Paraoxonase-1 Is Associated With Corneal Endothelial Cell Alterations in Patients With Chronic Obstructive Pulmonary Disease

Núria Soler,1 Anabel García-Heredia,2 Judit Marsillach,3 Bharti Mackness,2 Michael Mackness,2 Jorge Joven,1 Pere Romero,1 and Jordi Camps2

1Ophthalmology Service, Hospital Universitari Sant Joan de Reus, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Catalonia, Spain
2Unitat de Recerca Biomèdica, Hospital Universitari Sant Joan de Reus, Institut d’Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Catalonia, Spain
3Departments of Medicine (Division of Medical Genetics) and Genome Sciences, University of Washington, Seattle, Washington

Correspondence: Jordi Camps, Unitat de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, 45201 Reus, Catalonia, Spain; jcamps@grupsagessa.com.
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PURPOSE. To investigate the relationships between the levels of the antioxidant enzyme paraoxonase-1 (PON1) and corneal endothelial alterations in patients with chronic obstructive pulmonary disease (COPD) undergoing cataract surgery.

METHODS. We studied 172 patients with cataract attending our ophthalmology clinic. Based on spirometric analysis, they were segregated into two groups, 110 (64%) with COPD and 62 (36%) without COPD. Corneal endothelial cell morphology was examined by widefield noncontact specular microscopy, which allows measurements of endothelial cell density (ECD), hexagonality, and endothelial cell size coefficient of variation (ECCV). Corneal thickness was measured by noncontact pachimetry. PON1 and plasma TNFα concentrations were analyzed by ELISA. Serum PON1 activity was analyzed by spectrophotometry.

RESULTS. Patients with COPD had significant decreases in ECD, hexagonality, and corneal thickness, and a significant increase in ECCV. They also had significant decreases in serum PON1 activity but not in PON1 concentration. Serum PON1 activity showed a significant direct association with ECD, and an inverse association with corneal thickness.

CONCLUSIONS. Results of the present study suggest that PON1 may be involved in the pathophysiology of corneal endothelial alterations in patients with COPD.

Keywords: specular microscopy, cataracts, corneal endothelial cells

Chronic obstructive pulmonary disease (COPD) is a major health disorder. It is estimated to be the fourth highest cause of morbidity and mortality, worldwide.1 The derangement is defined as a nonreversible limitation of airway cell efflux, and has a multifactorial etiology involving lifestyle habits and genetic predisposition.2,3 In COPD, neutrophils, macrophages, eosinophils, and CD8+ lymphocytes infiltrate into lung tissues; stimulating free radical production and activating pro-inflammatory cytokines.4 These changes contribute to irreversible damage of parenchymal and airway cells, and to the development of a fibrogenetic reaction.5,6 Patients with COPD have a significant risk of developing other diseases including cardiovascular disease, osteoporosis, and diabetes mellitus; a risk that seems to be related to oxidative stress.7,8 Hypoxemia is frequently observed in patients with advanced COPD, and produces a chronic cellular metabolic lesion by activation of the anaerobic pathways.9 Treatment of COPD may also be related to the development of systemic complications, since corticosteroid inhalation can exacerbate metabolic alterations via several mechanisms.10 Some population-based studies have reported a dose-related increased risk of cataracts associated with the use of corticosteroid inhalers by patients with COPD.11 Other studies have suggested that the changes within the lens associated with cataracts may be caused by an excessive free radical production that exceeds the detoxifying capacity of the antioxidant defense systems.12

Paraoxonase-1 (PON1), an enzyme with lactonase and esterase activities, degrades lipid peroxides and plays an antioxidant role.13 In mammals, PON1 gene and protein expressions are observed in many cell types14,15 and the enzyme is found in the circulation bound to high-density lipoproteins (HDL).16 Previous studies from our group, as well as others, observed a strong PON1 expression in the lung bronchiolar epithelium of mice and humans,15 while serum PON1 activity has been shown to be decreased in COPD patients.17,18 To the best of our knowledge of the literature, the fact that PON1 alterations may be associated with the presence of cataracts in patients with COPD has not been investigated to date. Hence, the aim of the present study was to investigate PON1, hypoxemia and inflammation in relation to corneal endothelial alterations in patients with COPD undergoing cataract surgery.

METHODS

Experimental Design and Clinical Analyses
We undertook a cross-sectional study of 172 patients attending the Ophthalmology Clinic of Hospital Universitari de Sant Joan
de Reus (Catalonia, Spain) in preparation for cataract surgery. All patients underwent a detailed anamnesis and a slitlamp analysis (Diatom I-2000 slit lamp; Sibemed, Barcelona, Spain) to evaluate and to characterize the degree of COPD. After broncodilatation, and based on the 1 second forced expiratory volume (FEV1), they were segregated into two groups: 110 (64%) with COPD and 62 (36%) without COPD. Further, the patients with COPD were segregated into mild (FEV1 > 50%; n = 68; 62% of the total with COPD) and severe disease (FEV1 ≤ 50%; n = 42; 38% of the total number with COPD). Of the 110 patients with COPD, 95 (86%) were being treated with inhaled corticosteroids. Cataracts are a common adverse effect of corticosteroids. Of the 110 patients with COPD, 95 (86%) were being treated with inhaled corticosteroids. Corneal endothelial cell density (ECD) to be measured, as well as hexagonality and cell size coefficient of variation (ECCV). Corneal thickness was measured by noncontact pachimetry with an Orbscan II corneal topographer (Orbtek; Bausch & Lomb, Houston, TX). The degree of hypoxemia was estimated by measuring the partial pressure of oxygen (pO2) using a pulse oximeter connected to a Marquette DASH 2000 Multi Monitor (General Electric, Fairfield, CT). The only inclusion criterion was to be in need of cataract surgery and to be 60 years of age or older. All the procedures were in compliance with the Declaration of Helsinki.

### Table 1. Corneal Characteristics and Biochemical Parameters in Patients With Cataracts

<table>
<thead>
<tr>
<th></th>
<th>No COPD Median (95% CI)</th>
<th>Mild COPD Median (95% CI)</th>
<th>Severe COPD Median (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cell density, cells/mm²</td>
<td>2.45 (2.05–3.08)</td>
<td>2.11 (1.56–2.67)§</td>
<td>2.25 (1.03–2.91)‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hexagonality, %</td>
<td>60.0 (40.0–76.3)</td>
<td>53.0 (30.8–70.0)‡</td>
<td>47.0 (31.0–77.50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endothelial cell coefficient of variation, %</td>
<td>30.0 (21.0–41.1)</td>
<td>33.0 (25.4–63.2)§</td>
<td>37.0 (27.0–100.9)§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Corneal thickness, µm</td>
<td>556.0 (481.6–600.8)</td>
<td>550.0 (490.2–648.5)</td>
<td>540.0 (433.8–607.0)</td>
<td>0.045</td>
</tr>
<tr>
<td>pO2, mm Hg</td>
<td>99.0 (95.2–99.0)</td>
<td>99.0 (93.0–99.0)</td>
<td>98.0 (90.2–99.0)</td>
<td>0.286</td>
</tr>
<tr>
<td>Cataract PON1, mg/L</td>
<td>45.9 (13.8–77.7)</td>
<td>43.1 (18.8–55.3)</td>
<td>48.6 (28.2–59.0)</td>
<td>0.484</td>
</tr>
<tr>
<td>Serum PON1, mg/L</td>
<td>30.6 (0.14–105.0)</td>
<td>37.6 (7.6–66.6)</td>
<td>46.5 (9.6–88.5)</td>
<td>0.230</td>
</tr>
<tr>
<td>TBBase, U/L</td>
<td>7.0 (3.2–11.0)</td>
<td>5.4 (1.7–9.6)†</td>
<td>5.1 (0.3–9.2)‡</td>
<td>0.005</td>
</tr>
<tr>
<td>Paraoxonase, U/L</td>
<td>271 (140–768)</td>
<td>221.7 (107.7–429.7)‡</td>
<td>201.0 (91.0–429.2)†</td>
<td>0.042</td>
</tr>
<tr>
<td>TNF-α, ng/L</td>
<td>55.9 (2.7–507.9)</td>
<td>48.7 (10.0–447.5)</td>
<td>133.5 (9.99–295.2)</td>
<td>0.884</td>
</tr>
</tbody>
</table>

* ANOVA or Kruskal-Wallis test.  † P < 0.05.  ‡ P < 0.01.  § P < 0.001, with respect to patients without COPD by Student’s t-test or Mann-Whitney’s U test.  TBBL, 5-thiobutyl butyro lactone.

### Cataract Homogenization

To investigate the possible association between serum PON1 concentration and PON1 in the lens of the eye, 18 cataract fragments surgically aspirated and collected into the cartridge of the phacoemulsifier were centrifuged at 30,000g for 10 minutes. The pellets were resuspended in 400 µL of Tris-HCl buffer, pH of 7.0, homogenized in a Precellys24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), and recentrifuged. Supernatants were decanted for PON1 concentration measurement.

### Biochemical Analyses

Serum and aqueous humor PON1 concentrations were determined by in house ELISA with rabbit polyclonal antibodies generated against a synthetic peptide with the sequence CRNHQSSYQTRNLREVQ, which is specific for mature PON1. Details of this method have been previously reported.21 Serum and aqueous humor PON1 activities were analyzed by two different spectrophotometric methods that measured the lactonase and the esterase (paraoxonase) activities of this enzyme. Lactonase activity was measured as the hydrolysis of 5-thiobutyl butyro lactone (TBBL), as previously described.22,23 The activity was measured in an assay reagent containing 1 Mm CaCl₂, 0.25 Mm TBBL, and 0.5 Mm 5-thio-bis-2-nitrobenzoic acid (DTNB) in 0.05 Mm Tris-HCl buffer, pH of 8.0. The change in absorbance was monitored at 412 nm. Activity was expressed as units per liter (1 U = 1 µmol of TBBL hydrolyzed per minute). Paraoxonase activity was measured as the rate of hydrolysis of paraoxon at 410 nm and 37°C in a 0.05 Mm glycine buffer, pH of 10.5 with 1 mM CaCl₂.24 Activities were expressed as units per liter (1 U = 1 µmol of paraoxon hydrolyzed per minute). Plasma TNF-α was measured by ELISA (Peprotech, Inc., Rocky Hill, NJ) as an index of inflammation.

### Statistical Analyses

All statistical evaluations were performed with the SPSS 18.0 statistical package (SPSS, Inc., Chicago, IL). Normality of distributions was determined with the Kolmogorov-Smirnov test. Differences between two groups were assessed with the Student’s t-test (parametric) or the Mann-Whitney U test (nonparametric). Differences between several groups were assessed with ANOVA (parametric) or the Kruskal-Wallis test (nonparametric). Spearman correlations were used to evaluate the degree of association between variables. Multiple regres-
sion models were fitted to evaluate the associations between several variables. Results are shown as medians and 95% confidence interval (CI). A value of $P$ less than or equal to 0.05 was considered statistically significant.

**RESULTS**

Patients with COPD had significant decreases in ECD, hexagonality, and corneal thickness, and a significant increase in ECCV. These changes were more evident in the individuals...
with a severe form of the disease (Table 1). A representative example of the changes in corneal endothelium morphology in patients with COPD is shown in Figure 1. The serum, or cataract PON1 activities (TBBLase and paraoxonase), were significantly decreased, but not the serum or cataract PON1 concentrations (Table 1).

Serum TBBLase and paraoxonase activities showed direct significant associations with ECD, and inverse associations with the corneal thickness (Fig. 2). There was a significant direct association between \( pO_2 \) and endothelial cell hexagonality, and an inverse association with ECCV (Fig. 3, top and middle). Plasma TNF\( \alpha \) concentrations were inversely related to corneal thickness (Fig. 3, bottom). Of note was that there was a significant direct association between serum and cataract PON1 concentration (\( r = 0.46; P = 0.05 \)).

Linear regression analyses showed significant, and independent, associations between serum TBBLase and paraoxonase activities and ECD when adjusted for age, sex, presence of COPD, hypoxemia, corticosteroid treatment, and TNF\( \alpha \) concentrations (Table 2). Similarly, plasma TNF\( \alpha \) concentrations and paraoxonase activities were significantly, and independently, associated with corneal thickness (Table 3). Aqueous humor PON1 activities and concentrations were below the limit of the respective assays in all patients analyzed.

### Table 2. Multiple Regression Analyses of the Influence of Biochemical Variables on ECD Alterations

<table>
<thead>
<tr>
<th>Dependent Variable: ECD</th>
<th>B</th>
<th>P</th>
<th>B 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1: TBBLase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBBLase</td>
<td>0.055</td>
<td>0.007</td>
<td>0.016–0.094</td>
</tr>
<tr>
<td>( pO_2 )</td>
<td>0.000</td>
<td>0.997</td>
<td>0.000–0.000</td>
</tr>
<tr>
<td>TNF( \alpha )</td>
<td>0.000</td>
<td>0.365</td>
<td>0.000–0.000</td>
</tr>
<tr>
<td>Age</td>
<td>0.003</td>
<td>0.709</td>
<td>0.014–0.020</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.222</td>
<td>0.057</td>
<td>-0.451 to 0.007</td>
</tr>
<tr>
<td>COPD severity*</td>
<td>0.117</td>
<td>0.299</td>
<td>-0.107 to 0.341</td>
</tr>
<tr>
<td>Corticoid treatment†</td>
<td>-0.416</td>
<td>0.016</td>
<td>-0.751 to -0.081</td>
</tr>
<tr>
<td>Model 2: Paraoxonase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraoxonase</td>
<td>0.001</td>
<td>0.011</td>
<td>0.000–0.001</td>
</tr>
<tr>
<td>( pO_2 )</td>
<td>0.009</td>
<td>0.793</td>
<td>-0.058 to 0.075</td>
</tr>
<tr>
<td>TNF( \alpha )</td>
<td>0.000</td>
<td>0.791</td>
<td>-0.001 to 0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.006</td>
<td>0.424</td>
<td>-0.011 to 0.027</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.253</td>
<td>0.099</td>
<td>-0.492 to 0.013</td>
</tr>
<tr>
<td>COPD severity*</td>
<td>0.085</td>
<td>0.458</td>
<td>-0.142 to -0.308</td>
</tr>
<tr>
<td>Corticoid treatment†</td>
<td>-0.431</td>
<td>0.017</td>
<td>-0.779 to -0.083</td>
</tr>
</tbody>
</table>

Model 1: PON1 activity measured by the TBBLase assay; Model 2: PON1 activity measured by the paraoxonase assay

* Coded as 0: No COPD; 1: Mild; 2: Severe.
† Coded as 0: No; 1: Yes.

**Figure 2.** Relationships between PON1 TBBLase and paraoxonase activities with ECD and corneal thickness in patients with cataracts.
DISCUSSION

COPD is considered a systemic multi-organ disease with deleterious effects in peripheral tissues that are related to inflammation, oxidative stress, and hypoxemia. Several lines of evidence suggest that PON1 alterations are involved in the pathophysiology of COPD. The exact biological role of PON1 is not, as yet, completely elucidated since its physiologic substrate(s) is(are) not completely characterized. Indeed, PON1 was discovered by its ability to hydrolyze organophosphate xenobiotics such as paraoxon, but more recent evidence showed that PON1 is a lipolactonase that hydrolyzes lipid peroxides, and plays a role in the antioxidant protection system of lipoproteins and cells.

Data on PON1 alterations in COPD are scarce, but preliminary studies have shown decreased PON1 paraoxonase activities related to increased oxidative stress in patients with this disease. The present study confirms these results in a wider series of patients, and extends them by ascertaining that a decrease in PON1 activity is observed when both lactonase and paraoxonase activities are measured and, thus, it is not a substrate-dependent effect. This is of considerable importance since PON1 paraoxonase activity is strongly dependent on genotype. Several polymorphisms in the promoter and the coding regions of the PON1 gene have been described, of which PON1_192 is the most significantly associated with changes in the enzyme’s activity. Hence, in case-control studies to evaluate enzyme activity, it is imperative that cases and controls are matched for genotype, or that a genotype-neutral substrate is employed. If not, it would not be possible to ascertain whether the observed changes were due to the disease per se or whether the findings were coincidental to differences in allelic frequencies between cases and controls. Previous studies from our group have shown that TBBLase activity is considerably less influenced by PON1 polymorphisms than the polymorphism’s influence on the enzyme’s paraoxonase activity (i.e., is a more genotype-neutral substrate). As such, our present results confirm and validate those earlier preliminary reports.

The decrease in serum PON1 activity in COPD was related to an inactivation of the enzyme, and not to a decrease in enzyme synthesis; serum PON1 concentrations remaining unaffected by the presence, or the severity, of COPD. An explanation for this observation is that when PON1 hydrolyzes oxidized lipids, the enzyme becomes irreversibly inactivated. This was shown by Aviram et al. who demonstrated that, when incubated in vitro with oxidized palmitoyl arachidonoyl phosphatidylcholine,
lyso phosphatidylcholine, oxidized cholesteryl arachidonate, and oxidized low-density lipoproteins (LDL), the concentration of PON1 remained unaltered while the enzyme’s activity became inactivated. Indeed, previous studies observed decreased serum PON1 activities with normal or elevated PON1 concentrations in several diseases involving increased oxidative stress.13

In the current study, we also showed, to the best of our knowledge for the first time in the literature, an association between PON1 activity and the degree of corneal endothelium alterations in COPD patients. A decrease in serum PON1 activity has previously been reported in patients with diabetic retinopathy,42–44 and in patients with macular degeneration.35,36 Hashim et al.37,38 observed a decreased paroxonase activity in the serum of diabetic patients and senile individuals suffering from cataracts, and protein and mRNA PON1 expression in human cataractous lens tissue. Liton et al.39 reported that the gene expression of PON3 (another antioxidant enzyme, genetically related to PON1) was down-regulated in POMG trabecular meshwork cells. The present study reports direct significant associations between serum PON1 activities and ECD, as well as inverse associations with corneal thickness in patients with cataracts, with or without COPD. Cataracts are a multifactorial disorder in which normal vision is impaired owing to the loss of lens transparency. One of the mechanisms that leads to the development of lens opacity is oxidative insult generated as a result of an imbalance between antioxidants and reactive oxygen species.37 Earlier studies observed associations between antioxidant enzymes such as catalase, superoxide dismutase, and glutathione in cataracts.40,41 Our study suggests that PON1 also plays a role in the protection of corneal endothelial cells from free radical-induced oxidative damage. In the present study, we were unable to determine PON1 (activity or concentration) in aqueous humor. This is not surprising since PON1 is carried in the circulation bound to HDL particles, and this lipoprotein is present in this fluid at very low concentrations.42 The question arising is how peripheral PON1 can be associated with corneal endothelium alterations. The most likely explanation is that low serum PON1 activities do not efficiently protect from oxidative stress, and free radical species cross the blood–cornea barrier and, thus, affect endothelial cells.43 Free radicals can cause alterations in corneal endothelial cells via oxidation of cytoskeleton proteins, mainly vimentin and F-actin.44,45 Along with oxidative stress, inflammation is one of the main pathophysiologic findings in COPD.46,47 Indeed, oxidative stress and inflammation are interdependent phenomena, and exacerbate each other’s effect.25,48 In our study, we analyzed plasma TNFα concentrations as a marker of inflammation. This cytokine has been associated, in earlier studies, with other eye diseases such as glaucoma and cornea transplant rejection.49,50 Evidence suggests that, similarly to oxidative stress, TNFα alters the actin filaments of the endothelial cell cytoskeleton.51 Taken together, these changes in the cytoskeleton structure and function may cause morphologic and functional changes in the endothelial cells, and would ultimately produce cell death by apoptosis and a decrease in ECD.52

The finding of the present study, that corneal pachymetry decreased with increasing severity of COPD compared with patients without COPD, appears contra-intuitive considering the decrease in hexagonality and the increase in ECCV. Hexagonality and ECCV reflect the functional state of the corneal endothelium, and are indicators of its functional reserve. Normally, alterations in the endothelium structure and function would be associated with a diminished dehydration of the stroma and an increase (not a decrease) in pachymetry. We are unable to provide a mechanistic explanation for our findings, but we can speculate that the observed decrease in pachymetry is associated with the characteristics of COPD as a chronic disease. The time course of corneal alterations in our patients is not known and, perhaps at some point, the long term sustained ischemia associated with the COPD,53 caused the edema that was reabsorbed later. This would result in an unstructured stromal architecture, loss of collagen and, subsequently, stromal thinning.

In summary, the present study shows strong associations between serum PON1 activity and corneal endothelial cell alterations in patients with COPD. This suggests that this enzyme may be involved in the defense mechanisms against oxidative stress in the cornea and, as such, may participate in the metabolic alterations leading to the development of cataracts. Identification of such alterations could be valuable for early diagnosis and/or indication of the individual’s predisposition to cataract formation.

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