RESEARCH ARTICLE

Oxidative stress and PON1 (T172A/L55M) polymorphism: Potential risk factors for Osteoarthritis

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Abstract

Objective: To investigate the role of oxidative stress in the progression of osteoarthritis with the genetic determinant of paraoxonase-1 enzyme L55M in osteoarthritis patients.

Method: The case control study was carried out at the University of Karachi from April to November 2020, and comprised blood samples of female osteoarthritis patients aged >50 years and healthy controls matched for age and gender. Oxidative stress was assessed by measuring lipid peroxidation product and protein carbonyl content. Activities of paraoxonase-1 paraoxonase and arylesterase were evaluated in the subjects. Protein expression of paraoxonase-1 was also analysed using western blot method. Paraoxonase-1 L55M (rs854560) polymorphism was determined using tetra-amplification-refractory mutation system polymerase chain reaction. Data was analysed using SPSS® Statistics 20.0.

Results: Of the 103 subjects, 50(48.5%) were patients and 53(51.5%) were controls. The overall age range was 50-70 years. The extent of malondialdehyde ($p<0.001$) and protein carbonyl content ($p<0.05$) were increased significantly in the patients compared to the controls. Activity of paraoxonase and arylesterase was found decreased ($p<0.001$) in patients compared to the controls. The prevalence of genotype MM was higher in the patients than the controls($p=0.001$). L55M was more pronounced in patients suffering than the controls ($p=0.01$).

Conclusion: The elevated levels of malondialdehyde and cabonylated protein content might be associated with osteoarthritis progression. Decreased serum paraoxonase-1 activity with L55M was the major consequence of oxidative stress in female osteoarthritis patients.

Keywords: Osteoarthritis, Oxidative stress, Paraoxonase, Polymorphism. (JPMA 72: 1971; 2022)

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Introduction

Osteoarthritis (OA) is the degenerative disease of the locomotor system characterised by the morphological changes in joints cartilage, synovial membrane and subcondral bone.[1] However, the aetiology of disease manifestation is not entirely understood even though biochemical, genetics and mechanical aspects are taken as major factors. This joint disease progresses slowly, which, in turn, results in severe and long-term instability.[1] Another potential explanation is oxidative stress (OS) that stimulates cartilage deprivation and pro-inflammatory mediators, such as radical oxygen species (ROS) in OA.[2] OS-mediated lipid peroxidation may play a vital role in OA advancement.[3] Lipid peroxides cause severe damage to biological molecules (deoxyribonucleic acid [DNA], proteins, lipids), alter their functional integrity with loss of cellular structure and metabolic function.[4] Several studies have reported that oxidised lipids induce chondrocyte senescence and play a major role in OA pathogenesis.[2,4]

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Carbonyl content is another marker for measurement of OS-induced protein oxidation. Carbonylated proteins are generated directly by oxidative modification of residual amino acids, and indirectly by lipid peroxidation-mediated products which form protein adducts.[5] Carbonylation is a stable and irreversible modification, occurs early-on in the disease process, and leads to damage of protein function.[6]

Human serum paraoxonase-1 (PON1) is glycoprotein of 45-kDa synthesised in liver and secreted in blood stream in conjugation with high-density lipoprotein (HDL). PON1 is calcium ion (Ca^{+2}) dependent esterase with a wide variety of substrate specificity, and, hence, possesses paraoxonase and arylesterase activities. PON1 has been shown to hydrolyse the organophosphorous compounds and carboxylic esters.[7] This enzyme has a capability to hydrolyse lipid peroxidation products and prevents their accumulation. Therefore, PON1 plays a significant role in anti-oxidative and anti-inflammatory processes.[8] It has been shown that increased PON1 activity is associated with decreased levels of OS that behaves like a shield in diseased condition.[9] However, reduced PON1 activity has been reported in different diseases, including arthritis.[10]

PON1 gene is located on the long arm of chromosome 7 and contains several polymorphisms in the promotor region, including C-108T and G-909C, and coding region Q192R. Due to the genetic changes, the single nucleotide polymorphism (SNP) at coding region L55M (leucine to methionine substitution) of PON1 gene has a substantial effect on serum activity and concentration.[11] There is limited data available regarding PON1 L55M polymorphism with PON1 enzyme activity in OA. However, PON1 Q192R polymorphism has been reported in coronary artery disease (CAD), familial hypercholesterolaemia and in cataract patients from Pakistani population.[12-14] Recently, polymorphism in resistin gene -420C>G, +299G>A, interleukin-6 (IL-6) (IL-6-174G/C), transforming growth factor-beta1 (TGF-β1-29C/T) and calmodulin 1 gene-16C/T (CALM1-16C/T) has been reported in OA patients from Pakistani population.[15,16]

The current study was planned to investigate the role of OS in OA progression with the genetic determinant of PON1 enzyme L55M in OA patients.

Patients and Methods

This case control study was carried out (Probability sampling) at the University of Karachi from April to November 2020. After approval from the institutional bioethics committee, female OA patients aged >50 years and healthy controls matched for age and gender were enrolled. Patients with other inflammatory disease / autoimmune disorders, such as rheumatic arthritis, psoriatic arthritis, traumatic arthritis and gout, were excluded. Also excluded were patients with history of bone fracture and / or any malignant or benign tumour, and those taking disease-modifying anti-OA medications.

After taking written informed consent from the subjects, 5mL blood was collected through venipuncture for serum and genotype analysis. The serum was then separated by centrifugation at 3000xg for 10 minutes and was stored at -70°C until analysed.

Total serum protein content was determined by bicinchoninic acid (BCA) method using Pierce BCA protein assay kit (Thermo Scientific Fisher, United States), according to the manufacturer's protocol.

Lipid peroxidation was ascertained by measuring the formation of malondialdehyde (MDA) and thiobarbituric acid (TBA) complex. Briefly, 50µl serum was combined with assay mixture containing 750µl trichloroacetic acid (TCA), 750µl thiobarbituric acid (TBA) and 100µl sodium dodecyl sulphate (SDS). Reaction mixture was incubated for 1hour in boiling water and then the tubes were cooled on ice. Further, 1.5mL n-butanol was added in each tube and the mixture was centrifuged for 10 minutes at 3000rpm to isolate the organic layer. The absorbance of reaction product was read at 532nm. Next, 1,1,3,3-tetramethoxypropane was used as the standard, and the level of MDA was expressed as µM/µl serum.

The extent of carbonyl level in proteins is the marker of protein oxidative damage. Carbonyl content was quantified at 370nm, 2,4-dinitrophenylhydrazine (DNPH) reagent reacts with protein carbonyl groups to form Schiff, producing hydrazone adduct.[17] Also, 200µl serum was taken in a tube (marked as test) containing 10mmol 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5m hydrochloric acid (HCl). The same amount of serum was marked as control. The tubes were incubated for an hour in the dark at room temperature and then kept on ice for 10 minutes. Further, 1.5 mL TCA (20%) was added in each tube and centrifuged at 3000rpm. The obtained pallet was washed twice with 10% trichloroacetic and 1ml ethanol-ethyl acetate (1:1) respectively. After washing, the protein pallet was re-suspended in 0.5mL of 6M guanidine hydrochloride solution and incubated at 37°C for 10 minutes. Followed by incubation, the tubes were centrifuged and the supernatant was collected for measurement. Level of cabonylated protein was calculated using 22,000 M^{-1} cm^{-1} molar extinction coefficient and was expressed as nmol/mg protein.

Serum paraoxonase activity was assayed using the established protocol.[18] PON1 enzyme activity was measured using substrate paraoxon by quantifying the generation of p-nitrophenol over 3 minutes (60sec interval) at 412nm. Briefly, 25µL serum was added into reaction buffer containing 50mM glycine-sodium hydroxide (NaOH) buffer, potential hydrogen (pH) 10.5, 1mM calcium chloride $CaCl_2$ and 1mM paraoxon. The degree of substrate hydrolysis in the course of enzyme-substrate conjugation was estimated using molar absorptivity of 18290 $M^{-1}cm^{-1}$. Enzyme activity was expressed as unit per mL (U/mL) serum.

Arylesterase activity of PON1 was assessed using the protocol described in literature.[18] The assay tube contained 20mM Tris-HCL buffer pH 8.0, 1.0mM phenylacetate as a substrate, 1.0mM CaCl2 and 7µl of serum sample. The absorbance was monitored spectrophotometrically at 270nm. PON1 arylesterase activity was calculated using the molar extinction coefficient of 1310 $M^{-1}cm^{-1}$ and expressed as unit per liter of sera.

Genomic DNA was extracted from the whole blood using a protocol described previously.[14] Tetra-amplification-

refractory mutation system polymerase chain reaction (ARMS PCR) for PON1 L55M gene polymorphism was carried out using 1x PCR buffer (0.2M potassium chloride [KCl], 0.1M Magnesium chloride ($MgCl_2$), 0.1M tris-pH 8.3) in 850µl nuclease-free water. Reaction volume 20µl contained 15µl 1xPCR buffer, 10mM deoxynucleoside triphosphate (dNTP) mix, 10µM forward and reverse primers, 1U taq polymerase and 3.5µl DNA template was used to amplify the desired sequence. The following PCR program was used: denaturation at 94°C, annealing at 58°C, extension at 74°C, and total number of PCR cycles were 35. The amplified PCR products were separated on 2% agarose gel. Four sets of primers, two outer and two inner, for position L55M were used as described previously.[19]

Western blot analysis was performed for the validation of PON1 protein in OA patients and controls. Total protein was separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membrane (PVDF). Followed by blocking with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer for 1hour at room temperature. Membrane was incubated overnight with anti-PON1 antibody mouse monoclonal (ab24261, abcam, Cambridge, MA) diluted with (1:100) 3% blocking solution. Membrane was washed with TBS-Tween20 (TBST) 3 times for 2 minutes each. Horse raddish peroxidase conjugated goat anti-mouse immunoglobulin G (IgG) (cat no. 81-6520) secondary antibody (1:2000 in TBS) was used to incubate the membrane at room temperature for 1hour. The band was visualised by adding diaminobensidine in hydrogen peroxide (H_2O_2) (1mg/ml). Band intensity was measured by using Microtek Bio-6000 gel scanner.

Data was analysed using SPSS® Statistics 20.0. Results were described as mean ± standard deviation using independent t-test for the measurement of MDA, protein carbonyl and enzymatic activity of PON1 between the case and control groups. Pearson correlation coefficient (r) was used to determine the relation between PON1 enzyme activity and MDA level. Gene counting method and chi-square test were used to calculate PON1 L55M genotypic and allelic frequencies among experimental groups. MedCalc software was used for calculating odds ratio (OR) and 95% confidence interval (CI). P<0.05 was considered statistically significant.

Results

Of the 103 subjects, 50(48.5%) were patients and 53(51.5%) were controls. The overall age range was 50-70 years.

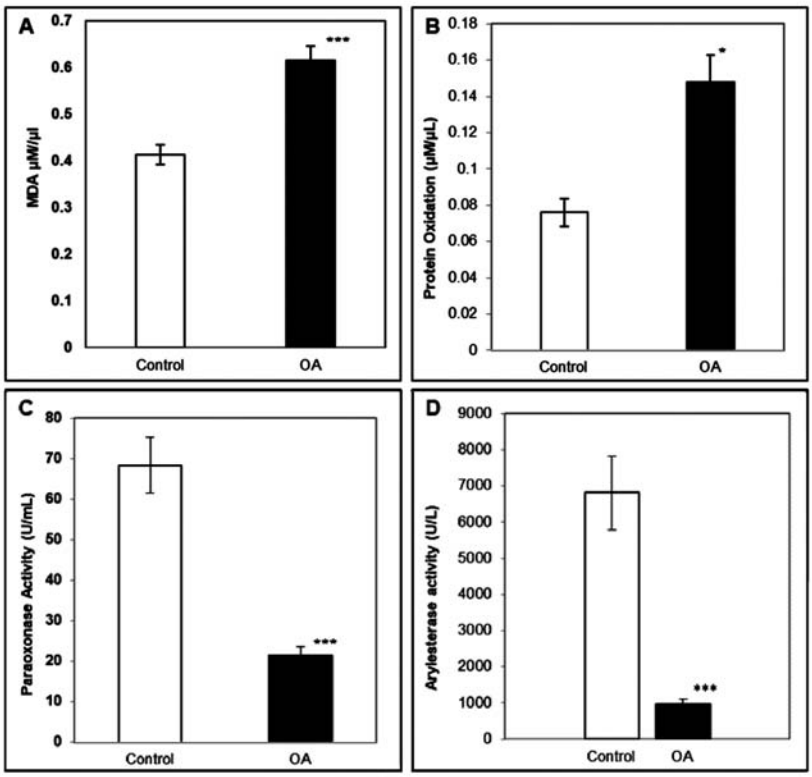

Figure-1: Levels of MDA (A), Protein oxidation (B), Activities of paraoxonase-1 PON1 paraoxonase (C) and Arylesterase (D) in serum samples of osteoarthritis OA (n=50) and control (n=53). Data represents the mean ± S.D. using independent T-test ***p< 0.001 for MDA, paraoxonase activity and arylesterase activity; *p < 0.05 is for protein oxidation.

Table: Distribution of paraoxonase-1 (PON1) T172A (L55M) genotype and allele type in osteoarthritis (OA) patients and control subjects.

Genotype	OA Patients	Control group	OR	95% CI	P-value	Chi-square
TT (LL)	18/50(36%)	24/53(45%)	0.679	0.3081-1.4994	<0.001	χ^2 =55.9
AT (LM)	7/50(14%)	15/53(28%)	0.412	0.1521-1.1184		
AA (MM)	25/50(50%)	14/53(26%)	2.785	1.2211-6.3551		
Allele Type						
T allele (L allele)	43/100(43%)	63/106(59.4%)	0.514	0.2958-0.8961	0.0189	
A allele (M allele)	57/100 (57%)	43/106(40.5%)	1.942	1.1159-3.3801	0.0189	

T: Thymine Nucleotide, L: Leucine amino acid, A: Adenine nucleotide, M: Methionine amino acid, OR: Odds ratio, CI: Confidence interval.

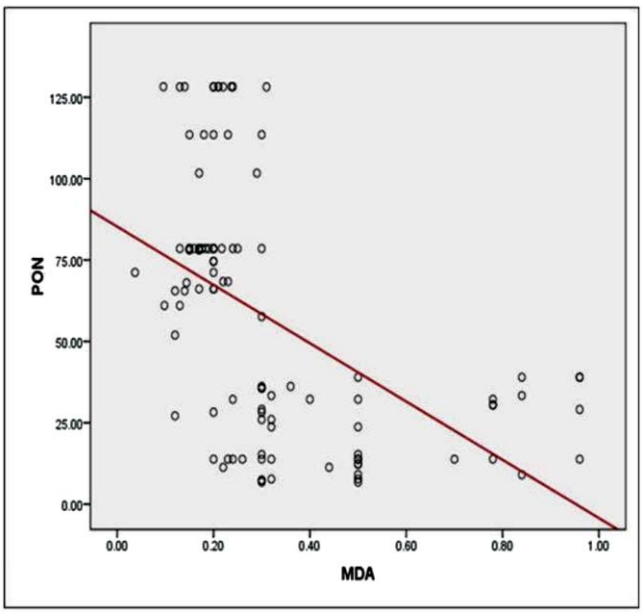

Figure-2: Inverse correlation between malondialdehyde (MDA) and paraoxonase-1 (PON1) enzyme activity.

Levels of MDA and Protein oxidation were significantly higher in the patients as compared to the controls (Figure-1 A: p<0.001; Figure-1 B: p<0.05). The association of OS with OA was significantly different between the groups. Serum paraoxonase and arylesterase activities were significantly lower (p <0.001) in OA patients (Figure-1C-1D). There was a significant inverse relation between MDA levels and serum PON1 activity (p<0.05) (Figure-2).

The gene frequencies of L55M in OA and healthy individuals are represented in Table. The wild-type genotype homozygous LL (TT) in the OA group was found to be 18(36%), heterozygous LM (AT) 7(14%), and homozygous MM (AA) 25(50%), whereas in the control group the corresponding frequencies were 24(45%), 15(28%) and 14(26%) respectively (Table). The occurrence of 55M allele was significantly higher in OA patients (p<0.01) (Table).

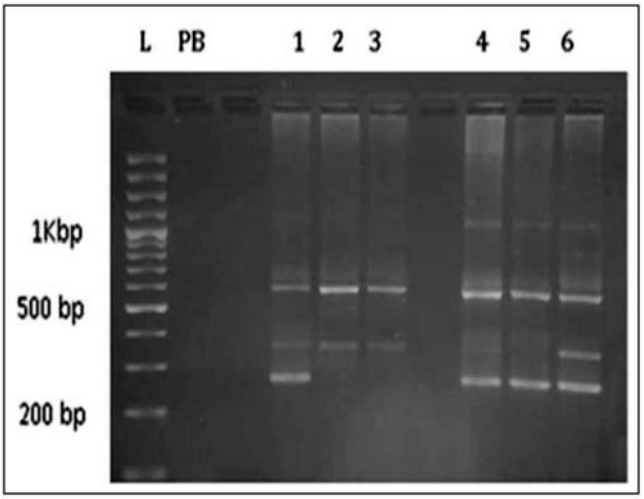

Figure-3: Tetra-amplification-refractory mutation system polymerase chain reaction (ARMS PCR) for paraoxonase-1 (PON1) L55M polymorphism. Lanes 1, 2 and 3 represent the heterozygous LM, homozygous LL and LL for controls. Lanes 4, 5 and 6 represent homozygous MM, MM and LM for osteoarthritis (OA) patients.
L: Ladder, PB: PCR blank.

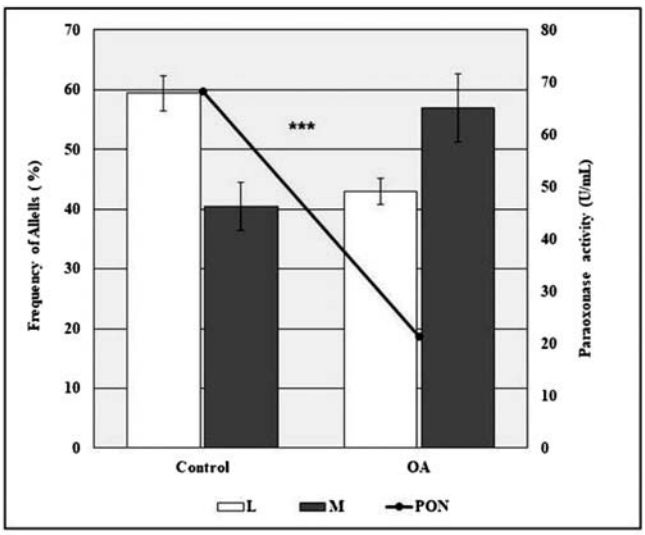

Figure-4: The correlation between allele frequencies and paraoxonase-1 (PON1) enzyme activity among osteoarthritis (OA) and control groups. Primary Y-axis represents allele frequency (%) and Secondary Y-axis shows PON1 activity. X-axis represents experimental groups control and OA.
L allele was wild type, while M allele was mutant. (***p<0.001).

Figure-3 shows PCR product size: two outer primers 571 bp; A allele (allele M) 262 bp; T allele (allele L) 351 bp.

The M allele was found to be reduced in the control group with concomitant increase in serum PON1 activity (p<0.001), while among the OA patients, M alleles and PON1 activity were found to be decreased significantly (p<0.001) (Figure-4).

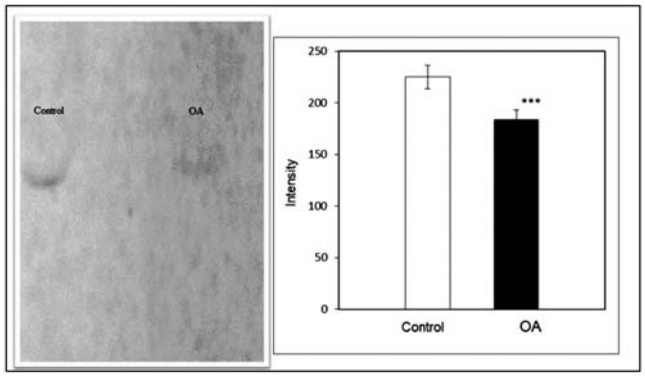

Figure-5: Western blot of paraoxonase-1 (PON1) related to osteoarthritis (OA) and control groups. ***p<0.001.

Western blot analysis demonstrated decreased ($p<0.001$) expression of PON1 in the patients compared to the controls (Figure-5).

Discussion

Osteoarthritis, known as a degenerative disease of the joints, is a progressive deterioration of articular cartilage and osteophyte formation at the surface of the joint. Pathologically, the disease is characterised by loss and destruction in cartilage tissue.[20] It has been suggested that ROS are actively produced by human articular chondrocyte. These ROS are unconfined and release during inflammatory process in the synoviocyte. ROS play a significant role to alter metabolism in chondrocytes and act as the effecters and mediators of the cartilage damage.[21] This detrimental effect is originated via chain reactions that provide free radical species continuously which in turn initiates peroxidative damage.[22]

The present study showed high OS in OA patients owing to increase extent of lipid peroxidation and protein oxidation or might be due to decreased PON1 antioxidant activity.

The MDA levels were found significantly increased ($p<0.001$) in OA patients compared to the healthy controls. This indicates the association of lipid peroxidation with OA patients. The results are in agreement with those of Vyas et al.[22] Sharma et al. also reported increased MDA level in synovial fluid of OA patients, suggesting that synovial fluid MDA level plays an important role in the aetiopathogenesis of OA.[23] Continuous exposures of ROS causes protein damage by oxidising residual amino acids, resulting in the formation of protein-protein cross-links, protein aggregation and proteolysis.[24]

The present study found increased carbonyl content in OA patients compared to the controls ($p<0.05$). Similar results were reported by Tetik et al.[25] It can be suggested that there is an inter-reliant relationship between free radicals intensity and the progression of the disease.

PON1 is an HDL-conjugated antioxidant enzyme that inhibits low-density lipoprotein (LDL) oxidation in human serum. PON1 provides protection against free radicals damage by restricting the oxidation of phospholipids and its activity is lost in the oxidative environment.[26] The current study determined that PON1 activity was significantly ($p<0.001$) reduced in OA patients compared to the healthy subjects which might be due to increased OS markers, such as carbonyl content and lipid peroxidation. It also demonstrated that serum PON1 arylesterase activity towards its substrate phenylacetate was also significantly decreased ($p<0.001$) in OA patients than in controls. The findings support the data of various studies that the reduction in antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and PON1 activity is correlated with increased parameters of OS, such as lipid hydroperoxides, total oxidant status and OS index in OA patients, and suggested that low PON1 activity is valuable adjunctive indicator of OA severity.[27] Furthermore, the current study also found negative correlation ($p<0.05$) between serum PON1 enzyme activity and MDA levels that indicate decreased PON1 activity might be associated with increased oxidative environment in OA patients.

The current study also established an association of the MM genotype of the L55M polymorphism with a two-fold amplified risk of OA. To the best of our knowledge, there is no study reporting PON1 polymorphism on OA patients. The frequency of mutant allele M in the PON1 gene in OA patients was significantly higher ($p<0.01$.) Evidence showed an association between L55M polymorphism and PON1. Garin et al. revealed that allele 55L had significantly higher paraoxonase concentration than the 55M allele in patients suffering with diabetes and cardiovascular diseases.[28] Leviev et al. also reported lower concentration of the PON1 messenger ribonucleic acid (mRNA) expression and enzyme activity in allele 55M than the L-type in atherosclerotic patients[29] due to the fact that efficacy to protect LDL against oxidative damage is affected by polymorphism at 55M in leucine-methionine.[30] It has been reported that allele 55M is found to be a risk factor for rheumatoid arthritis as increased concentration of serum paraoxonase is expressed in the L allele than in the M allele.[19] The current study also found significantly decreased paraoxonase activity in 55M allele compared to allele 55L.

Conclusion

Lipid peroxidation and protein oxidation-mediated OS played a crucial role in OA advancement. Significant decreased in PON1 activity in patients suffering from OA might be the major consequence of OS. There was a strong association between PON1 L55M polymorphism and OA, suggesting that anti-oxidants could be beneficial in delaying OA progression.

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Disclaimer: The text is based on an academic thesis, and part of it has been presented at a conference.

Conflict of Interest: None.

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