BASELINE DIENE CONJUGATION IN LDL LIPIDS FROM NEWLY DIAGNOSED TYPE 2 DIABETIC PATIENTS

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SUMMARY - Background: To determine whether the susceptibility of low-density lipoproteins (LDL) to oxidation was altered in subjects with newly diagnosed type 2 diabetes mellitus and impaired glucose tolerance using the measurement of LDL baseline diene conjugation (LDL-BDC) as an indicator of circulating mildly oxidized LDL in vivo.

Methods: LDL was isolated from sera of 26 newly diagnosed patients with type 2 diabetes mellitus (type 2 DM), 43 subjects with impaired glucose tolerance (IGT), and 67 subjects with normal glucose tolerance (NGT), who were matched for age, gender, body mass index and serum lipids. Twenty-seven healthy subjects with normal body mass index and normal values of lipids served as a control group. In all groups the LDL-BDC was compared with the production of conjugated dienes after copper-induced LDL oxidation, and with total antioxidative status.

Results: The LDL-BDC levels (µmol/l; mean ± SD) were similar in the three glucose tolerance categories (NGT 66.5 ± 6.9; IGT 67.9 ± 6.5; type 2 DM, 68.1 ± 7.1 p = 0.52) and there was no difference in comparison with the control group (68.9 ± 4.2 µmol/l). Also, no significant differences between the groups were observed in the susceptibility to LDL oxidation in vitro or total antioxidative status. There was no correlation between glyceremia and mildly oxidized LDL in vivo, nor with any of the parameters of the LDL oxidation in vitro.

Conclusions: In the examined population of newly diagnosed diabetic patients with mild hyperglycemia, as evidenced by the level of glycated haemoglobin (HbA1C), a lack of changes in the susceptibility of LDL to oxidation both in vivo and in vitro was established.

Keywords: diene conjugation, in vivo, low-density lipoprotein, type 2 diabetes.

RÉSUMÉ - Conjugaison basale des diènes dans les particules LDL de diabétiques de type 2 de diagnostic récent.

Contexte : Déterminer si la susceptibilité des lipoprotéines de basse densité (LDL) à l’oxydation est altérée chez des patients avec diabète de type 2 récemment diagnostiqués et chez des patients intolérants au glucose par la mesure de la conjugaison diène basale (LDL-BDC) choisie comme indicateur de LDL modérément oxydées circulantes in vivo.

Méthodes : Les LDL ont été isolées du sérum de 26 diabétiques de type 2 (DM), 43 intolérants au glucose (IGT), et 67 sujets normotolérants au glucose (NGT), appariés pour l’âge, le genre, l’index de masse corporelle et les lipides circulants. Le groupe contrôle a comporté 27 sujets sains avec index de masse corporelle normal et valeurs de lipides normales. Dans tous les groupes, le LDL-BDC a été comparé avec la production de diènes conjugués après oxydation des LDL induite par le cuivre, et avec le status antioxydant total.

Résultats : Les niveaux de LDL-BDC (µmol/l ; moyenne ± SD) étaient similaires dans les trois catégories de tolérance au glucose (NGT 66,5 ± 6,9 ; IGT 67,9 ± 6,5 ; type 2 DM, 68,1 ± 7,1 p = 0,52) et il n’y avait pas de différence par comparaison avec le groupe contrôle (68,9 ± 4,2 µmol/l). Il n’y avait pas non plus de différences significatives entre les groupes dans la susceptibilité des LDL à l’oxydation in vitro ni dans le status antioxydant total. Il n’y avait pas de corrélation entre la glycémie et le taux de LDL modérément oxydées in vivo, ni avec les différents paramètres de l’oxydation des LDL in vitro.

Conclusions : Dans cette population de patients nouvellement découverts diabétiques avec hyperglycémie modérée, reflétée par le niveau d’hémoglobine glyquée (HbA1C), nous observons l’absence de modification de la susceptibility des LDL à l’oxydation tant in vivo que in vitro.

Mots-clés : conjugaison diène, in vivo, lipoprotéine de basse densité, diabète de type 2.
diabetes is associated with increased oxidative stress and there is plenty of evidence that oxidative damage may play a role in the development of diabetic micro and macrovascular complications [1-6]. One of the consequences of increased oxidative stress may be increased lipid peroxidation. Among the various molecular targets, probably affected by peroxidation during oxidative stress, LDL is one of the most important [7]. The response of LDL to copper-induced oxidation in vitro has been proposed as a surrogate measure of the predisposition of LDL to the subendothelial oxidative stress in vivo and is the most widely used model in clinical studies. It has been suggested that diabetes is associated with an increased in vitro susceptibility of LDL to oxidation [8-10]. This, however, remains controversial, since difference in LDL oxidizability between healthy subjects and those with diabetes has not been confirmed by some studies [11, 12]. Due to the heterogenous nature of the chemistry of LDL oxidation, an attempt was conducted to estimate in vivo LDL oxidation [13]. The determination of autoantibodies to oxidized LDL is currently considered to be the method of choice, however, it is complex and not practical for clinical purposes [9, 14]. Another clinically applicable method, which measures the amount of baseline diene conjugation (BDC) in LDL as an indicator of mildly oxidized form of circulating LDL in vivo, has been recently proposed [15]. Strong correlation between the titre of autoantibodies against oxidized LDL and results of high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) studies indicated that BDC-LDL was a specific measure of oxidized LDL in vivo [15, 16]. Although the cause and site of oxidation of the minimally oxidized fatty acids as measured in the LDL-BDC assay are not known, the authors suggested that not the origin but the amount of the “preoxidized” LDL would be crucial for determining the atherogeneity of LDL [15]. Thus, it seems of interest to evaluate the validity of BDC-LDL measurement in high-risk subjects such as diabetic patients.

In this study we examined the level of baseline diene conjugation in LDL among subjects with newly detected type 2 diabetes, impaired glucose tolerance and normal glucose tolerance. Data were compared with parameters of LDL susceptibility to copper-induced oxidation in vitro and total antioxidative status in plasma and isolated LDL.

**PATIENTS AND METHODS**

**Patients**

This study included subjects with elevated fasting glucose levels detected at routine medical examinations who had been referred by their primary care physicians to the Vuk Vrhovac Clinic for the evaluation of their metabolic status. An oral glucose tolerance test (oGTT) with 75-g glucose load was performed in all subjects. Diabetes mellitus and IGT were diagnosed according to the 1999 WHO criteria for capillary plasma [17]. Subjects with impaired glucose tolerance (IGT) were defined as having a 2-hr capillary plasma glucose between 8.9 and 12.2 mmol/l (and normal fasting capillary plasma glucose < 7 mmol/l) and subjects with type 2 diabetes mellitus as having 2-hr capillary plasma glucose ≥ 12.2 mmol/l.

Selection of patients for the study was made by the same diabetologist on the basis of their medical history, a history of hypertension and current medication, physical examination, absence of retinopathy assessed by fundoscopic evaluation by an ophthalmologist and normal albumin excretion rate. The urinary albumin concentration was determined by immunoturbidimetric assay and only subjects with the excretion rate < 30 mg/24 hr were included in the study. No subjects were taking vitamins or antioxidants and had no inflammatory or malignant diseases. Subjects with clinical or electrocardiogram evidence of macrovascular disease or previous cerebrovascular diseases were excluded from the study. Before performing the oGTT, weight (kg) and height (m) were recorded and the body mass index (BMI) calculated (kg/m²).

The study was comprised of twenty-six subjects with newly diagnosed type 2 diabetes mellitus, forty-three subjects with impaired glucose tolerance (IGT) and sixty-seven age-, gender- and BMI-matched subjects with normal pattern during oral glucose tolerance test.

Twenty-seven healthy normotensive individuals with body mass index < 26 kg/m² and lipid levels within normal range, were recruited among the laboratory staff and served as the control group.

Informed consent was obtained from all study subjects and the study was approved by the ethical committee at the Vuk Vrhovac Clinic.

**Laboratory methods**

Blood specimens were obtained after a 10-h fast and 120 min after a 75-g glucose load. Glucose was measured by glucose-oxidase method using commercially available reagents (Trace Scientific, Melbourne, Australia), and triglyceride and cholesterol levels using standard enzymatic methods (Trace Scientific, Melbourne, Australia). High-density cholesterol fractions were determined after precipitation with polyethylene glycol [18]. Low-density lipoprotein (LDL) was calculated using Friedwald’s formula [19]. Glycated haemoglobin (HbA₁c) concentration was measured by immunoturbidimetric procedure, using commercially available reagents (Bayer Diagnostica, Tarrytown, NY, USA) and the reference range was 3.5-5.7%. The within- and between-run imprecision expressed as coefficient of variation (CV) ranged between 3.1-4.6% and 3.7-4.8%, respectively [20].
Precipitation of low density lipoproteins

It was postulated that LDL-BDC values in LDL isolated by heparin precipitation did not differ from those isolated by sequential ultracentrifugation [16]. Blood samples were collected by venipuncture before the oGTT test, allowed to stand for 30 min at room temperature (protected from UV light) and centrifuged at 3,000 g for 15 min. Serum samples, to which 1 mg/ml of EDTA was added, were allowed to equilibrate to room temperature and 1 ml of the sample was added to 7 ml of the heparin-citrate buffer (50,000 IU/l heparin in 0.064 M trisodium citrate adjusted to pH 5.05 with 5 N HCl). After vortexing, the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1,000 g for 10 min. The pellet was resuspended in 1 ml of 0.1 M sodium-phosphate buffer, pH 8.0, containing 0.9% NaCl.

Analysis of baseline diene conjugation in LDL

LDL oxidation was estimated by the baseline level of diene conjugation in lipid fraction of LDL [16]. Lipids were extracted from LDL samples (100 µl) by adding chloroform-methanol (2:1), vortexed vigorously, then centrifuged for 5 min at 1,000 g and the lower organic layer was removed and dried under nitrogen. The dry residue was redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm. It was suggested that simply measuring absorbance at 234 nm could not be generally applied for absolute quantification of conjugated diene formation because of interference of other biological compounds [21]. Ahotupa et al. have found that lipophilic substances of plasma that were transported with the LDL fraction and could absorb light at 234 nm, such as intermediates of the cyclooxygenase pathway as well as vitamin E, did not interfere with the assay [16]. Thus, obtained absorbance units were converted to molar units using the molar extinction coefficient 2.95 × 10^4 M^-1 cm^-1. The results were expressed as µmol/l. The within- and between-run imprecision expressed as coefficient of variation (CV) was 4.9% and 5.1%, respectively.

In vitro oxidizability of LDL

Prior to experiments, LDL samples were dialyzed overnight against EDTA-free phosphate buffer saline (0.01 M; pH 7.4). LDL (100 µg LDL protein) was incubated in PBS (0.01 M; pH 7.4) with CuSO₄ (final concentration 10 µmol/l) for 3 h at 30 °C [22]. The kinetics for the formation of conjugated dienes was monitored by the increase in absorbance at 234 nm at 30 min-intervals in a UV visible spectrophotometer (model SP-8, Pye Unicam). The following parameters were evaluated: the lag phase, which represents the resistance to oxidation and defined as the intercept of the linear least square slope of oxidation curve with the initial-absorbance axis (min) and the rate of conjugated diene formation (V) expressed in µmoles of dienes formed per minute (µmol/min). The coefficients of variation were 1.8% for lag phase determination and 7.2% for conjugated diene formation rate. In addition, with a molar absorbance for conjugated dienes of 2.95 × 10^4 M^-1 cm^-1, the maximum amount of dienes (µM) before the onset of decomposition were calculated by the maximum increase of the absorbance multiplied by 33.8.

Total antioxidant status

A method for assessing the antioxidants available in a biological fluid was performed using an adaptation of Miller’s [23] method [24]. The principle of the method is based on the incubation of ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) with a peroxidase (metmyoglobin) and H₂O₂ to produce the radical cation ABTS⁺, which is a blue-green chromogen with absorbance at 660 nm. Antioxidants in the added sample prevent the radical formation, and this antioxidant capacity can be compared to a known concentration of Trolox, a synthetic tocopherol analogue. A standard curve of Trolox was constructed for each assay and the results are expressed as mmol/l of Trolox units. The coefficient of variation was 4.9%.

Statistical analysis

Statistical analysis was performed by using the Complete StatSoft CSS (Tulsa, USA). Descriptive data are expressed as means ± standard deviation. Between-group differences were assessed by using Kruskal-Wallis test for skewed data. Mann-Whitney U-test was used to assess differences between normolipidemic control group and the glucose tolerance category groups. Spearman’s correlation coefficients were calculated to determine relation between LDL-BDC and lipids.

RESULTS

Table I shows the clinical characteristics of the studied groups. Age, gender distribution and body mass index did not differ significantly among the glucose tolerance categories. Body mass index was significantly higher in glucose tolerance groups than in control group. A higher prevalence of hypertensive subjects with IGT and type 2 DM can be seen. Table II summarizes the biochemical characteristics of the control and three glucose tolerance groups. The glucose data are shown and significant differences are predicted by classification of the groups. The values of HbA₁c in type 2 diabetic patients amounted to 6.15 ± 0.6%, which is the near upper limit of normal HbA₁c for control population (5.7%). Measurement of the lipid patterns revealed that LDL cholesterol was increased in type 2 diabetes (3.81 ± 0.94 mmol/l) and...
impaired glucose tolerance (3.91 ± 0.82 mmol/l) in comparison to reference value (<3.37 mmol/l), but did not differ from normal glucose tolerance group (3.83 ± 1.05 mmol/l) (Table II). The same table shows slightly elevated triglycerides as compared to the control group and no difference in their levels in the glucose tolerance groups.

Oxidizability markers in isolated LDL are presented in Table III. The LDL-BDC level (µmol/l; mean ± SD) in NGT, IGT and type 2 diabetes group was not significantly different from that of healthy control subjects. To ensure that similar results of baseline levels of oxidation in all groups were not due to ineffective protection against ex vivo oxidation, we measured LDL-BDC in ten samples with and without the addition of a lipid-soluble antioxidant butylated hydroxytoluene (BHT) in addition to water soluble antioxidant EDTA, as proposed in the original procedure. The results showed that there was no significant difference between EDTA and EDTA + BHT samples (67.6 ± 3.7 vs 65.9 ± 4.3 µmol/l, respectively: p = 0.2, n = 10).

Parameters of the copper-induced in vitro oxidation of LDL (lag time, maximal rate and maximal diene concentration) from both type 2 and impaired glucose tolerance subjects was not significantly different from subjects with normal glucose tolerance and control subjects (Table III). Regarding the potential link between the LDL-susceptibility to in vitro oxidation (lag phase) and LDL-BDC, there was a correlation only in the NGT group (r = 0.30; p < 0.01). Total antioxidative potential in plasma and isolated LDL failed to differ between the groups (Table III).

<table>
<thead>
<tr>
<th>Table I. Clinical characteristics of the studied subjects.</th>
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<tbody>
<tr>
<td><strong>Variables</strong></td>
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<td>-----------------------------------------------------------</td>
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<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Body mass index (kg/m²)*</td>
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<td>Normo: hypertensive</td>
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<td>Data are means ± SD. * Body mass index was significantly higher in glucose tolerance categories (GT) in comparison with the control group (p &lt; 0.02).</td>
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<th>Table II. Biochemical measurements in the studied subjects.</th>
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<td><strong>Parameter</strong></td>
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<td>-----------------------------------------------------------</td>
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<tr>
<td>HbA₁c</td>
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<tr>
<td>Fasting glucose [mmol/l]</td>
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<tr>
<td>Glucose 120 min [mmol/l]</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
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<tr>
<td>HDL cholesterol (mmol/l)</td>
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<td>HDL₂ cholesterol (mmol/l)</td>
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<td>HDL₃ cholesterol (mmol/l)</td>
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<td>LDL cholesterol (mmol/l)</td>
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<tr>
<td>VLDL cholesterol (mmol/l)</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
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<tr>
<td>Data are means ± SD; p-values were calculated by Kruskal-Wallis ANOVA. Between group differences to HDL₂ cholesterol and triglycerides was calculated by Mann-Whitney U-test. * p &lt; 0.01 vs control group.</td>
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</table>
Since there was no difference in LDL-BDC level in the three glucose category groups, we have examined the association of LDL-BDC with lipids in a combined group consisting of 136 subjects. A positive correlation between LDL-BDC and total cholesterol ($r = 0.17$, $p < 0.04$), LDL ($r = 0.21$, $p < 0.01$) and a negative correlation with HDL and HDL$_2$ cholesterol ($r = 0.11$, $p < 0.001$, for HDL and HDL$_2$, respectively) were detected.

### DISCUSSION

Given the oxidative theory of atherosclerosis, the amount of “preoxidized” LDL would be crucial for determining atherogenicity of LDL, especially in high-risk subjects such as diabetic patients. A number of recent reviews have examined the influence of hyperglycemia and/or vascular disease on increased LDL susceptibility to oxidation in vitro and in vivo [25]. Our study was designed to determine the LDL susceptibility to oxidation in vivo and in vitro at the time of the detection of diabetes and impaired glucose tolerance in comparison with the control healthy group. We have found no significant differences in the LDL-BDC when the groups were compared with each other or with the control group with normal BMI and triglyceride levels (Table III). As opposed to this, a group of researchers using similar technique for measuring LDL-BDC has demonstrated connections between LDL-BDC and hypertriglyceridemia as well as obesity in type 2 diabetic patients [26]. This discrepancy could probably be explained by the fact that secondary hypertriglyceridemia occurs in poorly controlled diabetes mellitus, while our examined population of newly detected type 2 diabetic subjects exhibits mild hyperglycemia and borderline triglyceride levels [27]. Thus, the duration of diabetes mellitus and/or glycemic regulation in the above mentioned study probably accounted to changes in LDL-BDC level. The same explanation could also be applied to the finding that LDL from newly detected type 2 diabetes does not differ in susceptibility to in vitro oxidizability compared with LDL from subjects with normal glucose tolerance. It is known that hyperglycemia itself can stimulate free-radical production. In newly onset type 2 patients without treatment examined in this study HbA$_1c$ levels averaged 6.15 ± 0.7%, which corresponds to good glycemic control in diabetic patients [27]. Thus, our results are consistent with other studies on susceptibility of LDL to oxidation in vivo, in which diabetic groups were matched for glycemic control [11, 12]. No significant difference in LDL lag time and conjugated diene production in vivo between well controlled type 2 diabetic patients (HbA$_1c$ = 6.2%) and subjects with normal glucose tolerance has recently been reported [28]. Examination of the lipid patterns revealed that, according to the American Diabetes Association categories of coronary heart disease (CHD) risk based on lipoprotein levels in adults with diabetes [29], our subjects belonged to the category of low CHD risk regarding the levels of triglycerides and HDL (<2.3 mmol/l and >1.15 mmol/l, respectively). Positive correlation between LDL-BDC and total cholesterol and LDL cholesterol and negative with HDL and HDL$_2$ cholesterol was detected in the group comprised of all three glucose tolerance category groups, which is in accordance with data obtained from the healthy subjects [30, 31]. As in our type 2 diabetic patients, LDL levels were slightly elevated, it could be speculated that their composition was changed [32]. Recently, it has been found that fatty acid composition of the core of the LDL particle is the main determinant of its susceptibility to in vitro oxidation in well controlled and normolipemic type 2 diabetic patients [28]. It has been postulated that diabetic patients may present a decrease in antioxidant defences [33], and reduced antioxidative potential in the plasma along with increased susceptibility of LDL to oxidation in vivo was dem-

### TABLE III. Baseline diene conjugation in LDL, parameters of the copper-induced in vitro oxidation of LDL and total antioxidative status of serum (TAS) and isolated LDL (TAS-LDL).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n = 27)</th>
<th>Normal-GT (n = 67)</th>
<th>Impaired-GT (n = 43)</th>
<th>Type 2 diabetes (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-BDC (µmol/l)</td>
<td>68.9 ± 4.2</td>
<td>66.5 ± 6.9</td>
<td>67.9 ± 6.5</td>
<td>68.1 ± 7.1</td>
</tr>
<tr>
<td>Lag time [min]</td>
<td>121.1 ± 19.8</td>
<td>120.7 ± 31.2</td>
<td>120.4 ± 25.2</td>
<td>121.0 ± 15.9</td>
</tr>
<tr>
<td>Max. rate (µmol/min)</td>
<td>0.37 ± 0.09</td>
<td>0.34 ± 0.1</td>
<td>0.36 ± 0.1</td>
<td>0.34 ± 0.009</td>
</tr>
<tr>
<td>Max. diene conc. (µmol)</td>
<td>33.9 ± 5.0</td>
<td>38.7 ± 9.7</td>
<td>36.7 ± 8.5</td>
<td>39.0 ± 7.9</td>
</tr>
<tr>
<td>TAS (mmol/l)</td>
<td>1.61 ± 0.13</td>
<td>1.55 ± 0.13</td>
<td>1.56 ± 0.11</td>
<td>1.55 ± 0.14</td>
</tr>
<tr>
<td>TAS LDL (mmol/l)</td>
<td>nm</td>
<td>0.23 ± 0.05</td>
<td>0.25 ± 0.008</td>
<td>0.26 ± 0.09</td>
</tr>
</tbody>
</table>

Data are means ± SD. nm: not measured. There were no significant differences between groups.
onstrated in poorly controlled diabetic patients [34]. We were unable to detect changes in total antioxidative status (Table III) which may possibly be related to good glycaemic control of our diabetic patients and/or to newly diagnosed diabetes.

In conclusion, the level of BDC-LDL, as an indicator of mildly oxidized LDL in the circulation in vivo does not change in newly diagnosed diabetic patients who have HbA1c level near the upper limit of its normal value. Further follow-up study of the same patients at different time-points, including measurement of the baseline levels of conjugated dienes extracted from LDL, would be of value in our understanding of the relationship between diabetes and markers of oxidative stress.

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REFERENCES


6 Haffner SM. Clinical relevance of the oxidative stress concept. Metabolism, 2000, 49 (suppl 1), 30-34.


35 Tsai EC, Hirsh IB, Brunzell JD, Chait A. Reduced plasma peroxyl radical trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM. Diabetes, 1994, 43, 1010-1014.