Main Determinants of PON1 Activity in Hemodialysis Patients

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Abstract

Background/Aims: Cardiovascular diseases are the major cause of morbidity and mortality in hemodialysis (HD) patients. These patients present reduced paraoxonase 1 (PON1) activity that depends on genetic and non-genetic factors; however, how these factors influence PON1 activity in HD patients is poorly clarified. Our aim was to evaluate the influence of two polymorphisms and non-genetic factors on PON1 activity in HD patients. Methods: We evaluated 183 HD patients under recombinant human erythropoietin (rhEPO) treatment and 22 healthy individuals. The lipid profile [total cholesterol, triglycerides, HDL-c, LDL-c, apolipoprotein (Apo) A-I, Apo B, lipoprotein(a) and oxidized low-density lipoprotein (Ox-LDL)], inflammatory markers [adiponectin, interleukin-6 (IL-6) and C-reactive protein (CRP)], PON1 activity and PON1 gene polymorphisms (L55M and Q192R) were evaluated. Results: HD patients presented higher levels of IL-6, CRP and Ox-LDL/LDL-c, and lower PON1 activity, total cholesterol, HDL-c, LDL-c, Apo A and Apo B; the most frequent genotype was heterozygosity for L55M polymorphism and homozygosity for the Q allele, the more frequent genotype of Q192R polymorphism. Multiple regression analysis identified heterozygosity and homozygosity for L55M and Q192R polymorphisms, very low-density lipoproteins, LDL-c, Apo A and CRP levels, time on dialysis and rhEPO dose, as the independent variables significantly associated with PON1 activity. The associations with CRP, rhEPO and time on dialysis were negative. Conclusion: Our results show that the reduced PON1 activity in HD patients who are not under statin therapy is strongly associated with inflammation, longer time on dialysis and high rhEPO doses, suggesting that the reduction in PON1 activity may worsen the prognosis of these patients.
Introduction

Human serum paraoxonase 1 (PON1) is an enzyme primarily synthesized by the liver that has been associated with lipoproteins, mainly with high-density lipoproteins (HDL). PON1 seems to have antiatherogenic, anti-oxidative and anti-inflammatory properties as it protects HDL and low-density lipoproteins (LDL) from oxidation, and hydrolyzes oxidized lipids [1]. In vitro studies showed that PON1 is also associated with very low-density lipoproteins (VLDL) and still presents antiatherogenic properties; however, its activity is lower than that presented when associated with HDL [2].

Cardiovascular diseases (CVD) are the leading cause for the high morbidity and mortality observed in hemodialysis (HD) patients [3]. Usually, these patients present with high levels of inflammation and oxidative stress that are known to favor the atherosclerotic processes and, therefore, may contribute to the increased cardiovascular risk in HD patients [3]. Recently, it has been reported that patients undergoing HD present a reduced PON1 activity, which could contribute to the high rate of CVD in these patients [4, 5].

The activity of this enzyme is influenced by genetic and non-genetic factors [5–7]. Two polymorphisms in the coding region of PON1 gene, namely L55M (leucine to methionine substitution at position 55) and Q192R (glutamine to arginine substitution at position 192), reduce the activity of this enzyme [6, 7]. The L55M polymorphism is associated with a decrease in mRNA expression and, therefore, with decreased PON1 activity [8]. Moreover, Q192R polymorphism is also associated with a decrease in PON1 activity, as this polymorphism affects the kinetics of hydrolysis of PON1 substrates [8].

How genetic and non-genetic factors influence PON1 activity in HD patients remains to be elucidated. The aim of our study was to evaluate the influence of genetic (L55M and Q192R polymorphisms) and non-genetic factors on PON1 activity in a group of HD patients.

Subjects and Methods

Patients

We studied a group of 183 HD patients (102 males, 81 females; mean age 66.23 ± 13.96 years old) under recombinant human erythropoietin (rhEPO) treatment [0.43 (0.20 – 0.75) µg/kg/week of darbepoetin-α]. The patients were also under intravenous iron supplementation according to the European Best Practice Guidelines for the management of anemia in patients under HD and rhEPO therapies [9]. All participants gave their informed consent to participate in this study that was previously approved by the Ethics Committee of the Clinics of Hemodialysis. Patients with autoimmune disease, malignancy, and acute or chronic infection were excluded. Some patients (40.44%) were under statins therapy.

HD patients were under therapeutic HD three times per week for 3–5 h for a median of 2.13 (0.81–5.24) years. All patients used the high-flux polysulfone FX-class dialyzers of Fresenius. The main causes of renal failure in this population were as follows: diabetic nephropathy (n = 66), hypertensive nephrosclerosis (n = 21), nephritic syndrome (n = 9), polycystic kidney disease (n = 8), obstructive diseases (n = 7), hereditary nephropathy (n = 3), chronic interstitial nephritis (n = 2), benign prostate hypertrophy (n = 1), systemic lupus erythematosus (n = 1), other diseases (n = 4) and of uncertain etiology (n = 61). A group of 22 apparently healthy individuals, matched as far as possible for age and gender (10 males, 14 females; mean age 36.03 ± 10.34 years old), were selected as controls, based on no history of kidney, inflammatory or infectious diseases, no history of CVD and under no regular medications that could interfere with our results.

Blood Samples

Blood samples were obtained immediately before HD procedure and processed within 2 h of collection. Blood was collected to EDTA-containing tubes and to tubes without anticoagulant in order to obtain plasma, buffy coat and serum. Aliquots were immediately stored at –80°C until assayed.

Lipid Profile

Serum lipids, lipoproteins and apolipoprotein (Apo) analysis were performed in an auto-analyzer (Cobas Mira S, Roche) using commercially available kits. Serum total cholesterol (TC) and triglycerides (TG) concentrations were determined by enzymatic colorimetric tests (CHOD-PAP and GPO-PAP methods, Roche, respectively). HDL cholesterol (HDL-c) and LDL cholesterol (LDL-c) levels were measured by using enzymatic colorimetric tests after selective separation of HDL and LDL fractions (Direct HDL-Cholesterol and Direct LDL-Cholesterol, Roche). Serum Apo A-I and Apo B levels were evaluated by immunoturbidimetric assays (uni-kit apolipoprotein A-I- and B-specific antiseraums, Roche). Serum lipoprotein(a), Lp(a), was quantified by using an immunoturbidimetric method (Lp(a), Roche Diagnostics). Plasma concentration of oxidized low-density lipoprotein (Ox-LDL) was evaluated by using a standard commercial enzyme-linked immunoassay (Oxidized LDL ELISA, Mercodia, eBioscience).

Inflammatory Markers

Plasma concentrations of adiponectin and interleukin (IL)-6 were evaluated by using standard commercial enzyme-linked immunoassays (adiponectin, IL-6 ELISA High-Sensitivity, Merco-dia, eBioscience). C-reactive protein (CRP) was evaluated by immunoturbidimetry, using commercially available kits [CRP (latex) High-Sensitivity, Roche Diagnostics].

Activity and Screening for L55M and Q192R Polymorphisms on PON1 Gene

The activity of PON1 was assessed spectrophotometrically and expressed in nmol of p-nitrophenol/ml/min. Briefly, PON1 activity was measured by adding serum to 1 ml Tris/HCl buffer (100...
**Table 1. Biochemical data of HD patients and controls**

| Parameters                        | Controls (n = 22) | HD patients (n = 183) | p    | HD patients with statins (n = 74) | HD patients without statins (n = 109) | p    |
|-----------------------------------|-------------------|-----------------------|------|-----------------------------------|---------------------------------------|------|
| Creatinine, mg/dl                 | –                 | 8.50 (6.25–11.83)     |      | 8.54 (6.10–12.06)                 | 8.47 (6.30–11.80)                      | 0.716|
| Kt/V                              | –                 | 1.48 (1.30–1.60)      |      | 1.40 (1.20–1.60)                  | 1.50 (1.38–1.60)                       | 0.138|
| Urea, mg/dl                       | –                 | 140.20 ± 41.01        |      | 137.41 ± 41.05                    | 142.11 ± 41.06                        | 0.480|
| Albumin, g/dl                     | –                 | 3.90 (3.53–4.16)      |      | 3.99 (3.50–4.19)                  | 3.89 (3.54–4.11)                       | 0.266|
| BMI                               | 21.85 ± 2.05      | 25.87 ± 4.64          | <0.001| 27.66 ± 4.97                      | 24.63 ± 3.96                          | <0.001|
| IL-6, pg/ml                       | 0.39 (0.27–0.58)  | 2.24 (1.35–4.27)      | <0.001| 2.24 (1.36–3.93)                  | 2.28 (1.33–4.30)                       | 0.768|
| CRP, mg/dl                        | 0.06 (0.04–0.14)  | 0.49 (0.22–1.33)      | <0.001| 0.61 (0.23–1.27)                  | 0.46 (0.22–1.40)                       | 0.796|
| HDL-c, mg/dl                      | –                 | 46.00 (40.00–60.25)   |      | 37.34 (32.51–46.43)               | 35.88 (32.56–46.05)                    | 0.901|
| TC, mg/dl                         | 202.27 ± 27.77    | 154.10 ± 43.72        | <0.001| 150.79 ± 36.00                    | 156.40 ± 48.39                        | 0.448|
| TG, mg/dl                         | 98.50 (84.25–135.75) | 119.00 (90.00–177.00) | 0.043| 120.00 (97.00–183.00)             | 116.50 (86.25–173.75)                  | 0.286|
| HDL-c, mg/dl                      | 46.00 (40.00–60.25) | 42.60 ± 13.70         | 0.002| 42.08 ± 13.34                     | 42.95 ± 14.00                         | 0.664|
| LDL-c, mg/dl                      | 104.36 ± 25.82    | 73.23 ± 29.33         | <0.001| 71.16 ± 28.41                     | 74.67 ± 30.00                         | 0.369|
| HDL/LDL ratio                     | 0.52 ± 0.20       | 0.68 ± 0.39           | 0.033| 0.69 ± 0.36                       | 0.68 ± 0.43                           | 0.680|
| VLDL, mg/dl                       | 19.70 (16.85–27.15) | 23.80 (18.00–35.40)   | 0.048| 24.00 (19.20–36.60)               | 23.30 (17.25–35.15)                    | 0.378|
| Lp(a), mg/dl                      | 25.10 (14.10–62.03) | 45.40 (25.85–89.05)   | 0.021| 58.40 (33.25–107.10)              | 39.85 (24.05–77.33)                    | 0.008|
| Apo A, mg/dl                      | 150.73 ± 23.57    | 121.95 ± 30.41        | <0.001| 125.19 ± 30.60                    | 119.19 ± 30.10                        | 0.105|
| Apo B, mg/dl                      | 89.09 ± 13.48     | 72.33 ± 21.81         | <0.001| 71.59 ± 20.26                     | 72.85 ± 22.91                         | 0.821|
| PON1/HDL-c ratio                  | 10.48 (7.42–12.33) | 9.38 (7.70–12.03)     | 0.501| 9.12 (6.78–12.88)                 | 9.19 (7.72–11.26)                      | 0.904|
| PON1/Apo A ratio                  | 3.02 (2.56–3.67)  | 3.22 (2.64–3.99)      | 0.729| 2.92 (2.33–3.84)                  | 3.25 (2.73–4.05)                      | 0.055|
| OxLDL, UI                         | 39.66 ± 11.10     | 35.95 ± 15.63         | 0.058| 35.04 ± 10.55                     | 36.59 ± 18.37                        | 0.829|
| OxLDL/HDLC ratio, U/mg             | 0.040 ± 0.013     | 0.053 ± 0.018         | <0.001| 0.054 ± 0.017                     | 0.052 ± 0.019                        | 0.405|
| Adiponectin, mg/l                 | 5.24 ± 2.29       | 9.25 ± 4.73           | <0.001| 7.69 ± 3.97                       | 10.33 ± 4.92                          | <0.001|

Results are presented as mean ± SD or median (interquartile range) unless otherwise indicated. p values were obtained using Mann-Whitney U test or Pearson χ² test. BMI = Body mass index.

Statistical Analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences for Windows, version 19.0. Statistical significance was accepted at p < 0.05. The distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test to assess significant deviations from normality. Comparisons between groups were performed using the Mann-Whitney U test or the Kruskal-Wallis test. The association between categorical variables was analyzed using the χ² test or Fisher’s exact test. To evaluate the contribution of the different studied variables on PON1 activity, multiple regression analysis was performed using stepwise selection with an entry criterion of p < 0.05.

Results

Compared to controls (table 1), our HD patients presented significantly higher levels of the inflammatory markers IL-6 and CRP. The PON1 activity was significantly lower in HD patients compared to control subjects. Some differences were found in the lipid profile of HD patients, namely significantly lower levels of TC, HDL-c, LDL-c, Apo A and Apo B; Ox-LDL was not different from controls; however, the Ox-LDL/LDL-c ratio was significantly higher; no significant differences were found for PON1/HDL-c and PON1/Apo A ratios. Significantly higher levels of adiponectin were also found in HD patients.

The most frequent genotype found in HD patients (table 2) was LM for L55M polymorphism, which was associated to a PON1 activity higher than MM and lower than LL polymorphisms. QQ was the more frequent genotype for Q192R polymorphism, presenting the lowest PON1 activity, as compared to QR and RR genotypes. Moreover, the most frequent association of these two polymorphisms is LM/LL (27.9%), followed by LM/LR (21.3%) and LL/QR genotypes (15.9%). Significantly higher PON1

mmol/l, pH 8.0) containing 2 mmol/l CaCl₂ and 5.5 mmol/l paraoxon (O,O-diethyl-O-p-nitrophenylphosphate, Sigma Chemical, Co.). The rate of generation of p-nitrophenol was determined by reading the absorbance at 412 nm, 37°C, with the use of a continuously recording spectrophotometer (Beckman DU-68).

To screen for presence of PON1 gene polymorphisms, genomic DNA was extracted from peripheral blood leukocytes using the standard salting-out extraction method, as previously described [10, 11]. The genotype screening was performed with allele-specific fluorogenic oligonucleotide probes in an assay combining the polymerase chain reaction and the 5′ nuclease reaction.

Statistical Analysis

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PON1 Activity in Hemodialysis Patients
activity was associated with homozygosity for L and R alleles, and lower PON1 activity was associated with homozygosity for the M and Q alleles (table 2). Moreover, HD patients with the LL/RR genotype presented the highest PON1 activity, and patients with the MM/QQ genotype presented the lowest PON1 activity.

Statins are known for their capacity to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, and for their anti-inflammatory properties. Considering that 40.44% of the patients were under statins therapy, we studied a possible interference in our analytical studies, and no statistically significant differences were found (table 1).

Multiple regression analysis identified for all HD patients the heterozygosity and homozygosity for the L55M and Q192R polymorphisms, and VLDL, LDL-c, Apo A and CRP serum levels, time on dialysis and rhEPO dose as the independent variables significantly associated with PON1 activity (table 3). These associations were negative for CRP, rhEPO dose and time on dialysis. Considering the patients according to statin therapy, those who were not under this therapy presented the heterozygosity and homozygosity for the L55M and Q192R polymorphisms, CRP, TG and sTfR serum levels, time on dialysis and rhEPO dose as the independent variables significantly associated with PON1 levels, while for patients under statin therapy, only the heterozygosity and homozygosity for the Q192R polymorphism, CRP and TG serum levels were identified as the independent variables significantly associated with PON1 levels.

**Table 2.** Genotype frequencies of PON1 polymorphisms and activity in hemodialysis patients

| Genotype   | HD patients (n = 183) | PON1 activity nmol p-nitrofenol/ml/min |
|------------|----------------------|--------------------------------------|
|            | n       | %      |                                     |
| PON1 L55M  |         |        |                                     |
| LL         | 57      | 31.1   | 464.8 (386.6–539.6)                 |
| LM         | 89      | 48.6   | 359.7 (333.1–428.6)                 |
| MM         | 37      | 20.2   | 315.0 (302.5–326.6)                 |
| p          |         | <0.001 |                                     |
| PON1 Q192R |         |        |                                     |
| QQ         | 103     | 56.3   | 326.9 (307.9–353.5)                 |
| QR         | 68      | 37.2   | 463.3 (418.6–514.6)                 |
| RR         | 12      | 6.6    | 576.9 (479.6–670.7)                 |
| p          |         | <0.001 |                                     |
| PON1 L55M/Q192R |     |        |                                     |
| LL/QQ      | 15      | 8.2    | 357.4 (325.8–390.2)                 |
| LL/QR      | 29      | 15.9   | 483.6 (444.4–548.1)                 |
| LL/RR      | 11      | 6.0    | 554.2 (473.8–674.1)                 |
| LM/QQ      | 51      | 27.9   | 335.8 (315.3–356.8)                 |
| LM/QR      | 39      | 21.3   | 438.9 (402.0–489.7)                 |
| LM/RR      | 1       | 0.5    | 576.9                               |
| MM/QQ      | 37      | 20.2   | 315.0 (302.5–326.6)                 |
| MM/QR      | 0       | 0      | –                                   |
| MM/RR      | 0       | 0      | –                                   |
| p          |         | <0.001 |                                     |

Results are presented as absolute number and percentage, and median (interquartile range). p values were obtained using Kruskal-Wallis test.

**Discussion**

PON1 presents antioxidant properties contributing to control the development of oxidative stress at blood level. It is known that the oxidation of LDL is a crucial starting step for the atherogenic process; therefore, by preventing oxidative stress, PON1 contributes to protect LDL from oxidative modifications and to reduce foam cell formation, inhibiting atherosclerosis [12].

The lipid profile of our HD patients showed some CVD risk changes, as compared to control, namely significantly lower HDL-c and Apo A levels and significantly higher Lp(a) concentration. Despite the lower values of LDL-c and the similar values of Ox-LDL in HD patients, the Ox-LDL/LDL-c ratio was significantly higher, suggesting a higher proportion of oxidized LDL particles and, therefore, a lower antioxidant protection within LDL that might be due to the significantly lower activity of PON1. The trend towards lower levels of oxLDL observed in HD patients might result from the formation of immune complexes formed by oxidized LDL and Ox-LDL antibodies, as suggested by some authors [13, 14]; proatherogenic and proinflammatory properties have been attributed to these immune complexes and their formation seems to be more prevalent in diabetic nephropathy [13]; considering the high percentage of diabetic patients (36.1%) in our studied patients, this could be a reasonable explanation for the values of Ox-LDL that we observed.

The mortality and morbidity of HD patients is 10–20 times higher than that found in the general population, and remains high in spite of the technological development of HD procedures and of the medical support in the last years. Cardiovascular events are considered as the major cause of death (more than 50%). Advanced age, a lipid risk profile and diabetes mellitus common in these
patients per se do not explain the high number and severity of the observed cardiovascular events. The association and the value of oxidative stress, inflammation and dyslipidemia in the evaluation of risk for cardiovascular events are well known. More recently, it was proposed that PON1 could play a role in the cardiovascular risk of these patients, considering its antioxidant and antiatherogenic properties. Indeed, some studies reported that the activity of PON1 was reduced in HD patients [4, 5]. In accordance with those studies, we found in our group of HD patients a significantly lower PON1 activity compared to controls (table 1).

The activity and concentration of this enzyme is affected by several factors, but more than 60% of its activity seems to be explained by genetic factors, including some polymorphisms in the coding region of the PON1 gene [12], such as the Q192R and L55M polymorphisms. The Q192R polymorphism modulates PON1 activity through the R allele that has been associated with increasing activity. The L55M polymorphism seems to modulate enzyme’s concentration. Thus, subjects with RR and LL genotypes present the highest PON1 activity [15]. In accordance, we found that the HD patients who were homozygous for L and R alleles presented higher PON1 activities compared to the other genotypes. Moreover, HD patients with LL/QR and LL/RR genotypes showed median values of PON1 activity that were similar to those found in the control group. The relative prevalence of the different studied polymorphisms observed in our HD patients is similar to the prevalence reported by others in HD patient populations [5]. The most frequent genotypes in HD patients were LM and QQ, for L55M and Q192R polymorphisms, respectively. As we were not able to study a larger control group, it is not possible to know if there are differences in the genotype frequencies between HD patients and controls. Indeed, it was reported that the genotype frequency of the LM genotype is higher in HD patients than in controls [5], and this would support a reduction in PON1 activity.

Considering that PON1 is strongly linked to HDL-c, Apo A and LDL-c, and that their values are significantly lower in HD patients compared to controls, the decreased activity of PON1 seems to be related to Apo A, VLDL and LDL levels. The result observed for Apo A is of particular relevance, as this Apo seems to be important to stabilize the enzyme [16]; Apo A is directly related to the number of HDL particles, as it is the major protein component of HDL [17]; therefore, the lower levels of Apo A found in HD patients and, therefore, of HDL particles could explain the lower activity of PON1. Indeed, when calculating the ratios PON1/Apo A and PON1/HDL-c, we did not find significant differences for HD patients and controls, suggesting that the reduction in PON1 may be explained by lower HDL particles [7]. To further understand the importance of the genetic and non-genetic factors under study in PON1 activity, we performed a multiple regression analysis (table 3). We found that in HD patients, especially those that are not under statin

| Table 3. Main determinants of PON1 activity by multiple linear regression analysis |
|---------------------------------|--------------|--------------|--------------|
|                                | All HD patients | Patients without statins | Patients with statins |
|                                | R²  | β   | P   | R²  | β   | P   | R²  | β   | P   |
| Ln PON1 activity               | 0.893  | <0.001 | 0.910  | <0.001 | 0.877  | <0.001 |
| (Constant)                      |      |      |      |      |      |      |      |      |      |
| Q192R polymorphism (QR)         | 0.666  | <0.001 | 0.667  | <0.001 | 0.657  | <0.001 |
| Q192R polymorphism (RR)         | 0.558  | <0.001 | 0.332  | <0.001 | 0.841  | <0.001 |
| Ln CRP                          | -0.146 | <0.001 | -0.237 | <0.001 | -0.191 | 0.029 |
| L55M polymorphism (LL)          | 0.323  | <0.001 | 0.427  | <0.001 |      |      |
| Ln VLDL                         | 0.127  | <0.001 | -0.131 | 0.006 |      |      |
| RhEPO dose                      | -0.108 | 0.003 |      |      |      |      |
| Apo A                           | 0.109  | 0.002 |      |      |      |      |
| LDL-c                           | 0.096  | 0.007 |      |      |      |      |
| L55M polymorphism (LM)          | 0.139  | 0.006 |      |      |      |      |
| Ln time on dialysis             | -0.181 | 0.026 | -0.106 | 0.013 |      |      |
| Ln TG                           | 0.142  | 0.001 |      |      |      |      |
| Ln sTfR                         | 0.142  | 0.001 |      |      |      |      |

PON1 Activity in Hemodialysis Patients  
Am J Nephrol 2012;36:317–323  
321
therapy, a low activity of PON1 is significantly associated with increasing inflammation, rhEPO dose and time on HD. As statins improve the lipid profile and exert anti-inflammatory activity, for patients under statin therapy the heterozygosity and homozygosity for the Q192R polymorphism, CRP and TG serum levels were identified as the independent variables significantly associated with PON1 levels.

We must notice that inflammation, rhEPO dose and time on HD have been associated with a higher mortality rate in HD patients [18]. Due to its sensitivity to inflammatory conditions, PON1 has been considered as a negative acute-phase protein. Henning et al. [19] recently reported a reduction in plasma PON1 activity in HD patients who spent more time on dialysis, which is in accordance with our results. Treatment with rhEPO appears to reduce the oxidative stress in CKD patients and improve PON1 activity [20]. However, some patients do not respond properly to this treatment and need higher rhEPO doses. These patients resistant to rhEPO therapy present higher levels of oxidative stress and inflammatory markers [21] that can contribute to the reduction in PON1 activity.

As expected, increasing PON1 activity in HD patients was associated with the genotypes QR and RR for Q192R polymorphism, and with the LL and LM genotypes for the L55M polymorphism. Moreover, we found that the increase in PON1 activity is also associated with increasing levels of LDL-c, Apo A and VLDL, reflecting the need for oxidative protection.

In conclusion, our results strengthen the hypothesis that several genetic and non-genetic factors are important determinants of PON1 activity. The reduction in PON1 activity in HD patients who are not under statin therapy is strongly associated with inflammation, longer time on dialysis and high rhEPO doses, known as poor prognostic factors in HD patients, suggesting that the reduction in PON1 activity may worsen the prognosis of these patients. Moreover, some polymorphisms may further contribute to increase the risk in these patients by lowering PON1 activity and its protective antiatherogenic role.

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Disclosure Statement

There are no potential conflicts of interest.

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