responsible for the cytotoxicity of activated macrophages. It is well known that NO is a potent vasodilator and has a role in ischaemic processes, through its contribution to the basal tone in the retinal



**Figure 2** Mean serum IL-8 and TNF- $\alpha$  levels according to the stage of diabetic retinopathy (DR): no DR (NDR), non-proliferative DR (NPDR), proliferative DR (PDR) and controls. Levels of both cytokine and chemokine were significantly higher in PDR and NPDR patients than controls.

circulation. It has been demonstrated that functional changes in the synthesis and metabolism of NO occur in the vascular walls of diabetic rats with increased superoxide anions, resulting in BRB breakdown.<sup>18</sup> Diabetic vascular damage in the retina may be mediated in part by NO, and this regulates the amount of retinal blood flow.<sup>19</sup> In addition, it has been demonstrated that NO can be induced by cytokines during inflammatory and infectious processes resulting in large amounts of NO production.<sup>20</sup> From these properties one may speculate that this gas may be involved in the early and late systemic and retinal complications of DR. Increased serum NO production found in our study with the stage of DR may contribute to the increased vascular permeability in the retina, and therefore the progression of the disease.

The present study clearly showed that serum NO level was correlated with the stage of DR. We think that NO is at least partly involved in the pathogenesis of the systemic and retinal microvascular consequences of diabetes.

Cytokines are polypeptides involved in the communication between cells, and abnormal production of IL-1 $\beta$ , sIL-2R, IL-6, IL-8 and TNF- $\alpha$  has been implicated in the pathogenesis of various inflammatory and autoimmune diseases.<sup>21</sup> Their pro-inflammatory response has been demonstrated. We think that these potentially inflammatory cytokines and NO with its endothelial implications may act together in the progression of DR. Chemokines that induce chemotaxis such as IL-8 have been identified in vitreous from patients with PDR.<sup>22</sup> The sIL-2R is a smaller polypeptide than the membrane-bound IL-2R, and has a potential role in the modulation of immune responses by inhibiting IL-2.<sup>23</sup> We think that high serum levels of sIL-2R, IL-8 and TNF- $\alpha$  indicate the activation of the immune system in DR and may have a pathophysiological role in the course of the disease.

Since some cytokines are potent vascular permeability factors, there is a keen interest in determining whether cytokines and NO may contribute to the early systemic and retinal microvascular abnormalities induced by diabetes and to the characteristic capillary leakage responsible for hard exudates and macular oedema. TNF- $\alpha$ , like IL-6 and IL-8, is a pro-inflammatory cytokine that involves BRB breakdown by opening tight junctions of retinal vascular endothelial cells and pigmented epithelial cells, participating in the pathogenesis of diabetes.<sup>24</sup> The greater the diabetic complications, the more TNF- $\alpha$ -stimulated superoxide was produced by activated polymorphonuclear neutrophils, contributing to the progression of DR in NIDDM.<sup>25</sup> Tanaka *et al*<sup>26</sup> reported that TNF- $\alpha$  serum activity was chronically increased in a rat model of NIDDM. Our *in vivo* results are compatible with these experimental data. Therefore, the present study showed that TNF- $\alpha$  levels in the serum of patients with PDR was significantly higher than in patients with NPDR, NDR and controls. These observations suggest that abnormalities in TNF- $\alpha$  production and control may operate during the development of microvascular complications of diabetes.

It has been demonstrated that plasma IL-6 levels in human peripheral monocytes were significantly elevated in patients with poorly controlled diabetes. After normalisation of plasma glucose, IL-6 levels declined.<sup>27</sup> Another previous *in vitro* study did not demonstrate a significant glucose-dependent effect on the production of IL-1 and IL-6.<sup>28</sup> Our *in vivo* results

are consistent with these *in vitro* studies and both these cytokines were undetectable in the serum of diabetic patients, as in controls.

Taken together the findings of the present study imply significantly increased serum NO metabolite levels in diabetic patients compared with healthy controls. This observation is possibly due to the NO-generating cells such as endothelial cells, PMN and macrophages found in inflammation, since these cells are also known to be involved in the pathogenesis of diabetes.<sup>29</sup> Increased cytokine levels found in the present study may be involved in the upregulation of endothelial cells, and induce NO synthase and thereby increase NO production. Therefore, our results suggest that chemokine, cytokines and NO take part together in the pathogenesis and progression of DR.

It has been demonstrated that increased glucose concentrations change cytokine secretion, suggesting an important pathological effect of these molecules with long-term metabolic glycaemic control parameters.<sup>30,31</sup> However, our patients with diabetes were all in good metabolic control at the time of study and blood sampling. In addition, it has been demonstrated that smoking has no influence on the levels of cytokines.<sup>32</sup> Furthermore, Hofbauer *et al*<sup>33</sup> have also reported that cytokine levels are not correlated with gender, age or smoking status. Likewise, Satoh *et al*<sup>34</sup> investigated the influence of age, smoking and race on serum cytokine levels in a healthy population using a sensitive ELISA, and found no difference between smokers and non-smokers for any cytokine. In addition, no correlation between age and cytokines was demonstrated. Therefore, the age difference between the diabetic groups and control in this study would have had no effect on the measured levels of cytokines.

Foss *et al*<sup>35</sup> demonstrated that there was no correlation between serum cytokine levels and either duration of diabetes or indices of metabolic control. However, serum cytokine levels progressively increased from well to poorly controlled diabetic groups. Serum cytokine concentrations in diabetic patients with chronic complications or without complications were statistically higher than in control subjects. The progressive increase in serum cytokine levels from the well to the poorly controlled diabetic groups suggests a relationship between levels of this cytokine and protein glycosylation.

Since it is well known that diabetes is characterised by systemic endothelial dysfunction with immunoinflammatory events, we think that the lymphocytes and leukocytes participate in the course and progression of diabetes and its complications. The reason is that most cytokines are released from lymphocytes and leukocytes, and previous studies

have demonstrated, for instance, that there is a significant association between the released lymphokine levels (released from T-lymphocytes and recruits macrophages) and grades of proliferative diabetic retinopathy.<sup>25</sup> In addition, immunohistochemical studies of preretinal membranes in proliferative retinopathy showed the presence of activated T-lymphocytes, B-lymphocytes, macrophages and deposits of immunoglobulins and complement.<sup>3,5,6,22</sup> Our results also suggest this conclusion.

In conclusion, our results have demonstrated increased serum sIL-2R, IL-8, TNF- $\alpha$  and NO levels in diabetic patients, with a significant correlation between the levels and the grade of DR. This suggests that these molecules may participate in the development and progression of DR from NPDR to PDR. However, we cannot distinguish whether the increased levels are the cause or the result of the DR process. Future studies may be necessary to determine whether these measured cytokines, chemokine and NO occur naturally in the progression of DR or are elicited in response to treatment. Thus, further investigation of the mechanism of action is needed to understand the precise role of NO, sIL-2R, IL-8 and TNF- $\alpha$  in diabetes.

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