

# Influence of endothelial nitric oxide synthase gene intron-4 27bp repeat polymorphism on its expression in autoimmune diseases

Suad AlFadhli

*Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Kuwait University, Hawally, Kuwait*

**Abstract.** The purpose of this study was to analyse the effect of the T-786C polymorphism and intron 4 27 bp variable number tandem repeat (VNTR) eNOS markers for their potential association with Systemic Lupus Erythematosus (SLE), Hashimoto's thyroiditis (HT) and Rheumatoid arthritis (RA) as well as to explore their effect on eNOS mRNA expression and nitrate production (NO<sub>x</sub>). Kuwaitis ( $n = 383$ ) matched by age, gender and ethnicity were genotyped by fluorescent-labelled-restriction fragment length polymorphism (RFLP) and fragment analysis. Expression of eNOS mRNA was analysed using RT-PCR and sera from subjects were screened for NO<sub>x</sub> using ELISA. Analysis of the allelic frequency revealed a significant association of the 4b allele with susceptibility to SLE ( $p = 0.0092$ ,  $OR = 1.76$ ). The 4bb genotype was found to be associated with SLE ( $p = 0.0076$ ,  $OR = 1.97$ ) and HT ( $p = 0.05$ ,  $OR = 1.81$ ). Allelic and genotypic distribution did not differ between RA patients and healthy control subjects. The 4bb genotype resulted in reduced expression of eNOS mRNA in SLE, RA and HT, but only the reduction in HT was significant ( $p = 0.05$ ). The 4ab genotype revealed a significant association with increased eNOS expression in HT ( $p = 0.03$ ) and RA ( $p = 0.014$ ) patients, and elevated NO<sub>x</sub> levels were detected in the autoimmune disease cohorts ( $p < 0.05$ ) when compared to healthy control subjects. The T-786C SNP failed to show a significant association ( $p > 0.05$ ) with SLE, HT, and RA patients. This study is the first to reveal a significant association between the 4bb genotype of the 27 bp VNTR and susceptibility to HT. The expression of eNOS is related to the number of 27 bp repeats, with heterozygous 4bb repeats showing a decrease in eNOS expression.

eNOS – endothelial nitric oxide synthase.

Keywords: eNOS, SLE, RA, HT

## 1. Introduction

Nitric oxide (NO) is a gaseous free radical synthesised from L-arginine by the nitric oxide synthase gene (NOS), and it is a potent regulator of the immune response that acts by triggering a cascade of signal transduction pathways that play a significant role in the inflammatory and autoimmune responses.

The role of NO in the pathogenesis of autoimmune diseases was uncovered a decade ago. Overproduction of NO could contribute to tissue injury given its capacity to increase vascular permeability, generate toxic free radicals such as peroxynitrite, and induce cytotoxicity [1,2]. Normal endothelial function is characterized by a dynamic balance between NO and other oxidants [1]. Increased production of NO metabolites and endothelial dysfunction has been well documented in individuals with active SLE and animal models of lupus [3–6]. Similarly, an increased concentration of NO have been correlated with disease severity in RA patients and is, in turn, regarded as a key mediator of apoptosis within the joints of RA patients [7–9]. Addi-

---

\*Corresponding author: Suad AlFadhli, Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Kuwait University, Kuwait, Kuwait PO Box 31470 Sulaibekhat, Kuwait. Tel.: +965 6000 2088; Fax: +965 2498 3835; E-mail: s.alfadhli@hsc.edu.kw, suadq8@yahoo.com.

tionally, increased NO levels have also been reported in hashimoto's thyroiditis (HT) patients [10].

Markers of oxidative stress are well correlated with disease activity in both SLE and RA [11]. NOS inhibition was reported to decrease the disease activity in experimental RA [12]. One of the 3 identified isoforms of NOS, eNOS (endothelial nitric oxide synthase) is critical to vascular homeostasis and therefore participates in the pathogenesis of endothelial dysfunction [13]. The eNOS gene consists of 26 exons located on chromosome 7q36.1. It has been extensively studied in various ethnic populations for its association with several cardiovascular diseases [14–17]. Numerous single nucleotide polymorphisms and variable number tandem repeats (VNTRs) have been reported in the *eNOS* gene, some of which may affect gene expression, protein synthesis and enzymatic activity, including T-786C, located in the promoter region, Glu298Asp, located in exon 7, and a VNTR of 27 bp in intron 4. It has been hypothesised that the 27 bp repeat in intron 4 of *eNOS* gene is responsible for the plasma levels of NO and could, therefore, play a significant role in the pathogenesis of autoimmune diseases [18]. However, limited studies have been carried out to explore the role of the 27 bp repeat polymorphism in autoimmune diseases. These include lupus [18], diabetes mellitus [19] and primary biliary cirrhosis [20]. Our previous study revealed a strong association of the 27 bp VNTR of the *eNOS* gene with Alopecia areata in the Kuwaiti population [21]. Complete genome scanning studies have identified several SLE susceptibility loci, including the *eNOS* locus [22,23]. More recently, our research has revealed a significant haplotype association of the eNOS 27 bp VNTR, T-786C SNP and GLu298Asp polymorphism with SLE patients [24].

In an attempt to generate further evidence, we developed a research study to investigate whether the eNOS T-786C polymorphism located in the promoter region and the 27 bp repeat of intron 4 are associated with eNOS expression at the mRNA and protein level in Kuwaiti SLE, RA and HT patients. A combined genetic and functional approach was employed to explore the correlation between the T-786C SNP, 27 bp repeat, NO production and eNOS gene expression in the three different disease groups.

## 2. Materials and methods

A total of 383 subjects were recruited for this study. The subjects were comprised of 120 SLE (female;

male ratio 11:1, mean age  $38.36 \pm 11.68$ ), 48 RA (female; male ratio 4:1, mean age  $56.2 \pm 10.67$ ), and 67 HT patients (female; male ratio, mean age  $46.2 \pm 8.42$ ) from Mubarak Al-Kabeer Hospital, as well as 148 healthy subjects. Study conduct was approved by the Kuwait University's Ethics Committee and all of the participants provided a written informed consent. SLE and RA patients who were enrolled in the study had at least four of the Systemic Lupus International Collaboration Clinics/American College of Rheumatology (SLICC/ACR) criteria for classification of the disease [25]. Demographic and clinical information's are provided in Table 1. The diagnosis of SLE was made if four or more of the following manifestations are present, either one after the other or at the same time. These include malar rash, photosensitivity, hematological disorder [anemia, thrombocytopenia, low WBC, low platelets, immunologic disorder [low C3, low C4, antinuclear antibody, anti-dsDNA, mucosal ulcers, pulmonary, arthritis, renal disorders, serositis, vasculitis and neurological disorder. A combination of clinical, laboratory and thyroid imaging data were used to define the HT patients. Clinical and laboratory investigation such as titres anti-microsomal (anti-TPO) antibodies, anti-thyroglobulin antibodies, FT4 level and Thyroid stimulating hormone were detected.

Healthy volunteers were randomly selected from five provinces in Kuwait by conducting a thorough survey involving demographic and clinical parameters. Inclusion criteria consisted of good general health and no history of autoimmune disease within first degree relatives. Exclusion criteria included any recent history of acute or chronic debilitating illnesses. Subjects who failed to meet the above set of criteria were excluded from the study. Kuwaiti nationals are either of Arab or Persian ancestry. Only Kuwaitis with Arab ancestry were included in this study. Ethnic bias within the study population was minimised by excluding patients who were not of Arab origin. Each participant donated peripheral blood for DNA and RNA analysis and serum isolation. Patient and control sera were stored at  $-20^{\circ}\text{C}$  for up to 1 month or at  $-80^{\circ}\text{C}$  for long-term storage. DNA was isolated from peripheral nucleated blood cells using the Gentra kit (Minneapolis, USA). RNA was isolated using QIAamp RNA blood kits (QIAGEN, Germany) according to the manufacturer's instructions. cDNA was prepared from the isolated RNA using the Hi-Capacity Reverse transcription kit (Applied Biosystems, CA, USA).

Table 1  
Demographic and clinical features of patients

|  |                 |
|--|-----------------|
| SLE ( <i>n</i> = 120)  |                 |
| Female: male ratio   | : 11:1          |
| Mean age   | : 38.36 ± 11.68 |
| Age at onset of disease  | : 27.6 ± 11.11  |
| Nationality  | : Kuwaiti Arabs |
| Malar rash (47%), photosensitivity (14%), hematological disorder [anemia (16%), thrombo-cytopenia (19.2%), low WBC (17.5%), low platelets (31.7%)], immunologic disorder [low C3 (31.7%), low C4 (31.7%), antinuclear antibody (74%), anti-dsDNA (39.2%)], mucosal ulcers (9.2%), pulmonary (12.5%) arthritis (44%), renal disorders (7.5%), serositis (1.7%), vasculitis (7.5%) |                 |
| HT ( <i>n</i> = 67)  |                 |
| Female: male ratio   | : 15.8:1        |
| Mean age   | : 46.2 ± 8.42   |
| Age at onset of disease  | : 41.3 ± 7.89   |
| Nationality  | : Kuwaiti Arabs |
| Clinical and laboratory data showed that 41.8% had high titre anti-microsomal (anti-TPO) antibodies, 34.3% had high anti-thyroglobulin antibodies, 10.4% had low FT4 and 47.8% had high TSH.   |                 |
| RA ( <i>n</i> = 48)  |                 |
| Female: male ratio   | : 4:1           |
| Mean age   | : 56.2 ± 10.67  |
| Age at onset of disease  | : 42 ± 12.8     |
| Nationality  | : Kuwaiti Arabs |
| CRP  | : 8.3–39.5 mg/L |
| RF   | : 29–131 IU/L   |

### 2.1. Genotyping of the T-786C SNP by restriction fragment length polymorphism

Genotypes of the T-786C polymorphism (rs20707-44) located in the 5' flanking region of *eNOS* were determined by PCR using the primers 5'-TGGAGAGTGCTGGTGTACCCCA-3 (forward) and 5'-GCCTCCACCCACCCTGTC-3 (reverse), followed by restriction digestion. PCR amplification resulted in 180 bp products, which were then digested with Msp I (New England Biolabs, MA, US) at 37°C. Msp I digestion produced 140, 90, 50 and 40 bp fragments for CT heterozygotes, 140 and 40 bp products for TT homozygotes, and 90, 50 and 40 bp products for CC homozygotes. Digestion fragments were separated by electrophoresis using a 4% agarose gel containing ethidium bromide and visualised under UV light. Genotyping results were further confirmed by randomly selecting samples to repeat.

### 2.2. Genotyping of the 27 bp repeat polymorphism by fragment analysis

Analysis of the 27 bp repeat polymorphism in intron 4 of the *eNOS* gene was carried out using the fragment analysis method. cDNA was amplified using the up-

stream labelled primer 5'-VIC AGGCCCTATGGTAGTGCCTT-3' and the downstream primer 5'-TCTCTT-AGTGCTGTGG TCAC-3'. Polymerase chain reaction was carried out in a 10 µl reaction mix containing 5 pmol of each primer. The thermal amplification program consisted of an initial denaturation at 95°C (5 min), followed by 35 cycles of 94°C (1 min), 59°C (1 min) and 72°C (1 min) and a final extension at 72°C (8 min). One microliter of the PCR amplicon was mixed with 10 µl of Hi Di formamide and 0.3 µl of genetic marker LIZ600 (Applied Biosystems, USA). The mixture was then heated at 95°C for 3 minutes and kept on ice for 2 minutes. Fragment analysis was performed using POP4 in a 36-cm capillary of the ABI3100 genetic analyser (Applied Biosystems, USA). A band size of 420 bp indicated five repeats of the 27 bp sequence, while a 323 bp band represented four repeats, corresponding to the b and a alleles, respectively. Samples corresponding to a/a and a/b genotypes were selected at random and subjected to direct sequencing using the ABI-PRISM 3100 genetic analyser (Applied Biosystems, USA).

### 2.3. Real time PCR

Quantitative real-time PCR (TaqMan probe-based detection) was performed to quantify the expression of the human *eNOS* gene relative to human GAPDH. Primers and probes for *eNOS* mRNA were designed using the NCBI sequence NM\_005223 (<http://www.ncbi.nlm.nih.gov/hotbin-post/Entrez/>); glyceral-dehyde-3-Phosphatede-hydrogenase. GAPDH was used as the endogenous control. Primers and probes were purchased from Applied Biosystems, USA, and the reaction was carried out according to the manufacturer's instructions.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The Nitrate/Nitrite colorimetric assay kit (catalogue No. 780001) from Cayman Chemical was used to measure the total nitrate concentration in subjects enrolled in the study. The reaction was carried out according to the manufacturer's instructions.

### 2.5. Statistical analysis

Allele and genotype frequencies were calculated manually for each marker. Differences in the genotypic and the allelic frequency between patients and controls were assessed by Pearson's chi-squared tests. A

Table 2

Distribution of allele and genotype frequencies of eNOS T786C SNP

| Cases (n)    | Allele   | Cases (f)   | Control No. (f) total 66 | chi square | *p value |
|--------------|----------|-------------|--------------------------|------------|----------|
| SLE (n = 80) | T        | 64 (0.80)   |                          | 0.27       | 0.603    |
|              | C        | 16 (0.20)   | T-55 (0.83)              |            |          |
| RA (n = 54)  | T        | 42 (0.78)   | C-11 (0.17)              | 0.59       | 0.442    |
|              | C        | 12 (0.22)   |                          |            |          |
| HT (n = 52)  | T        | 38 (0.73)   |                          | 1.83       | 0.176    |
|              | C        | 14 (0.27)   |                          |            |          |
| Cases (n)    | Genotype | Case No (f) | Control No. (f) total 33 | chi square | *p value |
| SLE (n = 40) | TT       | 30 (0.75)   | 25 (0.76)                | 0.01       | 0.92     |
|              |          | 19 (0.70)   |                          |            |          |
| RA (n = 27)  |          | 16 (0.62)   |                          | 1.39       | 0.238    |
| HT (n = 26)  |          | 4 (0.10)    | 5 (0.15)                 | 0.444      | 0.505    |
| SLE (n = 40) | TC       | 4 (0.15)    |                          | 0.001      | 0.975    |
| RA (n = 27)  |          | 4 (0.15)    |                          |            |          |
| HT (n = 26)  |          | 6 (0.23)    |                          | 0.602      | 0.438    |
| SLE (n = 40) | CC       | 6 (0.15)    | 3 (0.09)                 | 0.584      | 0.445    |
| RA (n = 27)  |          | 4 (0.15)    |                          |            |          |
| HT (n = 26)  |          | 4 (0.15)    |                          | 0.551      | 0.458    |

\*p-value was calculated using  $\chi^2$  test (2x2 contingency table).  
 \*SLE – systemic lupus erythematosus, RA – rheumatoid arthritis, HT – hashimoto's thyroiditis, HC – healthy control. \*T-786C SNP were genotyped for 40 SLE, 27 RA, 26 HT and 33 HC.

P value of < 0.05 was defined as significant. Odds ratios (ORs), and confidence intervals (CIs) were calculated using online statistical programs. The distribution of the genotypes for each polymorphism was assessed for deviation from Hardy–Weinberg equilibrium using the GenePop software. Deviation from hardy Weinberg equilibrium was observed for the studied population. Hence Cochran armitage trend test was used to analyze the data and association was still found to be significant. eNOS mRNA expression and differences in serum nitrate concentration between patients and healthy subjects were analysed using Student's *t*-test (Graph Pad Prism).

### 3. Results

Samples from 383 subjects were chosen as cases and controls for studying the allelic and genotypic distribution for each polymorphism in the Kuwaiti population. Two selected markers from the eNOS gene, the T-786C SNP located in the eNOS promoter region and the 27 bp repeat polymorphism in intron 4, were examined in SLE, RA and HT patients.

A total of 126 subjects were genotyped for the eNOS T-786C SNP. Allele C of the eNOS T-786C SNP, which is suspected for susceptibility, was found to be less frequent (20%) in the Kuwaiti population when compared to the T allele, which dominated the population. Allelic

Table 3

Distribution of allele and genotype frequencies of eNOS 27 bp VNTR

| Cases (n)     | Allele | Cases (f)    | Control No. (f) n = 296 | chi square | *p value |
|---------------|--------|--------------|-------------------------|------------|----------|
| SLE (n = 240) | 4b     | 200 (0.83)   |                         | 6.79       | 0.0092   |
|               | 4a     | 40 (0.17)    | 4b-219 (0.74)           |            |          |
| RA (n = 96)   | 4b     | 75 (0.78)    | 4a-77 (0.26)            | 0.66       | 0.417    |
|               | 4a     | 21 (0.22)    |                         |            |          |
| HT (n = 134)  | 4b     | 109 (0.81)   |                         | 2.76       | 0.097    |
|               | 4a     | 25 (0.19)    |                         |            |          |
| Cases (n)     | Repeat | Cases no (f) | Control No. (f) n = 148 | chi square | *p value |
| SLE (120)     | 4bb    | 81 (0.675)   | 76 (0.51)               | 7.12       | 0.0076   |
| RA (48)       |        | 28 (0.58)    |                         |            |          |
| HT(67)        |        | 44 (0.66)    |                         | 3.84       | 0.05     |
| SLE (120)     | 4aa    | 1 (0.008)    | 5 (0.034)               | 1.96       | 0.16     |
| RA (48)       |        | 1 (0.02)     |                         |            |          |
| HT(67)        |        | 2 (0.03)     |                         | 0.02       | 0.88     |
| SLE (120)     | 4ab    | 38 (0.32)    | 67 (0.45)               | 5.15       | 0.02     |
| RA (48)       |        | 19 (0.40)    |                         |            |          |
| HT(67)        |        | 21 (0.31)    |                         | 3.7        | 0.05     |

\*p-value was calculated using  $\chi^2$  test (2x2 contingency table).  
 \*SLE – systemic lupus erythematosus, RA – rheumatoid arthritis, HT – hashimoto's thyroiditis, HC – healthy control. \*The eNOS 27 bp VNTR were genotyped for 120 SLE, 48 RA, 67 HT and 148 HC.

and genotypic frequencies of the T-786C SNP in SLE, HT and RA patients failed to show any significant differences compared to healthy subjects (Table 2). The TC and CC genotypes were less frequently represented in both case and control subjects.

The allelic and genotypic frequencies of the 27 bp VNTR polymorphism were detected in a total of 383 subjects (Table 3). As expected, analysis of this marker revealed two alleles, 4a and 4b, representing the four and five repeats, respectively. The 4b allele was found to be predominant in both the case (> 78%) and control (74%) populations. Analysis of allelic frequency revealed a significant difference between SLE patients and healthy subjects only for the 4b allele ( $p = 0.009$ ,  $OR = 1.76$ ). The 4b allele (five repeats) confers susceptibility to SLE.

Three genotypes of the 27 bp VNTR polymorphism were detected: 4aa, 4ab and 4bb (Table 3). The 4bb genotype was found to be predominant in both case and control populations, followed by the 4ab genotype. The homozygous 4aa genotype was rare in all of the cohorts tested. The homozygous 4bb genotype revealed a significant association with susceptibility to SLE ( $p = 0.0076$ ,  $OR = 1.97$ ,  $CI = 1.19-3.24$ ) and HT ( $p = 0.05$ ,  $OR = 1.81$ ,  $CI = 0.9-3.3$ ) when compared to healthy control subjects. The frequency of occurrence of the 4bb genotype was higher in SLE (68%) and HT patients (66%) compared to healthy subjects

(51%). Similarly, the 4ab genotype revealed a significant association with protectivity to SLE ( $p = 0.023$ ,  $OR = 0.56$ ,  $CI = 0.34-0.93$ ) and HT ( $p = 0.05$ ,  $OR = 0.55$ ,  $CI = 0.3-1.01$ ) when compared individually with healthy control subjects. The 4ab genotype was observed in 32% of SLE and 31% of HT patients. No significant difference was observed between the allelic and genotypic distribution of RA patients and healthy controls.

We further investigated the functional involvement of the T-786C SNP and 27 bp VNTR on eNOS gene expression. A slight increase in the expression of eNOS mRNA was observed in SLE ( $\Delta Ct 8.01 \pm 1.85$ ) and RA patients ( $\Delta Ct 8.10 \pm 1.19$ ) when compared to healthy control subjects ( $\Delta Ct 8.56 \pm 1.5$ ), but the difference was not statistically significant ( $p > 0.05$ ). Similarly, HT patients ( $\Delta Ct 8.53 \pm 1.55$ ) and healthy subjects gave analogous results, failing to show any significant association. When samples were stratified according to genotype, no significant association was observed between the T-786C SNP and mRNA expression in any of the tested cohorts. However, an increase in the expression of mRNA was found to be common in all of the autoimmune patients with wild type and mutant alleles.

A significant difference in eNOS expression was observed between the 4ab genotype of RA ( $\Delta Ct 7.68 \pm 1.06$ ,  $p = 0.0144$ ) and HT ( $\Delta Ct 7.82 \pm 1.07$ ,  $p = 0.0293$ ) patients when compared individually to healthy subjects ( $\Delta Ct 9.32 \pm 0.77$ ). Fold differences of 1.5 to 6.5 and 1.4 to 5.9 were observed in the expression of target mRNA in both RA and HT patients, respectively, relative to healthy subjects. In addition, a suggestive significance ( $\Delta Ct 8.95 \pm 0.77$ ,  $p = 0.0556$ ) was observed between the 4bb genotype of HT patients and healthy controls ( $\Delta Ct 7.78 \pm 1.86$ ), where a 0.25- to 0.75-fold reduction in eNOS mRNA expression was observed. Furthermore, the differences in mRNA expression between the 4bb and 4ab genotypes were found to be statistically significant in SLE ( $p = 0.025$ ) and HT patients ( $p = 0.0067$ ). An increase in the expression of eNOS mRNA was observed in autoimmune patients with the 4ab genotype when compared to the 4bb genotype.

The serum concentration of nitrate in a total of 90 patients and 35 healthy subjects was evaluated. The results are expressed in micromolar ( $\mu M$ ). The serum nitrate concentration was found to be extremely significant ( $p < 0.0001$ ) in autoimmune patients from the SLE ( $12.12 \pm 6.55$ ), HT ( $12.31 \pm 7.77$ ) and RA ( $16.48 \pm 8.13$ ) cohorts when compared to healthy control sub-

jects ( $5.24 \pm 3.18$ ). Average serum concentration was found to be higher in RA patients compared to SLE and HT patients. A 3-fold difference in nitrate concentration was observed between RA patients and healthy subjects irrespective of genotype. Furthermore, the influence of the 27 bp repeat on nitrate production was explored. Both the 4ab and 4bb genotypes were found to be significantly associated with nitrate production in the autoimmune cohorts tested when compared to healthy control subjects ( $p < 0.05$ ). No significant difference was observed between the 4ab and 4bb genotypes of autoimmune patients.

#### 4. Discussion

The allele and genotype frequencies of the T-786C SNP failed to show any significant association with any of the tested cohorts. The lack of an association between the T-786C SNP and autoimmune diseases in the present study could be due to the predominance of the T allele within the Kuwaiti population. Table 4 shows data from different population studies in Kuwait [24], China [26,27], Columbia [28] and the USA [29] that also failed to find a significant association between the T-786C SNP and SLE, suggesting that it is unlikely to be a genetic susceptibility factor for SLE. Association of the T-786C SNP with HT has yet not been reported in any population study. Recently, the T-786C SNP was shown to be associated with RA in Chinese [30] and German populations [31,32]. Conflicting results demonstrating no association between the T-786C SNP and RA have been reported in Brazilian [33] and Italian [34] populations.

Studying the association of the eNOS-27 bp VNTR marker with autoimmune diseases revealed a significant association with susceptibility to SLE ( $p = 0.0092$ ) and HT ( $P = 0.05$ ). Analysis of allelic frequency revealed a significant association of the 4b allele with susceptibility to SLE ( $p = 0.009$ ) and the 4bb genotype to SLE ( $p = 0.008$ ) and HT ( $p = 0.05$ ). As shown in Table 4, this polymorphism (4b/4bb) has been associated with SLE in Colombian populations [28], while three other populations (USA, Korea and Greece) failed to show a significant association [18,29,35]. Conflicting results were reported for the Turkish population in which the 4a and 4aa alleles were shown to be associated with susceptibility to SLE [36]. A single study of the Greece population by Vazgiourakis et al. 2007 showed a significant association of the 27 bp VNTR with RA [18]. Previously,

Table 4  
Literature summary of eNOS 27 bp VNTR and T-786C SNP in SLE and RA

| Study                  | Population       | No.      | Marker               | Findings  |
|------------------------|------------------|----------|----------------------|---|
| SLE                    |                  |          |                      |   |
| Alfadhli et al.        | Kuwait (AR)      | 152/184  | 27 bp VNTR<br>T-786C | VNTR b allele (OR = 1.89, $p = 0.023$ )<br>NS                                       |
| Li et al.              | China            | 225/232  | T-786C               | NS  |
| Tang et al.            | China            | 90/86    | T-786C               | NS  |
| Vazgiourakis et al.    | Greece           | 190/145  | 27 bp VNTR           | VNTR allele (NS), but for LN vs. non LN (OR = 1.96, $p=0.025$ )                     |
| Serrano et al.         | Colombia         | 193/106  | 27 bp VNTR<br>T-786C | VNTR b allele (OR = 2.2, $p = 0.002$ ),<br>NS                                       |
| Doughs et al.          | USA              | 227/275  | 27 bp VNTR<br>T-786C | NS  |
| Lee et al.             | Korea            | 88/95    | 27 bp VNTR           | VNTR b allele (NS), but for LN vs. non LN (OR for ab genotype = 3.28, $p = 0.004$ ) |
| Maria I. Zervou et al. | Turkey           | 158/155  | 27 bp VNTR           | a allele ( $p = 2.7 \times 10^{-2}$ ) and a/a ( $p = 2.6 \times 10^{-2}$ ) SLE      |
| RA                     |                  |          |                      |   |
| An JD et al.           | China            | 196/201  | T-786C               | C-allele AS with RA ( $p < 0.05$ ).   |
| Inga Melchers et al.   | Germany          | 596/1160 | T-786C               | C/C AS with RA ( $p < 0.0001$ ).  |
| Gonzalez et al.        | North West Spain | 170/117  | T-786C               | NS  |
| Brenol et al.          | Brazil           | 105/100  | T-786C               | NS. However, C allele AS with extracellular manifestation of RA ( $p = 0.03$ ).     |
| Melchers et al.        | German           |          | T-786C               | C/C AS with Endothelial dysfunction in RA   |
| Vazgiourakis et al.    | Greece           | 202/235  | 27 bp VNTR           | Susceptibility to RA (OR: 2.0, 95% CI: 1.3–3.1).                                    |

LN = Lupus nephritis, \*NS: not associated, AS: associated.

a possible relationship between the synthesis of nitric oxide by the *eNOS* gene and immune dysregulation in the thyroid gland was reported by Varul et al. [10]. Our study further strengthens this finding by demonstrating a statistically significant association between HT patients and the *eNOS*-27 bp repeat polymorphism.

In general, no significant difference was observed for eNOS mRNA expression between the tested patients and healthy subjects. Our study revealed that T-786C SNP does not have an effect on the expression of the eNOS gene in the Kuwaiti population. When the influence of the 27 bp VNTR was explored parallel, it was found that the genotype of the 27 bp VNTR tends to play an influential role in eNOS expression. A significant increase in the expression of eNOS mRNA was observed in RA ( $p = 0.01$ ) and HT ( $p = 0.03$ ) patients with the 4ab genotype when compared to healthy control subjects. Conversely, a reduction in the expression of eNOS mRNA was observed in all autoimmune patients with the 4bb genotype compared to healthy control subjects, though it only reached a significant level in HT patients ( $p = 0.05$ ). Although no significant differences in eNOS mRNA expression were observed between SLE patients and healthy subjects for each individual genotype, the differences between the 4bb and 4ab genotypes within the patient group were found to be statistically significant in SLE ( $p = 0.03$ ) and HT ( $p = 0.007$ ). Both SLE and HT patients with the 4bb genotype had reduced eNOS mRNA expression.

Recently, Zhang et al. have shown that the 27 bp repeats in eNOS intron 4 produce small 27 bp micro RNAs [37,38]. This intron-based micro RNA can induce gene specific transcriptional suppression by modifying histone acetylation and DNA methylation, which may serve as an effective negative feedback regulator of eNOS expression. These data may help explain the conflicting results obtained in our study. Although a strong association was evident between the 4bb genotype and SLE at genomic level, the failure to obtain the same association at the mRNA level may be explained by increased production of the 27 bp micro RNA. A higher number of 27 bp repeats present in eNOS intron 4 will result in more micro RNA being produced and lead to reduced expression or degradation of eNOS mRNA. This, in turn, strengthens our finding that expression of the eNOS gene is related to the number of 27 bp repeats, with homozygous 4bb showing decreased eNOS mRNA expression and heterozygous 4ab showing increased expression of eNOS mRNA.

Additionally, a significant difference ( $p < 0.0001$ ) was observed when NOx concentrations in individual patients were tested and compared to healthy subjects. Regardless of the 4aa/4bb genotype, elevated levels of NOx were observed in all of the autoimmune patients tested. The increased serum NOx concentration in autoimmune patients provides direct evidence of excess

endogenous nitric oxide production. The production of nitric oxide by eNOS may offer a protective or anti-inflammatory function [39]. One of the characteristic features of SLE is the activation of endothelial cells, which have been revealed to be the source of the excess NO production [40]. Excessive nitric oxide production may alter complement mediated clearance of apoptotic cells in SLE patients, contributing to autoimmunity. Similarly, increasing evidence indicates that nitric oxide is a key regulator of apoptosis in RA [40]. Oxidative stress was reported to be increased and more profound in RA than SLE and could well reflect disease activity [11]. Increased nitric oxide levels have also been observed in HT patients [10]. Increased production of nitric oxide may disrupt T cell activation, differentiation and effector responses; each of which may contribute to autoimmunity in HT [41]. In our study, the lack of correlation between eNOS mRNA expression and NOx detection could be due to the fact that the chemical half-life of the mRNA might not reflect its functional half-life. Additionally, eNOS is not the lone source of nitric oxide. The two other isoforms of the NOS gene, nNOS and iNOS, are also expected to produce nitric oxide at high levels. Further studies are needed to explore the role of nNOS and iNOS in autoimmunity.

## 5. Conclusion

The aim of this study was to explore the potential role of the T-786C SNP and 27 bp VNTR in eNOS mRNA expression and nitrate production in SLE, HT and RA patients from the Kuwaiti population. Analysis of the 27 bp VNTR revealed a significant association of the 4bb genotype with susceptibility to SLE and HT. Our result highlights the possible relationship between the 27 bp VNTR and eNOS mRNA expression in autoimmune patients. Regardless of the genotype, the levels of nitric oxide were elevated in all of the tested autoimmune patients, demonstrating its significance in immune dysregulation. No significant association was observed between the T-786C SNP and any of the cohorts tested. The effect of this SNP on eNOS mRNA expression was not found to be significant in the Kuwaiti population.

## Acknowledgements

This work was supported by Kuwait University Research Administration Grant NM01/07.

## Conflict of interest

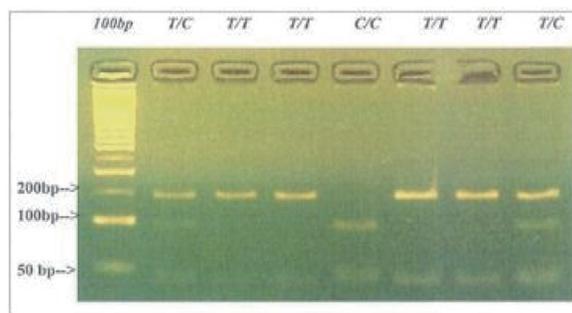
Author declares that no conflict of interest.

## References

- [1] J.D. Pearson, Normal endothelial cell function, *Lupus* **9** (2000) 183-8.
- [2] R.M. Clancy, S.B. Abramson, Nitric oxide: a novel mediator of inflammation, *Proc Soc Exp Biol Med* **210** (1995) 93-101.
- [3] J.C. Oates, E.F. Christensen, C.M. Reilly, et al, Prospective measure of serum 3-nitrotyrosine levels in systemic lupus erythematosus: correlation with disease activity, *Proc Assoc Am Physicians* **111** (1999) 611-621.
- [4] G. Gilkeson, C. Cannon, J Oates, et al, Correlation of serum measures of nitric oxide production with lupus disease activity, *J Rheumatol* **26** (1999) 318-324.
- [5] L. Brundin, E. Svenungsson, E. Morcos, et al, Central nervous system nitric oxide formation in cerebral systemic lupus erythematosus, *Ann Neurol* **44** (1998) 704-706.
- [6] G. Rolla, L. Brussino, M.T. Bertero, et al, Increased nitric oxide in exhaled air of patients with systemic lupus erythematosus, *J Rheumatol* **24** (1997) 1066-1071.
- [7] R.M. Clancy, A.R. Amin, S.B. Abramson, The role of nitric oxide in inflammation and immunity, *Arthritis Rheum* **41** (1998) 1141-1151.
- [8] St. Clair E.W., W.E. Wilkinson, T. Lang, et al, Increased expression of blood mononuclear cell nitric oxide synthase type 2 in rheumatoid arthritis patients, *J Exp Med* **184** (1996) 1173-1178.
- [9] R.J. Van't Hof, L. Hocking, P.K. Wright, et al, Nitric oxide is a mediator of apoptosis in rheumatoid joints, *Rheumatology* **39** (2000) 1004-1008.
- [10] P. Vural, S. Degirmencioglu, S. Erden, A. Gelincik, The relationship between transforming growth factor, nitric oxide and Hashimoto's thyroiditis, *Intl Immunol* **9** (2009) 212-215.
- [11] S.Z. Hassan, T.A. Gheita, S.A. Kenawy, et al, Oxidative stress in systemic lupus erythematosus and rheumatoid arthritis patients: relationship to disease manifestations and activity, *Int J Rheum Dis* **14** (2011) 325-31.
- [12] N. McCartney-francis, B.J. Allen, D.E. Mizel, Suppression of arthritis by an inhibitor of nitric oxide synthase, *J Exp Med* **178** (1993) 749-754.
- [13] B.E. Fenster, P.S. Tsao, S.G. Rockson, Endothelial dysfunction: clinical strategies for treating oxidant stress, *Am Heart J* **146** (2003) 218-26.
- [14] Y. Asakimori, N. Yorioka, J. Tanaka, et al, Association between ENOS gene polymorphism and cardiovascular events in nondiabetic hemodialysis patients: a prospective study, *Am J Kidney Dis* **44** (2004) 112-20.
- [15] J. Li, X. Wu, X. Li, et al, The endothelial nitric oxide synthase gene is associated with coronary artery disease: a meta-analysis, *Cardiology* **116** (2010) 271-8.
- [16] C. Dafni, N. Drakoulis, O. Landt, et al, Association of the eNOS E298D polymorphism and the risk of myocardial infarction in the Greek population, *BMC Med Genet* **20**(11) (2010) 133.
- [17] G. Ragia, E. Nikolaidis, A. Tavridou, et al, Manolopoulos, Endothelial nitric oxide synthase gene polymorphisms -786T > C and 894G > T in coronary artery bypass graft surgery patients, *Hum Genomics* **4** (2010) 375-83.

- [18] V. Vazgiourakis, P. Sidiropoulo, G. Bertisia, et al, Association of the nitric oxide synthase (eNOS) gene polymorphism with increased risk for both lupus glomerulonephritis and rheumatoid arthritis in a single genetically homogeneous population, *Lupus* **16** (2007) 867-874.
- [19] E. Galanakis, D. Kofteridis, K. Stratigi, et al, Intron 4 a/b polymorphism of the endothelial nitric oxide synthase gene is associated with both type 1 and type 2 diabetes in a genetically homogeneous population, *Hum Immunol* **69** (2008) 279-83.
- [20] A. Mantaka, G.N. Goulielmos, M. Koulentaki, et al, Polymorphisms of genes related to endothelial cells are associated with primary biliary cirrhosis patients of Cretan origin, *Hum Immunol* **73** (2012) 829-35.
- [21] S. AlFadhli, N. Kharrat, B. AlTememy, et al, Susceptible and protective endothelial nitric oxide synthase gene polymorphism in alopecia areata in the Kuwaiti population, *Autoimmunity* **41** (2008) 522-5.
- [22] J.J. Manson, D.A. Isenberg, The pathogenesis of systemic lupus erythematosus, *Neth J Med* **61** (2003) 343-346.
- [23] P.M. Gaffney, W.A. Ortmann, S.A. Selby, et al, Genome screening in human systemic lupus erythematosus: results from a second Minnesota cohort and combined analysis of 187 sib-pair families, *Am J Hum. Genet* **66** (2000) 547-556.
- [24] S. AlFadhli, B. AlTamimy, K. AlSaeid et al, Endothelial nitric oxide synthase gene haplotype association with systemic lupus erythematosus, **20** (2011) 700-8.
- [25] Hochberg MC, Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus, *Arthritis Rheum* **40** (1997) 1725.
- [26] X. Li, J. An, R. Guo, et al, Association of the genetic polymorphisms of the ACE gene and the eNOS gene with lupus nephropathy in northern Chinese population, *BMC Med Genet* **11** (2010) 94.
- [27] F.Y. Tang, X.W. Xie, G.H. Ling, et al, Endothelial nitric oxide synthase and nicotinamide adenine dinucleotide phosphate oxidase p22phox gene (C242T) polymorphisms and systemic lupus erythematosus in a Chinese population, *Lupus* **19** (2010) 192-6.
- [28] N.C. Serrano, C. Paez, P.A. Correa, J.M. Anaya, Endothelial nitric oxide synthase gene polymorphism is associated with systemic lupus erythematosus, *J Rheumatol* **31** (2004) 2163-8.
- [29] G. Douglas, C. Reilly, M.A. Dooley, et al, Angiotensin-converting enzyme (insertion/deletion) and endothelial nitric oxide synthase polymorphisms in patients with systemic lupus erythematosus, *J. Rheumatol* **31** (2004) 1756-62.
- [30] J.D. An, X.Y. Li, J.B. Yu, et al, Association between the eNOS gene polymorphisms and rheumatoid arthritis risk in a northern Chinese population, *Chin Med J* **125** (2012) 1496-9.
- [31] I. Melchers, S. Blaschke, M. Hecker, et al, The -786C/T single-nucleotide polymorphism in the promoter of the gene for endothelial nitric oxide synthase: Insensitivity to physiologic stimuli as a risk factor for rheumatoid arthritis, *Arthritis & Rheumatism* **54** (2006) 3144-3151.
- [32] I. Melchers, M. Cattaruzza, A gene defect in the promoter of the endothelial NO synthase as a risk factor for rheumatoid arthritis, *Z Rheumatol* **66** (2007) 326-327.
- [33] C.V. Brenol, J.A. Chies, J.C. Brenol, et al, Endothelial nitric oxide synthase T-786C polymorphism in rheumatoid arthritis: association with extra articular manifestations, *Clin Rheumatol* **28** (2009) 201-5.
- [34] M.A. Gonzalez-Gay, J. Llorca, E. Sanchez, M.A., et al, Inducible but not endothelial nitric oxide synthase polymorphism is associated with susceptibility to rheumatoid arthritis in northwest Spain, *Rheumatol* **43** (2004) 1182-5.
- [35] Y.H. Lee, H.J. Kim, Y.H. Rho, et al, Intron 4 polymorphism of the endothelial nitric oxide synthase gene is associated with the development of lupus nephritis, *Lupus* **13** (2004) 188-91.
- [36] I. Maria, M. Zervou, V. Vazgiourakis, N. Yilmaz, et al, TRAF1/C5, eNOS, C1q, but not STAT4 and PTPN22 gene polymorphisms are associated with genetic susceptibility to systemic lupus erythematosus in Turkey, *Hum Immunol* **72** (2011) 1210-1213.
- [37] M.X. Zhang, H. Ou, Y.H. Shen, J. Wang, et al, Regulation of endothelial nitric oxide synthase by small RNA, *Proc Natl Acad Sci USA* **102** (2005) 16967-1672.
- [38] M.X. Zhang, C. Zhang, Y.H. Shen, et al, Effect of 27nt Small RNA on Endothelial Nitric-Oxide Synthase Expression, *Mol Biol Cell* **19** (2008) 3997-4005.
- [39] S.B. Abramson, A.R. Amin, R.M. Clancy, et al, The role of nitric oxide in tissue destruction, *Best Practice & Research Clinical Rheumatology* **15** (2001) 831-845.
- [40] R. Clancy, G. Marder, V. Martin, et al, Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy, *Arthritis Rheum* **44** (2001) 1203-1208.
- [41] M.B. Grisham, C.G. Johnson, M.D. Gautreaux, R.D. Berg, Measurement of nitrate and nitrite in extracellular fluids: a window to systemic nitric oxide metabolism, *Methods: A companion to methods in enzymology* **7** (1995) 84-90.

### Supplementary file



**Figure 1:** A 3% agarose gel electrophoresis with ethidium bromide dye presenting *MspI* treatment of 180 bp of 5' flanking region for eNOS gene -786T/C SNP. TT homozygotes produce 140 and 40 bp bands (Lane 3, 4, 6, and 7). CC homozygotes produce 90bp, and 50 and 40 bands, which overlap to appear as a single band (Lane 2 and 8). CT heterozygotes produce all bands of 140, 90, 50, and 40 bp (Lane 5). Lane 1: 100 bp DNA ladder.

Fig. 1. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/DMA-130983>)



**Hindawi**  
Submit your manuscripts at  
<http://www.hindawi.com>

