Plasma Malondialdehyde Levels Influenced by Endothelial Nitric Oxide Synthase Gene Intron-4 27BP Repeat Polymorphism in Rheumatoid Arthritis

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Abstract

Objective: The aim of this study was to investigate the association between plasma malondialdehyde (MDA) level and endothelial nitric oxide synthase gene intron-4 27bp repeat polymorphism in rheumatoid arthritis. Materials and Methods: We conducted a case control study in which 100 patients diagnosed with rheumatoid arthritis (RA) and 100 healthy controls were enrolled. DNA was extracted from peripheral blood and eNOS polymorphism was detected by PCR. Plasma samples from the subjects were screened for MDA. Results: Allelic and genotypic distribution did not differ significantly between RA patients and healthy control subjects. There was no significant difference between the mean values of MDA in controls and patients (p=0.67). Patients with ab genotype showed significantly high level of plasma MDA as compared to controls with ab genotypes (p < 0.05). Patients with bb and aa genotypes showed no significant difference in plasma MDA as compared to controls with bb and aa genotypes (p> 0.05). There was no significant difference in plasma MDA levels among patients and controls with bb, ab and aa genotypes (p> 0.05). Conclusions: Plasma MDA levels are influenced by eNOS gene intron-4 27bp repeat Polymorphism in Rheumatoid Arthritis.

Keywords
eNOS, Polymorphism, Rheumatoid arthritis, Malondialdehyde.
INTRODUCTION
Rheumatoid arthritis (RA) is a common systemic autoimmune disease associated with potentially debilitating joint inflammation as well as altered skeletal bone metabolism and co-morbid conditions [1]. Malondialdehyde (MDA) is a reactive aldehyde formed by the degradation of polyunsaturated lipids by reactive oxygen species. It causes stress in cells and forms protein and DNA adducts. Human ALDH1A1 aldehyde dehydrogenase is capable of oxidising malondialdehyde [2-5]. Malondialdehyde (MDA), a marker of oxidative stress, is reportedly to be increased in rheumatoid arthritis (RA) according to many studies [6-11]. Endothelial nitric oxide synthase (eNOS) is a constitutively expressed enzyme of 135 kDa in the vascular endothelial cells. The e-NOS gene, located on chromosome 7q35-36, is composed of 26 exons and spans 21 kb [12]. One of the common polymorphisms in the eNOS gene is 27bp repeat polymorphism in intron 4 of the gene [13]. This is a variable base pair repeat polymorphism in intron 4 (intron4b/a) that has 2 common alleles containing 4 variable nucleotide tandem repeats a and 5 variable nucleotide tandem repeats b, which make 1 heterozygous ab, and 2 homozygous aa and bb genotypes [14,15]. Although the biological impact of the VNTR 4a/b polymorphism is unclear, it has been suggested that this polymorphism would regulate the expression of eNOS by the formation of small RNAs (siRNAs). Endothelial cells containing five copies present higher quantities of siRNA and lower levels of mRNA of eNOS, when compared with cells that contain four copies [16,17]. Although many studies have shown the association between eNOS polymorphism and some autoimmune diseases, the results are conflicting [13]. One study found association between enos polymorphism and adiponectin level [18]. Moreover, there is no study that shows the effect of eNOS gene intron-4 27bp repeat polymorphism upon plasma MDA levels in RA. Therefore, our aim was to investigate the association between plasma MDA level and eNOS gene intron-4 27bp repeat polymorphism in RA.

MATERIALS AND METHOD
Subjects
100 rheumatoid arthritis patients were recruited from the Department of Rheumatology (Medicine) of the All India Institute of Medical Sciences (AIIMS), New Delhi. All the patients met the American College of Rheumatology 1987 revised criteria [19], were 20 to 60 years old (mean age (39.05±11.45) years). All the patients were on treatment for RA before recruitment. 100 age- and ethnicity-matched normal volunteers comprising patient’s relatives, students of AIIMS and voluntary blood donors of Blood Bank, AIIMS were studied as controls. All the controls were 20 to 60 years old (mean age (29.5±7.42) years). Each participant donated peripheral blood for DNA analysis and plasma isolation. Plasma samples were stored at −20°C in aliquots until use. All study participants provided written informed consent and the study was approved by the local ethics committee.

DNA extraction
Genomic DNA was isolated from peripheral blood leucocytes by the Miller extraction method [20].

Analysis of the 27bp repeats polymorphism in intron 4 of the eNOS gene
Detection of 27bp repeat polymorphism in intron 4 of the eNOS gene was performed in all the subjects by PCR genotyping. Primer pairs used were as follows: sense-5’ AGG CCC TAT GGT AGT GCC TTT 3’ and antisense- 5’ TCT CTT AGT GCT GTG GTC AC 3’. Samples were amplified for 36 cycles, consisting of denaturation at 950°C for 45 seconds, annealing at 560°C for 1 minute and extension at 720°C for 1 minute with a final extension of 7 minutes. Amplified products were run on 2.5% agarose gels and visualized by ethidium bromide staining. 420bp size product denotes ‘b’ allele (five repeats) and 393 bp size product denotes ‘a’ allele (four repeats).

Estimation of Malondialdehyde (MDA)
Plasma level of MDA was measured according to the method of Ohkawa et al, 1979 [21].

Statistical Methods
Statistical analysis was performed according to SPSS (Statistical Package for Social Sciences) for windows (version 9.0.0, SPSS Inc., Chicago) and TFPGA (Tools for population genetic analysis) version 1.3 developed by Mark Miller from the department of biological science, North Arizona University. Frequency of genotypes (bb, ab, aa) and alleles (b, a) of 27bp repeat polymorphism in intron 4 of eNOS gene were assessed using Fisher’s exact test and chi-square test wherever applicable. Mann Whitney test and Kruskal Wallis test were used wherever applicable. Unpaired and two tailed t tests were used to analyze laboratory data. P value ≤0.05 was considered statistically significant.

RESULTS
Association of eNOS gene intron-4 27bp repeat Polymorphism and Rheumatoid arthritis
On comparative evaluation of the frequency of the intron-4 27bp repeat Polymorphism genotype and
alleles between the patients and healthy subjects (table 1), it was found that b allele and bb genotype were more prevalent in RA patients (0.84 & 71%) as compared to healthy subjects (0.75 & 58%). The OR of bb genotype was 1.77 (95% CI= 0.95-3.33, p=0.055) to develop Rheumatoid arthritis. The OR of ab genotype was 0.68 (95% CI= 0.35-1.31, p=0.22) to develop Rheumatoid arthritis. The OR of aa genotype was 0.36 (95% CI= 0.07-1.53, p=0.12) to develop Rheumatoid arthritis.

Levels of MDA in controls and patients
The plasma concentration of MDA in a total of 100 patients and 100 healthy subjects was evaluated (Table 2). The results are expressed in micro molar (μM). Values of MDA in controls and patients were between the range of 2.59 to 20.66, median 4.11, mean 4.29 and 2.33 to 44.73, median 4.17, mean 7.4 μmol/l (Table 2). There was no significant difference between the mean values of controls and patients (p=0.67). However, on further analysis (fig 1 and 2), it was observed that 11% of the patients had MDA values in the range of 15 and 30 μmol/l, 3% having NO beyond 30 μmol/while only 2% of the controls had MDA values in the range of 15 to 30 μmol/l and none of the controls had MDA beyond 30 μmol/l. In short, 14% of the patients had MDA values higher than 15 μmol/l whereas only 2% of the controls had MDA values above 15 μmol/l.

Relationship between eNOS VNTR intron 4 a/b polymorphism and plasma MDA in controls and patients
Plasma levels of MDA in individual genotypes in controls and patients are shown in Table 3. Patients with ab showed significantly high level of plasma MDA as compared to controls with ab genotypes (p < 0.05). Patients with bb and aa genotypes showed no significant difference in plasma MDA as compared to controls with bb and aa genotypes (p> 0.05). There was no significant difference in plasma MDA levels among patients with bb, ab and aa genotypes (p> 0.05). Likewise, there was no significant difference in plasma MDA levels among controls with bb, ab and aa genotypes (p>0.05).

Table 1: Distribution of eNOS VNTR intron 4 a/b Genotypes and Frequency of Alleles in Controls and Rheumatoid Arthritis Patients

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients (N=100)</th>
<th>Controls (N=100)</th>
<th>P value</th>
<th>O.R.</th>
<th>C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>bb</td>
<td>71</td>
<td>58</td>
<td>0.055</td>
<td>1.77</td>
<td>0.95 - 3.33</td>
</tr>
<tr>
<td>ab</td>
<td>26</td>
<td>34</td>
<td>0.22</td>
<td>0.68</td>
<td>0.35 – 1.31</td>
</tr>
<tr>
<td>aa</td>
<td>3</td>
<td>8</td>
<td>0.12</td>
<td>0.36</td>
<td>0.07 – 1.53</td>
</tr>
<tr>
<td>Ab+aa</td>
<td>29</td>
<td>41</td>
<td>0.08</td>
<td>0.59</td>
<td>0.31 – 1.10</td>
</tr>
</tbody>
</table>

| Alleles  | b              | 0.84           | 0.75    |      |     |
|          | a              | 0.16           | 0.25    |      |     |

Table 2: Plasma level of MDA in controls and RA patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± STD</th>
<th>Median</th>
<th>Range</th>
<th>Mean ± STD</th>
<th>Median</th>
<th>Range</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>4.92±2.63</td>
<td>4.11</td>
<td>2.59-20.66</td>
<td>7.4 ± 7.5</td>
<td>4.17</td>
<td>2.33-44.73</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Table 3: Plasma MDA levels in relation to eNOS intron 4 a/b polymorphism

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median</th>
<th>Mean± SD</th>
<th>Genotype</th>
<th>Median</th>
<th>Mean± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bb (71)</td>
<td>4.07</td>
<td>6 ± 5.5</td>
<td>bb (58)</td>
<td>4.37</td>
<td>5.26 ± 2.85</td>
<td>0.18</td>
</tr>
<tr>
<td>ab (26)</td>
<td>6.1</td>
<td>10.97 ± 10.79</td>
<td>ab (34)</td>
<td>3.89</td>
<td>4.63 ± 2.5</td>
<td>0.032</td>
</tr>
<tr>
<td>aa (3)</td>
<td>7.02</td>
<td>9.2 ± 7.1</td>
<td>aa (8)</td>
<td>3.59</td>
<td>3.8 ± 0.47</td>
<td>0.19</td>
</tr>
</tbody>
</table>
DISCUSSION
In this study, we investigated whether there is an association between plasma MDA levels and eNOS gene intron-4 27bp repeat polymorphism in RA. It was found that there was no significant difference in the frequencies of bb, ab and aa genotypes of the eNOS gene intron 4 among controls and RA patients. However, the distribution pattern obeyed Hardy Weinberg equilibrium thereby suggesting that the sample size was appropriate for the study on eNOS gene intron-4 27bp repeat polymorphism. This finding is in contrast to a previous finding referred to a Cretan cohort of RA patients implicating the polymorphism in susceptibility to RA [9]. This may be due to the differences in the genetic background between the populations studied.

We demonstrated that there was no significant difference in plasma MDA levels between RA patients and healthy controls. This finding is in contrast to other studies which found increased plasma MDA level in RA patients as compared to controls [6-11]. One of the reasons for this finding may due to collection of blood samples from RA patients who were already on drug treatment. Oxidative stress might have been decreased due to the drug treatment in RA patient and as a result MDA levels were not significantly increased.

Plasma MDA levels were correlated with eNOS gene intron-4 27bp repeat polymorphism. We found that patients with ab showed significantly high level of plasma MDA as compared to controls with ab genotypes (p < 0.05). However, patients with bb and...
aa genotypes showed no significant difference in plasma MDA as compared to controls with bb and aa genotypes (p > 0.05) and, there was no significant difference in plasma MDA levels among patients with bb, ab and aa genotypes (p > 0.05). This indicates that RA patients heterozygous for this polymorphism are likely to have increased plasma MDA levels and increased oxidative stress as compared to those who are homozygous for this polymorphism. Thus, we can conclude that plasma MDA levels are influenced by eNOS gene intron-4 27bp repeat polymorphism in RA. Similar studies are required to be conducted in other populations to verify and validate our findings in the present study. To the best of our knowledge, there is no previous study that showed interrelationship of MDA and eNOS gene intron-4 27bp repeat polymorphism in RA.

CONCLUSIONS
This study aimed at the elucidation of the relationship between eNOS gene intron-4 27bp repeat polymorphism and plasma MDA levels in RA. We demonstrated that patients with ab showed significantly high level of plasma MDA as compared to controls with ab genotypes (p < 0.05). This indicates that RA patients heterozygous for this polymorphism are likely to have increased plasma MDA levels and increased oxidative stress as compared to those who are homozygous for this polymorphism. Thus, we can conclude that plasma MDA levels are influenced by eNOS gene intron-4 27bp repeat polymorphism in RA. Similar studies are required to be conducted in other populations to verify and validate our findings in the present study.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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