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Comparison of Conventional Inflammatory Parameters with Tumor Necrosis Factor-α Levels in Different Stages of Diabetic Retinopathy and Non-Diabetic Controls

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Abstract

Aim of this study was to identify the risk factors associated with diabetic retinopathy in patients with Type 2 diabetes mellitus(DM), and to compare the levels of erythrocyte sedimentation rate (ESR), high sensitive CRP(hs-CRP) and tumor necrosis factor-α (TNF-α), in patients with every stage of diabetic retinopathy and non-diabetic healthy controls. A total of 79 individuals, 64 diabetic and 25 healthy controls were included in the study. Patients were divided into three groups. Group 1 consisted of those without diabetic retinopathy (DR) (n=26), group 2 with non-proliferative (NPDR) (n=19), and group 3 with proliferative diabetic retinopathy (PDR) (n=19). Even though the similar age distribution, disease duration were different as 6.9 ± 6.5, 10.3 ± 5.4, and 12.7 ± 5.1 years in Group 1, Group 2 and Group 3, respectively (p>0.001). The majority of the patients with PDR were male, had high levels of albuminuria and high percents of receiving insulin treatment (p<0.05). The levels of ESR, hs-CRP and TNF-α were not different either in or between the groups. A positive correlation was observed between hs-CRP and postprandial blood glucose (p<0.001 and rho=0.621). Disease duration and metabolic control were considered as the most important determining factors with regard to the DR. Presence of a positive correlation between hs-CRP and postprandial hyperglycemia indicates the importance of post absorptive glycemic excursions.

Keywords: Diabetic retinopathy, inflammation, hs-CRP, TNF-α, postprandial blood glucose

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Introduction

Urban living and technological innovations have brought about a rapid increase in sedentary lifestyles along with a parallel increase in obesity prevalence in both developed and developing countries. This negative development has led to a global diabetes epidemic. Extended life spans and developments in diabetic treatment have meant a marked increase in the number of patients affected by the chronic complications associated with diabetes mellitus (DM). As a result, both macro- and microvascular complications such as retinopathy, nephropathy, and neuropathy are more commonly observed; and there has been a significant rise in the mortality and morbidity rates related to true complications [1-4]. Diabetic retinopathy (DR) is the most common preventable cause of blindness among individuals 20–74 years of age in developing countries. Many risk factors have been identified for the development and progression of DR, including genetic predisposition, type of DM, disease duration, hyperglycemia, hyperlipidemia, hypertension, nephropathy, pregnancy, and puberty. There has been an increase in investigations aimed at identifying individuals at risk through various serological markers and periodic eye examinations, because these high-risk lesions can progress asymptomatically in these patients [1-6].

C-reactive protein (CRP) is the prototype acute-phase protein. Its level starts rising in the sixth hour after inflammation begins and falls back to normal after there is no more inflammation due to its short half-life [7,8]. Clinical studies demonstrated that subclinical chronic inflammation causes an increase in cardiovascular risk in diabetic and non-diabetic individuals. As a result, a close relationship was reported between the development of cardiovascular diseases and CRP levels [9-14] It was also reported that an increase in erythrocyte sedimentation rate (ESR) may indicate the presence of this subclinical inflammation due to its dependence on plasma fibrinogen levels and in light of the studies reporting increased fibrinogen levels in patients with DM [15].

Furthermore, tumor necrosis factor (TNF)-α, an endogenous angiogenic factor, has recently been identified as a multifunctional regulatory cytokine [16-18]. It has been demonstrated that TNF-α changes endothelial cell morphology and function, increases the synthesis of inflammatory markers such as CRP and other cytokines, mediates monocytic and fibroblastic chemotaxis, and increases extracellular matrix protein synthesis in patients with type 1 and

type 2 DM [19, 20]. Additionally, it has been demonstrated in various studies that TNF-α is an important mediator of obesity-associated insulin resistance, and its levels also increase together with insulin resistance in obese patients with type 2 DM [21-23].

The aim of this study was to identify risk factors related to DR development and to detect and compare the levels of conventional inflammatory parameters such as erythrocyte sedimentation rate (ESR), high-sensitivity CRP (hs-CRP), and TNF-α, during the various stages of DR in patients with type 2 DM and in non-diabetic controls.

Materials and Methods

This study was conducted in patients with type 2 DM, who were followed up in the Endocrinology and Metabolism Division of the Department of Internal Medicine at the Uludag University Faculty of Medicine, after obtaining approval from the local ethics committee. All patients with DM who agreed to sign written informed consent were scheduled for screening. Patients with diseases that could affect lipid and carbohydrate metabolism apart from diabetes; those with malignant diseases, systemic or local infections, diabetic foot lesions, traumatic musculoskeletal system diseases, immune deficiency, anemia or leucopenia, chronic liver disease, or end-stage renal failure; those using drugs that could affect inflammatory markers and measurements of cytokine levels; and those who smoked were excluded. Individuals in whom no systemic disease was detected through history, physical examination, or laboratory tests; who were not using steroids or non-steroidal anti-inflammatory drugs; and who had demographic characteristics similar to the patient groups were included in the control group.

Demographic data such as age and sex and antidiabetic treatment regimens of all patients were recorded. Detailed physical examinations of all patients were performed, and those with findings that necessitated exclusion from the study were not evaluated. Body mass index (BMI) was calculated by obtaining height and weight measurements and dividing the weight in kilograms by the square of the height in meters (kg/m^2). Blood pressure measurements were obtained while sitting, after a 30-minute rest, and 1-minute pulse rates were recorded.

The patients were later examined by the same physician at the Ophthalmology Department Outpatient clinic and divided into groups according to the specifications of the Diabetic

Retinopathy Study and Early Treatment Diabetic Retinopathy Study. **Group 1** consisted of patients without DR (n=26), **Group 2** consisted of patients with background non-proliferative DR lesions (n=19), and **Group 3** consisted of patients with neovascularization, pre-retinal hemorrhage, vitreous hemorrhage, pan-retinal photocoagulation scarring, and a history of vitrectomy (n=19). Healthy volunteers constituted the **control group** (n=25).

After an average of 10 hours of fasting, venous blood samples were obtained and biochemical, hematological, and immunological analyses were performed. Postprandial blood glucose (PPBG) measurements were performed 120 minutes after starting breakfast. A 24-hour urine sample of each patient was collected and the amount of quantitative albumin excretion was measured. The hs-CRP was measured using immunonephelometry reagents (Dade Behring Inc., Marburg, Germany). Quantitative TNF-α levels were measured using the Human TNF-α Elisa Kit (Endogen Il; Pierce, Rockford, IL, USA). The lower detection limit of TNF-α was 2 pg/mL. The intra-assay and interassay coefficients of variation were 4.2% and 4.5%, respectively.

Statistical Methods

Statistical analyses of the obtained data were performed using the IBM-Statistical Package for the Social Sciences (SPSS) version 21,0 program. Continuous variables are presented as median (min-max), disease duration was given mean (± standard deviation) and percentage of patients using insulin was given as (n, %).

The Shapiro–Wilk test was used to determine the normality of the data. Comparisons of the groups were performed using the Kruskal-Wallis nonparametric test. The Mann-Whitney *U* test was used for parametric comparison of two groups. The relationships between serum hs-CRP and TNF-α levels and independent variables were evaluated using the Spearman correlation coefficients. The significance level was considered as $p < 0.05$.

Results

Data comparisons of the patients with DM and the control group enrolled in our study are shown in Table 1.

Table1. Data comparisons of patients with type 2 diabetes and control group

Data	Control group (n=25)	Group 1 (n=26)	*p* 1	Group 2 (n=19)	*p* 2	Group 3 (n=19)	*p* 3
Age (years)	46(35-64)	53,5(38-77)	0,231	61(35-75)	0,001	60(47-74)	0,001
SBP (mmHg)	120(100-150)	125(100-150)	**0,030**	130(100-165)	**0,021**	130(110-165)	**0,019**
DBP (mmHg)	75(55-95)	77,5(60-95)	**0,022**	80(60-95)	**0,019**	75(55-95)	**0,65**
BMI (kg/m^2)	28(21-44)	27(18-38)	0,450	26(24-39)	0,506	27(23-43)	0,704
FBG (mg/dL)	99(57-119)	233(115-481)	**<0,001**	196(92-386)	<0,001	196(97-375)	**<0,001**
PPBG (mg/dL)	124(76-148)	281,5(152-551)	**<0,001**	233(150-526)	<0,001	249(125-421)	**<0,001**
HbHbA1c%	5,4(4,7-6,4)	10,4(6,8-14,1)	**<0,001**	8,9(7,5-12,8)	<0,001	9,3(4,9-13,6)	**<0,001**
Total C (mg/dL)	183(136-295)	196(148-279)	0,457	180(128-286)	0,569	196(76-249)	0,776
HDLC (mg/dL)	50(24-82)	50,5(34-89)	0,5400	43(32-61)	0,099	43(29-68)	0,180
LDLC (mg/dL)	111(65-213)	109(55-183)	0,366	105(46-202)	0,196	116(30-151)	0,400
VLDL (mg/dL)	24,4(9-65)	32,1(10,2-74,4)	**0,016**	31,2(16,4-75,2)	0,045	27,4(12,5-62,8)	0,690
TG (mg/dL)	122(45-324)	160,5(51-372)	**0,016**	156(82-376)	0,110	140(70-314)	0,387
MA (mg/24 h)	4,3(0-10,6)	12,7(0,2-389)	**<0,001**	10,1(2,8-88)	**<0,001**	16,9(3,9-751)	**<0,001**
ESR (mm/h)	9(2-37)	9(2-38)	0,538	13(2-40)	0,265	15(1-80)	0,109
hs-CRP (mg/dL)	0,33(0,05-3,77)	0,30(0,01-4,59)	0,559	0,12(0,02-2,06)	0,112	0,30(0,07-2,81)	0,644
TNF-α (pg/mL)	6,53(2,2-32,6)	8,62(2,2-39)	0,332	7,26(2,2-21,5)	0,098	6,85(2,2-18,5)	0,818

Abbreviations: ***DD:*** *disease duration;* ***SBP:*** *systolic blood pressure;* ***DBP:*** *diastolic blood pressure;* ***BMI:*** *Body mass index;* ***FBG:*** *Fasting blood glucose;* ***PPBG:*** *Postprandial blood glucose;* ***HbA1c%:*** *Percentage of glycosylated hemoglobin; ,* ***Total C :*** *total cholesterol* **,** ***HDL C****: high- density lipoprotein cholesterol* ***LDL C****: low-density lipoprotein cholesterol ,* ***VLDL C****: very low-density lipoprotein cholesterol;* ***TG****: Triglyceride,* ***MA****: Microalbuminuria,* **hs-CRP:** High sensitive c-reactive protein, ***TNF-α:*** tumor necrosis factor alpha, ***PUI:*** *Percentage of patients using insulin*

p1: Comparision of Control&Diabetic patients without retinopathy,

p2: Comparision of Control& Diabetic patients with non-proliferative retinopathy,

p3: Comparision of Control& Diabetic patients with proliferative retinopathy

Although there was a significant differences in terms of age distribution between control and group 2 and group 3 (p<0,001), no significant age distribution was found between group 1 (p>0,05). The median age was 46 (35-64), 53,5(38-77), 61(35-75) and 60(47-74) respectively. There was a wider distribution in terms of age in diabetic patients than controls. There were significant differences for measurement of SBP and DBP in between control and diabetic groups (p<0,005). Median SBP was higher in diabetic patients (125 mmHg,130 mmHg, 130 mmHg) than controls (120 mmHg) But, median DBP was high in group 1 and 2 (77,5 mmHg, 80 mmHg) when compared with control and third groups (75 mmHg in both groups).

As expected, statistically significant differences were found between the groups with regard to metabolic parameters. There were significant differences for measurements of Fasting blood glucose (FBG); postprandial blood glucose (PPBG); glycosylated hemoglobin (HbA1c); low-density lipoprotein (LDL), very-low-density lipoprotein cholesterol (VLDL); triglycerides (TRG); and 24 hour-urinary albumin excretion rate in between controls and diabetic groups. P value was <0,005 for all parameters as stated above. Un expectedly higher FBG (233), PPBG (281), and HbA1c (10,3%) levels were found in group 1. The patients in this group showed more significant differences when compared the control group than other diabetic groups. There were no significant differences in ESR, hs-CRP, and TNF- α levels. Median ESR was 9 mm/h, 9 mm/h, 13 mm/h and 15 mm/h respectively. While median hs-CRP was 0,33 mg/dL, 0,30 mg/dL, 0,559 mg/dL and 0,30 mg/dL for each group respectively, median TNF- α was 6,53 (2,2-32,6), 8,62 (2,2-39), 7,26(2,2-21,5) and 6,85(2,2-18,5).

Comparison of the data obtained from diabetic patients with no retinopathy and those with proliferative and non-proliferative retinopathy are shown in Table 2.

There was no significant difference in age distribution between the groups. However, there was a statistically significant difference with regard to diabetes age ($p < 0.001$). The disease duration was 6.9 ± 6.5 years for patients without retinopathy, 10.3 ± 5.4 years for patients with nonproliferative retinopathy, and 12.7 ± 5.1 years for patients with proliferative retinopathy. No statistically significant differences were found between the groups with regard to age, BMI, arterial blood pressure, HbA1c, and the other metabolic parameters, together with inflammatory parameters and cytokine levels ($p > 0.05$). However, in the proliferative retinopathy group, significantly higher urinary albumin excretion was found, compared with both the group without retinopathy and that with non-proliferative retinopathy ($p < 0.005$). Similarly, the percentage of patients who were receiving insulin treatment among patients with proliferative retinopathy (42%, 52%, and 68,4% respectively and p=0,003) was significantly higher than that of the other two groups.

Although not given in the table due to lack of meaningful data a positive correlation was found only between PPBG and hs-CRP levels. The correlation coefficient and p value for hs-CRP were 0.231 and 0.041, respectively. There was no correlation between hs-CRP and TNF-

α levels. Furthermore, apart from PPBG, no significant correlations were detected between metabolic measurements, demographic data, and inflammatory and cytokine measurements.

Table 2. Comparison of the characteristics, metabolic control and inflammatory parameters of diabetic patients

Data	Group 1 (n=26)	Group 2 (n=19)	Group 3 (n=19)	*p*
Age (years)	53,5(38-77)	61(35-75)	60(47-74)	0,233
DD (years)	6.9 ± 6.5 [a,b]	10.3 ± 5.4	12.7 ±5.1	**0,001**
SBP (mmHg)	125(100-150)	130(100-165)	130(110-165)	0,321
DBP (mmHg)	77,5(60-95)	80(60-95)	75(55-95)	0,587
BMI (kg/m^2)	27(18-38)	26(24-39)	27(23-43)	0,934
FBG (mg/dL)	233(115-481)	196(92-386)	196(97-375)	0,299
PPBG (mg/dL)	281,5(152-551)	233(150-526)	249(125-421)	0,193
HbA1c%	10,4(6,8-14,1)	8,9(7,5-12,8)	9,3(4,9-13,6)	0,099
Total C (mg/dL)	196(148-279)	180(128-286)	196(76-249)	0,776
HDLC (mg/dL)	50,5(34-89) [a,b]	43(32-61)	43(29-68)	0,059
LDLC (mg/dL)	109(55-183)	105(46-202)	116(30-151)	0,277
VLDL (mg/dL)	32,1(10,2-74,4)	31,2(16,4-75,2)	27,4(12,5-62,8)	0,117
TG (mg/dL)	160,5(51-372)	156(82-376)	140(70-314)	0,277
MA (µg/min)	12,7(0,2-389)	10,1(2,8-88)	16,9(3,9-751)	0,0,03
ESR (mm/h)	9(2-38)	13(2-40)	15(1-80) [a]	0,005
hs-CRP (mg/dL)	0,30(0,01-4,59)	0,12(0,02-2,06)	0,30(0,07-2,81)	0,141
TNF-α (pg/mL)	8,62(2,2-39)	7,26(2,2-21,5)	6,85(2,2-18,5)	0,374
PUI (%)	42	52	68.4	0,003

F/M: *female male ratio;* ***DD:*** *disease duration;* ***SBP:*** *systolic blood pressure;* ***DBP:*** *diastolic blood pressure;* ***BMI:*** *body mass index;* ***FBG:*** *fasting blood glucose;* ***PPBG:*** *postprandial blood glucose;* ***HbA1c%:*** *percentage of glycosylated hemoglobin; ,* ***Total C :*** *total cholesterol* **,** ***HDL C****: high- density lipoprotein cholesterol* ***LDL*** *C: low-density lipoprotein cholesterol ,* ***VLDL C****: very low-density lipoprotein cholesterol;* ***TG****: Triglyceride;* ***MA****: microalbuminuria;* **hs-CRP:** high sensitive c-reactive protein, ***TNF-α:*** tumor necrosis factor alpha, ***PUI:*** *Percentage of patients using insulin*

a: *Comparision of diabetic patients without retinopathy& with non-proliferative retinopathy*

b: *Comparision of diabetic patients without retinopathy & proliferative retinopathy*

Discussion

It is well-known that approximately 25% of the patients with type 2 DM, in whom the prevalence of DR is directly proportional to disease duration, develop DR during the diagnostic stage of the disease and that 60% of the patients without retinopathy may also develop DR 20 years later [1-4,6]. In parallel with data in the literature, the mean age of the patients without DR in our study was 54.69±11.58 years, and the mean disease duration was 6.96±6.58 years. In the group with non-proliferative retinopathy, the mean age was 59.79±10.36 years and their mean disease duration was 10.31±5.45 years. In the group with proliferative retinopathy, the mean age was was 58.84±7.89 years and their mean disease duration was 12.78±5.1 years. Although there was no significant difference between the groups with regard to age distribution, there was a statistically significant difference between the groups with regard to disease duration. This difference reveals that disease duration is an important risk factor for the development of DR.

The Diabetes Control and Complications Trial (DCCT) conducted in patients with type 1 DM and the United Kingdom Prospective Diabetes Study (UKPDS) conducted in patients with type 2 DM demonstrated that glycemic control is the most important factor that determines the development and progression of microvascular complications [4-6]. In our study, the patient group without DR mostly consisted of obese women with shorter disease durations, and their mean HbA1c levels were higher than those of the other groups. Despite the poor metabolic control of the patients in the other two groups, their HbA1c levels were lower. However, the metabolic data revealed significantly higher values in each of the three groups compared with the control group. The mean HbA1c measurements of the various groups were 10.27±1.99%, 9.47±1.84%, and 9.23±1.86% in Groups 1, 2, and 3, respectively, whereas it was 5.23±0.32% in the control group.

In patients with DR, the mean disease duration was longer and there were higher rates of insulin administration (42%, 52%, and 68.4% in Groups 1, 2, and 3, respectively). The probable explanation of the association between higher rates of insulin use and better glycemic control in these patients may be the experience in the disease control. Initially patients with type 2 DM use oral therapy and usually patients can't show substantial compliance to strict lifestyle changes during this period. Due to progressive nature of the

diabetes, these patients need further therapy as disease progress. In generally, insulin is added to treatment as the glycemic control getting worse; Then patient begin to adhere more closely to lifestyle changes after this stage and to make more frequent measurements of glucose. Consequently, these patients were more experienced in regulating their blood sugar.

Diabetic nephropathy is currently the most common cause of end-stage renal failure, and its presence leads to substantial increases in morbidity and mortality. Detection of microalbuminuria during the early stages in patients with type 1 and type 2 DM has been regarded as the most reliable predicting factor for the development of apparent diabetic nephropathy, and the prevalence of DR increases considerably in patients who develop nephropathy [24-26]. The microalbuminuria levels in our study revealed significant differences in the diabetic patient groups ($p < 0.001$). Furthermore, the number of patients diagnosed with macroalbuminuria and the albumin excretion rate in the proliferative retinopathy group were significantly higher compared with the other groups.

A positive correlation between an increase in ESR and the incidence of atherosclerotic heart diseases has been reported. Higher leukocyte counts, fibrinogen levels, and ESR levels were detected in patients who developed type 2 DM during the follow-up period and were included in large population studies [27,28]. However, in this study, the ESR was 15.2±15.4 mm/h in patients with diabetes, whereas it was 14.9±11.48 mm/h in the control group. Furthermore, the ESR values were similar in the groups with DR, and there was no statistically significant difference between the groups.

Various studies have suggested that there is a parallel increase in disease severity and CRP levels under conditions of impaired glucose metabolism and that there is a positive correlation between poor glucose control in diabetic patients and CRP. Every 1% increase in HbA1c leads to a 20% increase in CRP [29-34]. This correlation was not only specific for diabetes but also similar for the other components of the metabolic syndrome [9-13]. Furthermore, patients with major cardiovascular events presented with significantly higher levels of hs-CRP and HbA1ccompared with patients without any cardiovascular event [35]. Differently from these studies, we could not determine any statistically significant difference between the groups with regard to hs-CRP levels, neither between the control group and diabetic patients nor between the groups with DR. It was reported that many antidiabetic agents, statins, fibric

acid derivatives, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers may impede inflammation independently from their basic effects, and inflammation parameters may alter serum levels [36-38]. From this point of view, the use of these drugs by most of our patients may have impeded subclinical inflammation.

Despite findings of high FBG and poor metabolic control, determination of a positive correlation between chronic subclinical inflammation and PPBG in all groups was a very important finding of our study. The Diabetes Epidemiology Collaborative Analysis Of Diagnostic Criteria in Europe (DECODE) study, which revealed the importance of controlling PPBG to decrease the risk of cardiovascular events, indicated that PPBG levels were an important predictor of all-cause cardiovascular deaths. In this study, it was demonstrated that patients not considered diabetic according to their FBG levels, but whose second-hour plasma glucose levels were high, had a 50% higher death rate due to cardiovascular diseases compared with those with normoglycemia [39]. Similarly, results of other large studies such as the Diabetes Intervention Study (DIS), the Stop-NIDDM, and the Kumamoto Study also emphasized the importance of postprandial hyperglycemia. In the Risk Factors in IGT and Type 2 Diabetes (RIAD) study, Hanefeld and colleagues [40,41] measured the intima-media thickness and morphologically demonstrated that FBG levels were not much of a determining factor for atherosclerotic lesions compared with PPBG levels, and there was a strong positive correlation between an increase in the second-hour PPBG levels and carotid intima-media thickness. Similarly to the results of these large population studies, it was also demonstrated in our study that considering fasting glucose levels alone, with regard to strict glycemic control, might be insufficient in preventing the development of chronic complications.

TNF-α is a pleiotropic cytokine related to both normal and many pathological metabolic responses and is a highly expressed factor in adipose tissue [21-23]. A study in which patients with type 2 DM were subjected to a continuous 2-year follow-up with 3-month intervals demonstrated that serum levels of TNF-α also increased in parallel with an HbA1c increase [42]. In another study in which 40 patients with type 2 DM were divided into two groups, mild and severe insulin resistance, a significant TNF-α increase was found in 23% of the patients with mild insulin resistance and in 51% of the patients with severe insulin resistance [43]. In 44 patients with type 1 DM whose CRP levels and leukocyte counts were normal and had no pathology that could cause inflammation, a significantly higher level of TNF-α was

positively correlated with an increase of HbA1c and fructosamine levels and negatively correlated with HDL cholesterol [44]. The biological activity of TNF-α is regulated by the serum-soluble TNF receptor types 1 and 2. Increase of these serum-soluble receptors may alter free and active plasma cytokine levels by influencing the activity of TNF-α [45-47]. Differently from these studies that revealed an increase in TNF-α and its association with metabolic control in patients with type 1 and type 2 DM, there was no significant difference in our study between diabetic patients and the control group in terms of TNF-α levels. Limb et al. [43] reported that increased cytokine production also leads to an increase in receptor levels of diabetic patients with and without retinopathy, thereby making it difficult to measure cytokine levels. In another study, it was suggested that the concentration of soluble receptors also increased in vitreous fluid and that many drugs might affect the production and activity of TNF-α levels in patients with proliferative DR [48, 49]. The absence of a significant difference between the groups can be explained by the fact that our patients received antidiabetic treatment, statins, and antihypertensive drugs.

Conclusion

In our study, disease duration and nephropathy with macroalbuminuric levels were prominent in DR, whereas the positive correlation between PPBG and hs-CRP was prominent with regard to metabolic parameters. Comparison of classic inflammatory parameters such as ESR, hs-CRP levels, and proinflammatory cytokines, specifically TNF-α, demonstrated that there was no significant difference between diabetic patients and the control group. These results, which contrast with those found in the literature, may be explained by the medications used by our patients, such as antidiabetic, antihypertensive, and lipid-lowering drugs, and also by the fact that soluble TNF-α receptor levels could not be measured.

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