SERUM PARAOXONASE ACTIVITY AND PARAOXONASE GENE POLYMORPHISM IN TYPE 2 DIABETIC PATIENTS WITH OR WITHOUT VASCULAR COMPLICATIONS

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SUMMARY - Background: Serum paraoxonase (PON) activity and the relevance of PON gene polymorphism in vascular complications of type 2 diabetic patients were investigated in a case-control study.

Methods: The population included 105 control subjects, 96 diabetic patients without vascular complications and 71 diabetics with vascular complications.

Results: Serum PON activity was significantly decreased (p < 0.001) in diabetic patients without vascular complications: 207 IU (25-817) compared with the controls: 259 IU (24-950). Although serum PON activity was also decreased: 232 IU (34-797) in the population with vascular complications, the difference was not statistically significant (p = 0.11). The Q192 allele frequency is significantly higher (p < 0.005) in diabetics without vascular complications (77%), and with vascular complications (73%) than in the controls (63%). No significant association was found between either PON1L/M and PON2C/S gene polymorphisms and vascular complications.

Conclusions: The difference in allele frequency for the PON1Q/R 192 gene polymorphism may be the cause of the low paraoxonase activity observed in type 2 diabetes mellitus. Further studies need to be conducted to elucidate the role of the enzyme in the development of vascular complications in diabetes.

Key-words: paraoxonase, polymorphism, high density lipoprotein, type 2 diabetes mellitus, vascular complications.

RÉSUMÉ - Activité paraoxonase sérique et polymorphisme du gène de la paraoxonase chez des diabétiques de type 2 avec ou sans complications vasculaires.

Contexte : L’activité de la paraoxonase et la relation de son polymorphisme avec les complications vasculaires observées chez des patients diabétiques de type 2 ont été étudiées dans le cadre d’une étude cas-contrôle.

Méthodes : La population était constituée de 105 sujets témoins, 71 patients diabétiques avec des complications vasculaires à type de micro et macroangiopathie et 96 patients indemnes de complication.

Résultats : L’activité sérique de la paraoxonase était significativement (p < 0,001) plus faible chez les patients diabétiques sans complications : 207 UI (25-817) comparativement aux témoins : 259 UI (24-950). Dans la population diabétique avec complications l’activité de l’enzyme est également diminuée : 232 UI (34-797) sans que toutefois la significativité statistique soit atteinte (p = 0,11). La fréquence de l’allèle Q est significativement plus élevée (p < 0,005) chez les patients diabétiques sans complications (77 %), chez les patients avec complications vasculaires (73 %), en comparaison à celle obtenue chez les témoins (63 %). Aucune relation n’est retrouvée entre les autres polymorphismes de l’enzyme (PON1 L/M 55 et PON2 C/S 311) et les complications vasculaires du diabète.

Conclusion : La différence de fréquence des allèles retrouvée au niveau du polymorphisme (PON1 Q/R 192) de l’enzyme peut expliquer l’activité plus faible de la paraoxonase observée dans le diabète de type 2. D’autres études sont toutefois nécessaires pour démontrer le rôle joué par cette enzyme dans l’apparition des complications vasculaires du diabète.

Mots-clés : paraoxonase, polymorphisme, lipoprotein de haute densité, diabète de type2, complications vasculaires.
It is thought that oxidized low-density lipoproteins (LDL) play an important role in the initiation of atherosclerosis [1]. Numerous studies [2] suggest that fatty acid streaks develop in response to a series of events leading to the migration of oxidised-loaded monocytes into the sub-endothelial space of the arteries. High-density lipoproteins (HDL) have been shown to prevent oxidative modification of LDL both in vivo [3], and in vitro [4]. Human serum paraoxonase (PON) is a polymorphic enzyme that can catalyse the hydrolysis of organo-phosphate pesticides and nerve gases [5]. Recently, it has been shown that this enzyme plays a very important physiological role in addition to the detoxification of these compounds. Biochemical studies indicate that PON is located on HDL [5, 6] and it seems to protect LDL from oxidative stress by hydrolysing lipid peroxides, and these may provide HDL-associated protection against atherosclerosis [7].

Furthermore, serum PON activity has been found to be lower in diseases where the HDL concentration is reduced such as fish-eye disease [8] and Tangier disease [9]. The PON-like genes designated PON1, PON2, and PON3 have been identified and mapped to chromosome 7q21-q22 [10, 11]. Two polymorphic sites are known to exist at the PON1 locus in humans: one at position 192 (glycine (Q) to arginine (R) substitution and a second at position 55 (leucine (L) to methionine (M) substitution). Several case-control studies have shown that the PON1 192 Q/R gene variation is associated with an increased risk for coronary artery disease (CAD) [12, 13].

Recently, Sanghera et al. [14] reported that a common polymorphism at codon 311 (cysteine (C) to serine (S) substitution) in the PON2 gene was significantly associated with CAD in an Asian-Indian population and that it apparently increases the risk of CAD both alone, and in combination with the PON1 codon 192 polymorphism. The main causes of mortality and morbidity in type 2 diabetic patients are coronary heart disease and other vascular diseases. Low serum PON activity has been found in diabetic patients [15, 16] and an association between the paraoxonase 192 Arg allele and CAD has been described in French [17, 18] and Japanese patients [19] with type 2 diabetes. An increase in the frequency of the PON1_55 L allele has been reported in type 1 diabetic subjects with retinopathy compared with those without this complication [20]. Conflicting results have been reported [16, 21, 22], however. In order to investigate the relationship between PON gene polymorphism and the susceptibility to vascular complications in diabetes mellitus, we determined in this study paraoxonase activity and genotype distribution in controls, and in type 2 diabetic patients with or without vascular complications.

### MATERIALS AND METHODS

The subjects in this study were Caucasian diabetic patients diagnosed in accordance with National Data Group criteria [23]. Sera were collected from 175 type 2 diabetic subjects (59 women, 116 men) aged (27) to (80) years. All were on a diet and taking oral hypoglycaemic drugs supplemented in 2 cases with insulin. They were distributed as follows: 96 had no vascular complications and 71 had vascular disease (macroangiopathy and/or microangiopathy). Macroangiopathy (n = 36) was defined by clinical symptoms and/or ultrasonographic data. Coronary disease was diagnosed in the cardiology department and defined during the study by an history of myocardial infarction or angor. Microangiopathy (n = 35) was defined by diabetic retinopathy detected by fundus eyes examination and/or diabetic nephropathy characterised by microalbuminuria (30 - 300 mg/24h). The test characterizing a diabetic neuropathy was the loss of ankle jerk reflexes.

The main characteristics of the patients are summarised in Table I. None of the patients presented renal or hepatic insufficiency, thyroid disease, acute or chronic inflammation, or infection. 49 patients were undergoing lipid-lowering drug treatment. No information was available concerning oestrogen therapy.

A group of healthy subjects recruited from a Health Centre (53 women and 52 men), mean age 46.72 ± 10.9 years, served as the control group.

### METHODS

#### Sample collection

Blood was collected in Vacutainer tubes with or without anticoagulant (EDTA or heparin). After centrifugation (1500 g, 15 min) the sera and plasma were separated and stored at −20°C before further analysis. Whole blood was stored at −20°C for not more than two weeks before DNA extraction. All icteric or haemolytic blood samples were discarded.

#### Laboratory measurements

Fasting blood glucose was determined in heparinized plasma using a glucose-oxidase method (Glucose PAP®, Biomerieux, Marcy-l’Etoile, France) adapted for the Hitachi 717® analyzer (Roche Diagnostics, Meylan — France).

Total cholesterol (TC), HDL-Cholesterol (HDL-C) and triglycerides (TG) were measured in plasma with the CHOD-PAP®, HDL-C PLUS and GPO-PAP® Kits (Roche Diagnostics) on a Hitachi 717 analyser.

LDL-cholesterol (LDL-C) was calculated using Friedewald’s formula if the triglycerides were less than 4.5 mmole/l [24].

Glycated-haemoglobin (HbA1c) was determined in EDTA whole blood by high performance liquid phase
chromatography (HPLC) on a Variant analyser (Bio-rad, Ivry sur Seine, France).

PON enzyme-activity

Enzyme-activity was measured using paraoxon (Sigma) as the substrate after addition of serum (10 µl) in Tris-HCl buffer (990 µl, 20 mM, pH 7.8 containing CaCl₂ 2 mmol/l). The reaction was continuously monitored at 405 nm at 37°C (Kontron Uvikon 810 spectrophotometer). Results were expressed as IU. One unit of international enzyme activity is equal to 1 nmol of paraoxon hydrolysed per minute and per ml of serum.

DNA analysis

Genomic DNA was extracted from human blood cells using the Nucleon bac CC³ Kit (Amersham). The genotyping of paraoxonase was carried out with the restriction enzyme digestion pattern after PCR amplification.

The target region of the two genes were amplified by PCR using a mixture of 500 ng of genomic DNA, 0.3 µM of each primer, 5 µl of Mullis 30 buffer and 1.25 U of Taq DNA polymerase. After an initial incubation, the reaction mixture were subject to 35 cycles, each cycle comprising denaturation, annealing and extension. The PCR amplified product was digested with specific restriction enzymes and the fragments visualised with ethidium bromide after separation by electrophoresis on 4% agarose gel.

The technical protocol of paraoxonase genotype determination is summarized in Table II.

STATISTICAL ANALYSIS

Statistically significant differences between parameters having a Gaussian distribution were investigated by Student’s unpaired t test. Variables with a non-gaussian distribution were compared using the Mann-Whitney U-test. The χ² test was used to determine the significance of the difference in allele frequency. P < 0.05 was considered to be significant. The correlation between the variables was studied by the linear regression.

RESULTS

Age, BMI and the mean value (± SD) for each parameter in the various study groups are summarized in Table I. The type 2 diabetic patients had significantly elevated plasma triglycerides and low HDL-cholesterol compared with the control subjects.
**TABLE II.** Technical protocol of paraoxonase genotype determination.

<table>
<thead>
<tr>
<th>DNA analysis</th>
<th>CYCLES</th>
<th>RESTRICTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1, Gln 192 Arg (99bp)</td>
<td>94°C: 8°; 94°C: 1°, 58°C: 1°, 30°C: 2°</td>
<td>Alw1 3.2U</td>
</tr>
<tr>
<td></td>
<td>Allele Q: 99bp</td>
<td>Allele R: 65 and 44bp</td>
</tr>
<tr>
<td>PON1, Leu 55 Met (170bp)</td>
<td>94°C: 5°; 94°C: 30°, 62°C: 30°, 72°C: 30°, 72°C: 6°</td>
<td>NlaIII 5.5U</td>
</tr>
<tr>
<td></td>
<td>Allele L: 170bp</td>
<td>Allele M: 126 and 44bp</td>
</tr>
<tr>
<td>PON2, Cyst 311 Ser (262bp)</td>
<td>94°C: 4°; 94°C: 1°, 51°C: 1°, 30°C: 2°</td>
<td>dD1 7U</td>
</tr>
<tr>
<td></td>
<td>Allele C: 142 and 120bp</td>
<td>Allele S 120, 75 and 67bp</td>
</tr>
</tbody>
</table>

**TABLE III.** $PON_1$ activity, genotype distribution and allele frequencies in type 2 diabetic patients with or without vascular complications and in controls.

<table>
<thead>
<tr>
<th></th>
<th>Healthy donors</th>
<th>Diabetic patients without vascular complications</th>
<th>Diabetic patients with vascular complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON activity (IU)</td>
<td>259 (24-950)</td>
<td>207 (25-817)*</td>
<td>232 (34-797)</td>
</tr>
<tr>
<td>$R/Q192$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q/Q n (%)</td>
<td>40 (39)</td>
<td>p = 0.0045</td>
<td>55 (57)</td>
</tr>
<tr>
<td>Q/R n (%)</td>
<td>50 (48)</td>
<td>p = 0.467</td>
<td>29 (41)</td>
</tr>
<tr>
<td>R/R n (%)</td>
<td>14 (13)</td>
<td>p = 0.620</td>
<td>7 (7)</td>
</tr>
<tr>
<td>Q allele</td>
<td>0.63</td>
<td>p = 0.0016</td>
<td>0.77</td>
</tr>
<tr>
<td>R allele</td>
<td>0.37</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>$M/L 55$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/L n (%)</td>
<td>40 (38)</td>
<td>p = 0.620</td>
<td>31 (32)</td>
</tr>
<tr>
<td>L/M n (%)</td>
<td>52 (50)</td>
<td>p = 0.055</td>
<td>27 (38)</td>
</tr>
<tr>
<td>M/M n (%)</td>
<td>12 (12)</td>
<td>p = 0.23</td>
<td>10 (14)</td>
</tr>
<tr>
<td>L allele</td>
<td>0.63</td>
<td>p = 0.620</td>
<td>0.63</td>
</tr>
<tr>
<td>M allele</td>
<td>0.37</td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>$C/S 310$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/S n (%)</td>
<td>52 (49)</td>
<td>p = 0.097</td>
<td>62 (65)</td>
</tr>
<tr>
<td>S/C n (%)</td>
<td>46 (44)</td>
<td>p = 0.341</td>
<td>43 (60)</td>
</tr>
<tr>
<td>C/C n (%)</td>
<td>7 (7)</td>
<td></td>
<td>7 (10)</td>
</tr>
<tr>
<td>S allele</td>
<td>0.71</td>
<td>p = 0.041</td>
<td>0.8</td>
</tr>
<tr>
<td>C allele</td>
<td>0.29</td>
<td>p = 0.288</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* significant difference with healthy population (Mann-Whitney test)
(a) difference with healthy population; (b) difference with population without vascular complications.
In the group of diabetic patients with vascular complications, plasma creatinin was significantly higher than the group with no complications. Plasma PON activity was significantly decreased ($p < 0.001$) in the diabetic patients without vascular complications (207 IU (25-817)) compared with the controls (259 IU (24-950)). Although plasma PON activity was also decreased (232 IU (34-797)) in the population with vascular complications, the difference did not reach statistical significance ($p = 0.11$) (Table III).

No significant correlation was found between serum HDL-cholesterol concentration and PON activity ($r = 0.235$ in the control group and $r = 0.028$ in the diabetic group).

The genotype and allele distributions of the three polymorphisms are shown in Table II. There was a significant difference in the PON 1 192 genotype distribution and allele frequencies in the controls and in the diabetics without vascular complications; the Q192 allele frequency was higher ($p = 0.0016$) in patients (77%) than in controls (63%). In contrast, the genotype distribution was similar in the controls and the diabetic group with complications, whereas the Q192 allele frequency (63% vs 73%) was just significant ($p = 0.051$). There was no significant difference in the
distribution of PON1 genotype and allele frequencies between the two subgroups of diabetic patients with or without vascular complications. The PON1 polymorphism did have a major effect on serum PON1 activity (Fig. 1). In all of the groups studied PON1 activity was significantly higher in the RR genotype and lower in the QQ whereas the QR genotype had an intermediate activity.

No significant association was found between either PON1 55 L/M or PON1 311 C/S gene polymorphism and vascular complications. MM homozygotes had significantly lower PON1 activity than LM heterozygotes who in turn had lower activity than LL homozygotes. There was no difference in the extent of the 192 Q/R polymorphism in any of the groups studied (Fig. 1).

There were no significant differences between the population with microangiopathy or macroangiopathy and that without complications for any of the polymorphisms studied (Table IV).

**DISCUSSION**

These data confirm the previous observations [15, 16] of low PON activity in type 2 diabetes. The low PON activity could have a significant effect on the ability to metabolise lipid-peroxides in type 2 diabetic patients, and could therefore be one of the reasons for the increased lipid peroxidation often reported in this disease and the increased mortality due to coronary artery disease.

In our study, the only difference in the genotype distribution was between the controls and diabetic patients without vascular complications. The R192 allele frequency was significantly lower, however, in the two groups of patients studied (with or without vascular complications) than in the controls. The Q to R substitution at position 192 is determinant for activity since the R allele codes for a protein having a paraoxon hydrolysis activity that is several times greater than the Q allele. The protection by paraoxonase of LDL against peroxidation may be dependent on its esterase activity towards paraoxon and may be affected by the PON1 192 Q/R gene variation. These results contradict those of Mackness et al. [25] who presented evidence that HDL from PON Q allele carriers afforded greater protection against LDL lipid peroxidation than that from R allele carriers. Recently, Cao et al. [26] showed that the paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and unaffected by the PON1 192 Q/R gene variation. Thus, extensive research remains to be undertaken to elucidate how this polymorphism is linked to the disease susceptibility. There was no significant difference in allele frequency for the PON1 192 Q/R polymorphism between the two groups of diabetic patients studied. An association of the R allele with coronary heart disease was found in both patients with type 2 diabetes [27, 28] and non diabetic patients [13, 14]. In the largest case-control studies [29], however, such an association was not found.

In addition to the polymorphism in PON1 at amino acid position 192, a polymorphism of PON1 affects position 55. We concluded from our results that the PON1 55 gene variation may determine serum paraoxonase activity, with LL genotypes having the highest levels. These results are in agreement with those of Garin et al. [30] who investigated this polymorphism in 408 diabetic patients with or without vascular disease and concluded that the PON1 55 L/M polymorphism appeared to be of central importance to paraoxonase function in accordance with its associa-
tion with modulated concentrations. Mackness et al. [15] were unable to find, however, a significant difference between the PON,55 genotype with regard to PON activity in type 2 diabetic patients. In our study, there was no significant difference in genotype distribution and allele frequencies for the PON,55 polymorphism between the groups of diabetic patients with or without vascular complications compared with controls and between the two groups of patients studied. With the exception of Garin’s study [30] in which the PON,55 LL genotype was found to be an independent risk factor for coronary heart disease in diabetic French patients, other studies [15, 29] did not detect a link between the PON,55 L/M gene variant and the cardiovascular risk. Recently some authors reported [31] that lipid peroxidation is increased in paraoxonase L55 homozygote compared with M allele carriers, but the disease relevance of the 55 L variant has yet to be elucidated.

Two homologues of PON, PON, and PON, and the existence of a common polymorphism (A/G 148 and C/S 311) at the PON, locus has been described [32]. Few results have been reported concerning the association between the PON, C/S311 polymorphism and vascular complications. Recently, Sanghera et al. [14] reported that C/S polymorphism at PON 2 was associated with coronary artery disease in an Asian-Indian non diabetic population. In our study, we were unable to confirm association of this polymorphism with vascular complications, but inconsistent association may be attributed to difference in ethnic factors. All of the patients included in our study were Caucasians.

When we divided the group of diabetic patients with vascular complications into two sub-groups according to the nature of the complication, there were no significant differences in PON serum activity or PON genetic polymorphism between the diabetic population with microangiopathy, with macroangiopathy and that with no complications. These results are in agreement with those of Mackness [16] who studied PON192, PON55 and PON311 genotype distribution and allele frequencies in type 2 diabetic patients and did not find any significant differences between the population with retinopathy and that with no complications for any of the polymorphisms studied.

In conclusion, our data support the hypothesis that the Q/R192 gene polymorphism, rather than the two other polymorphisms (L/M 55 and C/S 311), is associated with diabetic status. The difference in allele frequency for this polymorphism between the diabetic patients and the controls may be the cause of the low paraoxonase activity found in type 2 diabetes mellitus, but further studies need to be conducted to elucidate the role of the enzyme in the development of vascular complications in diabetes.

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15. Mackness MI, Mackness B, Arrol S, et al. Paraoxonase 192 Gln/Arg gene polymorphism and paraoxonase activity and concentration with modulated concentrations. Mackness et al. [15] were unable to find, however, a significant difference between the PON,55 genotype with regard to PON activity in type 2 diabetic patients. In our study, there was no significant difference in genotype distribution and allele frequencies for the PON,55 polymorphism between the groups of diabetic patients with or without vascular complications compared with controls and between the two groups of patients studied. With the exception of Garin’s study [30] in which the PON,55 LL genotype was found to be an independent risk factor for coronary heart disease in diabetic French patients, other studies [15, 29] did not detect a link between the PON,55 L/M gene variation and the cardiovascular risk. Recently some authors reported [31] that lipid peroxidation is increased in paraoxonase L55 homozygote compared with M allele carriers, but the disease relevance of the 55 L variant has yet to be elucidated.

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