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Effect of Endothelial Nitric Oxide Synthase Gene Glu298Asp Polymorphism on Plasma Malondialdehyde Levels 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 eNOS gene Glu298Asp 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-RFLP. Plasma samples from the subjects were screened for MDA.*
Results: *There was a trend of increase of GG genotypic and G allelic frequencies of the eNOS gene Glu298Asp in patients when compared with controls. GT genotype emerged as major genotype in both controls and patients. Genotypes disobeyed Hardy-Weinberg equilibrium. There was no significant difference between the mean values of MDA in controls and patients (p=0.67). Patients with GG, GT and TT genotypes showed no significant difference in plasma MDA as compared to controls with GG, GT and TT genotypes (p> 0.05).*
Conclusions: *Plasma MDA levels are not influenced by eNOS gene Glu298Asp polymorphism in RA.*
Keywords: *eNOS, Polymorphism, Rheumatoid arthritis, Malondialdehyde*

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I. Introduction

Rheumatoid arthritis (RA) is a common systemic inflammatory disease, the exact mechanism of which is still unclear [1]. 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 reported to be increased in rheumatoid arthritis (RA) according to many studies[6-11].

Endothelial nitric oxide synthase (e-NOS) 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]. The potentially significant role of eNOS in the pathogenesis of various diseases has been examined previously. Genetically polymorphic variants are known as useful markers when examining the importance of various molecules in association with diseases. Several genetic polymorphisms of the eNOS gene have shown associations with a number of disease conditions; 3 polymorphisms, including -786*T/C in the 5' flanking region of the gene, exon 7 SNP and a VNTR polymorphism in intron 4, have been given the most attention in studying the eNOS gene. A single base substitution (G894 → T) in exon 7 of the human eNOS gene has been reported, and it causes a Glu→Asp amino acid exchange at residue 298 of the protein [13].

Although many studies have shown the association between this polymorphism and some autoimmune diseases, the results are conflicting [14]. One study found association between eNOS polymorphism and adiponectin level in metabolic syndrome [15]. To the best of our knowledge, there is no study that shows the effect of eNOS gene Glu298Asp polymorphism upon plasma MDA levels in RA. Therefore, our aim was to investigate the association between plasma MDA level and eNOS gene Glu298Asp polymorphism in Rheumatoid Arthritis.

II. Materials and Method

Subjects

One hundred 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 [16], were 20 to 60 years old (mean age (39.05±11.45) years). All the patients were on treatment for RA before recruitment. One hundred 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 [17].

Analysis of the Glu 298 Asp polymorphism in exon 7 of the e NOS gene

The genomic DNA was amplified by PCR using the following primers flanking the polymorphic region of eNOS gene: sense-5'TCC CTG AGG AGG GCA TGA GGC T-3' and anti-sense- 5' TGA CGG TCA CAC AGG TTC CT 3'. Samples were amplified for 30 cycles, consisting of denaturation at 950C for 1 minute, annealing at 610C for 1 minute and extension at 720C for 1 minute with a final extension of 10 minutes. Amplified products were run on a check gel with 1% agarose. The resulting 457-bp amplification product was digested with 8U of the Ban II restriction enzyme at 370C for at least 20 hours. *Ban* II digested the amplified fragments into smaller fragments (137 and 320 bp). The restricted fragments were resolved on 2% agarose gels and visualized by ethidium bromide staining.

Estimation of Malondialdehyde (MDA)

Plasma level of MDA was measured according to the method of Ohkawa et al, 1979.[18].

Statistical Methods

Statistical analysis were 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 (GG,GT, TT) and alleles (G,T) of Glu298Asp polymorphism in exon 7 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.

III. Results

Association of eNOS Glu298Asp Polymorphism and Rheumatoid arthritis

On comparative evaluation of the frequency of Glu298Asp genotype and alleles between the patients and healthy subjects (Table 1), it was found that G allele and GG genotype are more prevalent in RA patients (0.68 & 41%) as compared to healthy subjects (0.62 and 27%). Odd's ratio (OR) of GG genotype was 1.88 (95% CI=0.99-3.56, p= 0.036) to develop Rheumatoid arthritis. The individuals carrying TT genotype, the OR to develop RA was insignificant as compared to G allele carriers (GG +GT).

Table 1 Distribution of Glu298Asp Genotypes and Frequency of Alleles in Controls and Rheumatoid arthritis Patients

	Patients (N=100)	Controls (N=100)	P value	O.R.	C.I.
Genotypes					
GG	41	27	0.036	1.88	0.99 - 3.56
GT	54	70	0.02	0.5	0.27 – 0.94
TT	5	3	0.72	1.7	0.34 – 9.28
GT+TT	59	73	0.037	0.53	0.28 – 1.01
Alleles					
G	0.68	0.62			
T	0.32	0.38			

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 micromolar (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/. 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.

Table 2 Plasma level of MDA in controls and RA patients

	CONTROL			PATIENTS			
Variable	**Mean ± STD**	**Median**	**Range**	**Mean ± STD**	**Median**	**Range**	**P value**
MDA	**4.92±2.63**	**4.11**	**2.59-20.66**	**7.4 ± 7.5**	**4.17**	**2.33-44.73**	**0.67**

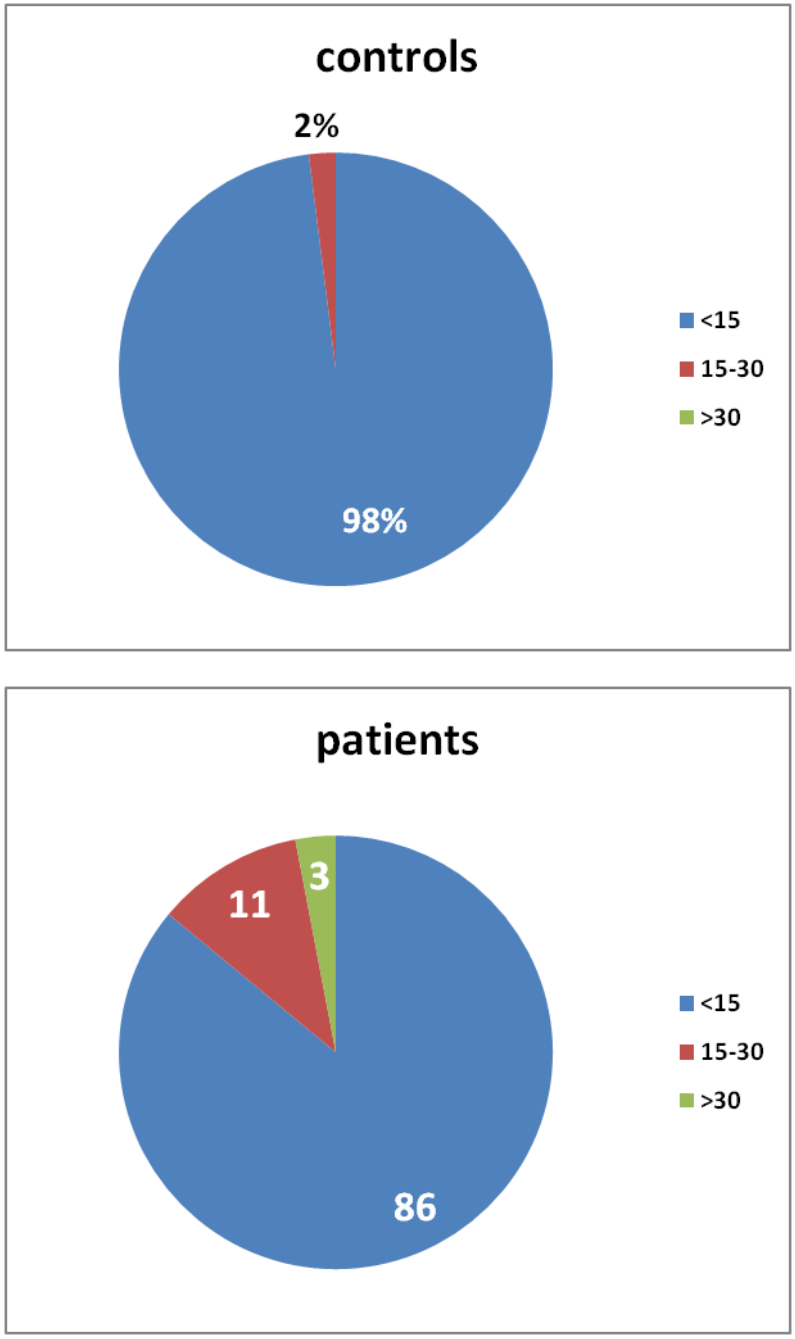

Fig 1. Distribution of plasma MDA levels in 100 controls and 100 patients.

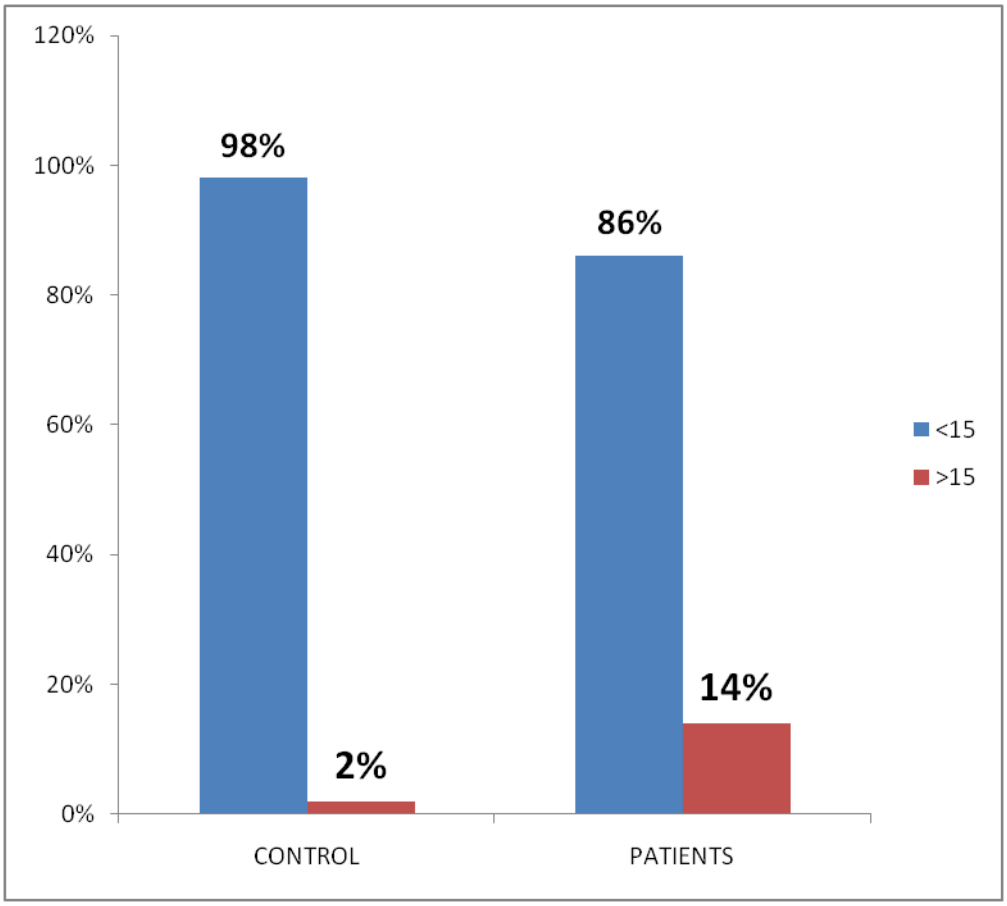

Fig 2. Distribution of plasma MDA levels in controls and patients.

Relationship between eNOS Glu298Asp 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 GG, GT and TT genotypes showed no significant difference in plasma MDA as compared to controls with GG, GT and TT genotypes (p> 0.05). There was no significant difference in plasma MDA levels among patients with GG, GT and TT genotypes (p> 0.05). Likewise, there was no significant difference in plasma MDA levels among controls with GG, GT and TT genotypes (p>0.05).

Table 3 Plasma MDA level in relation to eNOS Glu298Asp polymorphism

PATIENTS			CONTROLS			
Genotype	**Median**	**Mean± SD**	**Genotype**	**Median**	**Mean± SD**	**P value**
GG (41)	3.87	6.84 ± 7.7	GG (27)	4.09	4.96 ± 2.9	0.54
GT (54)	4.46	7.95 ± 7.7	GT (70)	4.11	4.83 ± 2.45	0.15
TT (5)	4.9	2.56 ± 3.7	TT (3)	3.66	6.3 ± 4.75	0.88

IV. Discussion

In this study, we investigated whether there is an association between plasma MDA levels and eNOS gene Glu298Asp polymorphism in RA. We demonstrated here that there was a trend of increase of GG genotypic and G allelic frequencies of the eNOS gene Glu298Asp, respectively, in RA patients when compared with controls. Odd's ratio (OR) of GG genotype was 1.88 (95% CI=0.99-3.56, p= 0.036) to develop Rheumatoid arthritis. This may suggest that majority of people are susceptible to RA. GT emerged as major genotype in both controls and patients suggesting that heterozygous are more protected against RA. However, in one study in our lab on the same polymorphism GG dominates over GT and TT genotypes [19]. Likewise in several other studies, GG dominates over GT and TT genotypes [20-22]. In our study, the distribution pattern did not obey Hardy Weinberg equilibrium. Therefore, not much importance can be ascribed to this finding. Moreover, previous findings referred to a Spanish cohort of RA patients did not support the implication of eNOS gene Glu298Asp polymorphism in susceptibility to RA [14]. Gonzalez et al revealed that inducible but not endothelial NOS polymorphism is associated with susceptibility to RA in Northwest Spain. There may be

several reasons for the distribution pattern not obeying HW equilibrium. These include restriction in sample size, presence of natural selection, mutation, non-random mating and migration [23-24]. Therefore, one of the strategies should be to extend the study to a larger cohort.

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 Glu298Asp polymorphism. We found that patients with GG, GT and TT genotypes showed no significant difference in plasma MDA as compared to controls with GG, GT and TT genotypes ($p > 0.05$). There was no significant difference in plasma MDA levels among patients with GG, GT and TT genotypes ($p > 0.05$). Likewise, there was no significant difference in plasma MDA levels among controls with GG, GT and TT genotypes ($p > 0.05$). Thus, we can conclude that plasma MDA levels are not influenced by eNOS gene Glu298Asp polymorphism. To the best of our knowledge, there is no previous study that showed interrelationship of MDA and eNOS gene Glu298Asp polymorphism in RA.

V. Conclusions

This study aimed at the elucidation of the relationship between eNOS gene Glu298Asp polymorphism and plasma MDA levels in RA. We demonstrated that there was no significant difference in plasma MDA levels between RA patients and healthy controls. Moreover, we found that patients with GG, GT and TT genotypes showed no significant difference in plasma MDA as compared to controls with GG, GT and TT genotypes ($p > 0.05$). This indicates that plasma MDA levels are not influenced by eNOS gene Glu298Asp polymorphism in RA. Further study is required in a larger cohort of treatment naïve RA patients.

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Conflict of interest

The authors declare that they have no conflict of interest

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