In this article, we present data on endothelial Nitric Oxide Synthase (eNOS) gene T786C and G894T polymorphisms in Greek steady-state Sickle Cell Disease patients in comparison to healthy controls. Moreover, eNOS mRNA levels were determined in peripheral blood samples from 18 patients and 9 controls. This article complements our recently published article named “Prognostic value of eNOS T786C and G894T polymorphisms in Sickle Cell Disease” (I. Armenis, V. Kalotychou, R. Tzanetea, Z. Kontogeorgiou, D. Anastasopoulou, M. Mantzourani, M. Samarkos, K. Pantos, K. Konstantopoulos, I. Rombos, 2016) [1].

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### Value of the data

- Genotype and allele distribution of eNOS T786C and G894T polymorphisms were studied for the first time in Greek Sickle Cell Disease patients.
- This is the first study to find higher prevalence of the C allele in comparison to T for the -786 position in Sickle Cell Disease.
- Peripheral blood eNOS mRNA levels, as an index of eNOS expression, were studied and analyzed according to the presence or absence of Sickle Cell Disease and eNOS promoter T786C polymorphism.

### 1. Data

Genotype and allele distribution for polymorphisms T786C and G894T in patients and controls are presented in Tables 1 and 2 respectively. No statistically significant differences were obtained [1]. eNOS mRNA levels normalized to GAPDH are presented in Fig. 1 (Sickle Cell Disease patients relative to controls) and Fig. 2 (patients of eNOS 786TC and CC genotypes relative to patients of TT genotype).

### 2. Experimental design, materials and methods

#### 2.1. Study population

Seventy nine consecutive Greek SCD patients (mean age: 48.8 ± 11.5 years, 25–76 years, 25 male, 54 female) and forty eight Greek healthy controls were enrolled. The study was approved by the Hospital Ethics Committee in accordance to Helsinki Declaration. All patients were at steady state at the time of sample collection (no painful crises or other acute complications during the last three months) and they had not been transfused for the last three months.

#### 2.2. DNA extraction and eNOS genotyping

Genomic DNA was isolated from peripheral blood using the PureLink Genomic DNA extraction kit (Invitrogen). Nucleotide substitutions T786C and G894T were detected using Sanger sequencing and PCR-RFLPs techniques respectively. The PCR primers used were as follows:

for T786C: 5′ATGCTCCCAACCAGGCCATCA′ (forward),
5′GTCTTTGAGTCTGACATTAGG3′ (reverse) covering a fragment of 237 bp.
Table 1
Genotype and allele frequencies for eNOS T786C polymorphism in Sickle Cell Disease patients and controls.

| Genotypes     | Sickle Cell Disease patients (n=79) | Controls (n=48) | P     |
|---------------|------------------------------------|----------------|-------|
| TT vs TC+CC   | 21.5% vs 78.5%                     | 10.4% vs 89.6% | 0.173 |
| CC vs TT+TC   | 46.8% vs 53.2%                     | 47.9% vs 52.1% | 0.906 |
| Alleles       | T vs C                             |                |       |
| T vs C        | 37.3% vs 62.7%                     | 31.3% vs 68.7% | 0.324 |

Table 2
Genotype and allele frequencies for eNOS G894T polymorphism in Sickle Cell Disease patients and controls.

| Genotypes     | Sickle Cell Disease patients (n=79) | Controls (n=48) | P     |
|---------------|------------------------------------|----------------|-------|
| GG vs GT+TT   | 50.6% vs 49.4%                     | 39.6% vs 60.4% | 0.226 |
| TT vs GG+GT   | 19.0% vs 81.0%                     | 12.5% vs 87.5% | 0.340 |
| Alleles       | G vs T                             |                |       |
| G vs T        | 65.8% vs 34.2%                     | 63.6% vs 36.4% | 0.712 |

Fig. 1. eNOS mRNA levels in Sickle Cell Disease patients (n=18) and healthy controls (n=9), relative to GAPDH mRNA. Sickle Cell Disease patients: 1.06 ± 0.84, Controls: 1.30 ± 0.92, P=0.105.

Fig. 2. eNOS mRNA levels relative to GAPDH in Sickle Cell Disease patients, according to their eNOS T786C genotype: TT: 1.22 ± 0.89, TC: 2.45 ± 1.88, CC: 2.10 ± 1.87, P=0.454.
Image 1. Sanger sequencing results from 3 samples of 786CC, TT and TC genotype respectively.
2.3. RNA extraction and quantitative real-time PCR

Eighteen SCD patients (5 male and 13 female) underwent eNOS expression study. Total RNA was isolated from peripheral blood of the above patients and 9 controls (4 male and 5 female), none of them smoking or receiving drugs apart from folate supplementation and acetylsalicylic acid. The RNeasy Mini Kit and Qiashredder homogenizer (Qiagen) after selective lysis of erythrocytes were applied. One microgram of total RNA was reverse-transcribed using the iScript™ cDNA SynthesisKit (Bio-Rad). Fifty nanograms of cDNA were used for eNOS quantification on a CFX-96 real-time PCR (Bio-Rad CFX96TouchTM) using hydrolysis probes for eNOS gene and for GAPDH as a reference gene (Applied Biosystems). Amplification was performed with the appropriate cycling parameters, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Measurements were performed in duplicate using the iTaq™ Universal Probes supermix (Biorad). Relative gene expression data were analyzed using the Livak $2^{-\Delta\Delta CT}$ method.

2.4. Statistical analysis

Frequencies of genotypes and clinical characteristics were compared using Pearson’s chi-square test and Fischer-exact test, where applicable. The general, dominant and recessive genetic models, as well as allele frequencies were used to compare patients and normal controls. Quantitative data were expressed as mean ± S.D. and were compared with Analysis of Variance (ANOVA) or Mann–Whitney U test, whether applicable. Levene’s F test was applied to estimate equality of variances and Kolmogorov–Smirnof test to assess deviation from normal distribution. A P-value lower than 0.05 was considered as statistically significant.
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Reference

[1] I. Armenis, V. Kalotychou, R. Tzanetea, Z. Kontogeorgiou, D. Anastasopoulou, M. Mantzourani, M. Samarkos, K. Pantos, K. Konstantopoulos, I. RombosPrognostic value of T786C and G894T eNOS polymorphisms in sickle cell disease, 2016. (in press).